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Superior thermal stability and fast crystallization behavior of a novel, biodegradable α -methylated bacterial polyester

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Abstract

Given their ubiquity in modern society, the development of biodegradable and renewably sourced plastics is essential for the creation of an environmentally sustainable society. One of the drawbacks for currently available biodegradable plastics such as poly(L-lactic acid) (PLLA) and polyhydroxyalkanoates (PHAs) is that it is difficult to simultaneously achieve mechanical flexibility and certain crystallization behavior in these materials, which limits their use as replacements for established petroleum-based plastics such as isotactic polypropylene (iPP). Here, we report the synthesis and characterization of a new biodegradable plastic, poly(3-hydroxy-2-methylbutyrate) [P(3H2MB)], which is a member of the bacterial PHA family whose members include an α -methylated monomer unit. Biosynthesis of P(3H2MB) was achieved using recombinant *Escherichia coli* expressing an engineered pathway. Biosynthesized P(3H2MB) exhibited the highest melting temperature (197 °C) among the biosynthesized PHAs and improved thermal resistance. It also exhibited improved crystallization behavior and mechanical flexibility nearly equal to those of iPP. The primary nucleation rate of P(3H2MB) was faster than that of P(3HB), and the spherulite morphology of P(3H2MB) was much finer than that of P(3HB). This crystal morphology may result in more rapid crystallization behavior, increased transparency, and enhanced mechanical properties. The superior physical properties of P(3H2MB) have the potential to open new avenues for the production of high-performance biodegradable plastics for replacing petroleum-based bulk commodity plastics.

Introduction

The accumulation and prevalence of microplastics, defined by the National Oceanic and Atmospheric Administration (NOAA) as plastic particles <5 mm in diameter, in the environment has become a global concern¹. As the supply, demand, and use of plastics increase, the probability of many nonbiodegradable plastic materials entering the environment also increases². Although these materials are gradually broken into smaller particles via physical fragmentation, these fragments do not

biodegrade, resulting in a wide distribution throughout the oceans as microplastics. During this process, the total surface area of microplastics increases, enabling the adsorption of hydrophobic organic pollutants on their surface. Most of the microplastics found in aquatic environments have terrestrial origins³ but are accidentally released via runoff following storms. Additionally, microplastics such as scrubbers in cosmetic products and fibers from textiles continually enter wastewater treatment plants (WWTPs)⁴ and cannot be effectively removed, resulting in the release of a considerable amount of microplastics into aquatic environments⁵. This growing threat has led to renewed interest in finding biodegradable alternatives that can be degraded to chemical compounds (e.g., CO₂ and H₂O) that can easily enter biogeochemical cycles to reduce their environmental impact.

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One key class of materials receiving attention as biodegradable alternatives to nonbiodegradable plastics are polyhydroxyalkanoates (PHAs). PHAs are aliphatic polyesters that can be produced in bacterial cells and act as carbon-storage materials in nature⁶. These materials can be produced via fermentation of biomass feedstocks, and most PHAs are satisfactorily biodegradable because they are provisional cellular storage materials that are easily catabolized by bacteria⁷. Importantly, PHAs are biodegradable in marine environments and have broad potential to combat the problems associated with nonbiodegradable microplastics⁸. The most widespread PHA found in nature is poly(3-hydroxybutyrate) [P(3HB)], which is a crystalline thermoplastic polyester that has thermal properties similar to those of polypropylene. However, due to their thermal instability above the melting temperature (T_m), poor mechanical properties, and slow crystallization rate, P(3HB) and 3HB-based PHA copolymers have limited use as replacements for petroleum-based plastics⁶. The crystallization behavior affects the cycle time or production rate of plastic molding, such as injection molding, sheet extraction, and fiber spinning. In other words, the crystallization behavior is an important factor influencing the production cost and CO₂ emission per unit product. Copolymerizing other PHA monomers, such as 3-hydroxyhexanoate (3HHx) or 4-hydroxybutyrate, may improve the mechanical properties of 3HB-based PHA copolymers, but this process considerably extends the crystallization time⁶. Generally, for plastics, there is a trade-off between mechanical flexibility and crystallization. Poly(L-lactic acid) (PLLA) is another biodegradable polymer that is produced from renewable sources, and it is currently the polymer most widely used in the manufacture of biodegradable plastics. However, the exposure of PLLA to temperatures above the glass transition temperature (T_g) induces shrinkage and deformation, and its thermal resistance is low. Inducing crystallization of PLLA enhances the thermal resistance; however, the crystallization of PLLA is quite slow, and consequently, its processability is regarded as poor. The high glass-transition temperature of PLLA leads to poor mechanical properties and poor biodegradability⁹, limiting its overall use by plastic manufacturers. PLLA is not regarded as biodegradable except under industrial composting conditions and exhibits biodegradability at only high temperatures exceeding its glass transition temperature¹⁰.

To address the deficiencies encountered with currently characterized biobased biodegradable plastics, we sought to identify and produce biodegradable plastics with novel structures and physical properties that could be more widely adopted by manufacturers. Some heterotrophic bacteria found in activated sludge in WWTPs produce PHAs with unique chemical structures^{11,12}. In addition to 3HB monomers, these naturally

produced PHAs are composed of 3-hydroxyvalerate (3HV) and 3HHx, and moreover, α -methylated monomers such as 3-hydroxy-2-methylbutyrate (3H2MB) and 3-hydroxy-2-methylvalerate (3H2MV) have been used in copolymers. A 3H2MB-containing PHA copolymer was produced by naturally occurring bacteria in a mixed culture from activated sludge, where the copolymer consisted of up to 13 mol% 3H2MB units as a monomer of a quaternary copolymer¹². Because of the relatively low concentration of the 3H2MB monomer in these naturally produced PHA copolymers, the properties of PHAs composed mainly of 3H2MB have not been well defined. Additionally, the bacterial species and the enzymes capable of incorporating 3H2MB into PHA have not been identified¹³.

In a previous study, we biosynthesized PHA copolymers of 3H2MB and 3HB by using genetically engineered *Escherichia coli*¹⁴. However, even in this case, the 3H2MB ratio was limited to 34 mol%, and the copolymer comprised a mixture of copolymers with various 3H2MB ratios. In another study, 3H2MB-containing PHA was biosynthesized via Claisen condensation by a thiolase, and the 3H2MB ratio of this polymer was also limited to 32 mol%¹⁵. In this study, we overcome the shortcomings of previous studies to control the 3H2MB composition of PHA polymers and develop a system to produce a novel P(3H2MB) homopolymer. By elucidating its precise thermal and mechanical properties, we demonstrate the great potential for this material to replace conventional nonbiodegradable plastics.

Materials and methods

Materials

The isotactic P(3HB) homopolymer used in this study was biosynthesized by *Ralstonia eutropha* H16. The molecular weight of P(3HB) was $M_n = 2.3 \times 10^5$ ($M_w/M_n = 2.3$), as measured by gel permeation chromatography (GPC). Isotactic polypropylene (iPP) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received. The molecular weights of iPP were $M_n \sim 97,000$ and $M_w \sim 340,000$. To obtain film samples, the iPP pellets were melted at 220 °C and pressed using a hot-press apparatus H300-01 (AsOne Corporation, Osaka, Japan). Poly(L-lactic acid) (Ingeo 3260HP; high-crystallinity grade, consisting of L-lactic acid >99.5 mol%) was obtained from NatureWorks LLC (Minnetonka, MN, USA). PLLA was dissolved in chloroform (CHCl₃) and cast to obtain a cast film. Further details for P(3H2MB) are given in subsequent sections.

Bacterial strains and plasmids

E. coli L5BJ, a *fadB fadJ* double-deletion mutant of *E. coli* LS5218 [*fadR601, atoC(Con)*], was used as the host strain for PHA biosynthesis^{16,17}. A plasmid harboring the PHA biosynthetic operon from *Aeromonas caviae* was

introduced into *E. coli* LSBJ. The operon consists of a PHA granule-associated protein gene (*phaP_{Ac}*), a PHA synthase gene (*phaC_{Ac}*), and an (*R*)-specific enoyl-CoA hydratase gene (*phaJ_{Ac}*). To efficiently polymerize P (3H2MB), N149S, and D171G point mutations were introduced in *PhaC_{Ac}*, and a D4N point mutation was introduced in *PhaP_{Ac}*^{14,17,18}. To enhance the supply of 3H2MB-CoA, a pTTQ vector containing the propionyl-CoA transferase (PCT) gene from *Megasphaera elsdenii* (*pct*) was also introduced into strain LSBJ¹⁹.

Biosynthesis of P(3H2MB)

Recombinant *E. coli* LSBJ was first cultivated for 15 h at 37 °C with reciprocal shaking (160 rpm) in a 50 mL flask containing 20 mL of lysogeny broth (LB) medium (Supplementary Scheme 1) until the OD reached ~5–6. LB contained 10 g/L Bacto-tryptone (Difco Laboratories, Detroit, MI, USA), 5 g/L Bacto-yeast extract (Difco Laboratories), and 10 g/L NaCl. In addition, 50 mg/L kanamycin and 50 mg/L carbenicillin were added for plasmid maintenance during preculture. A 5 mL aliquot of the preculture broth was inoculated into 100 mL (final volume) of M9-modified medium in a 500 mL shaking flask and cultivated for 4 h at 30 °C with reciprocal shaking (130 rpm). This M9²⁰-modified medium contained 17.1 g/L Na₂HPO₄·12H₂O, 3 g/L KH₂PO₄, 0.5 g/L NaCl, 2 mL of 1 M MgSO₄·7H₂O, 0.1 mL of 1 M CaCl₂, and 2.5 g/L Bacto-yeast extract. For plasmid maintenance during cultivation, 50 mg/L kanamycin and 50 mg/L carbenicillin were added. *trans*-2-Methylbut-2-enoic acid (tiglic acid) was used as the 3H2MB precursor. Tiglic acid inhibited cell growth, whereas a high glucose concentration caused catabolic repression of PHA biosynthetic genes induced by isopropyl-β-D-thiogalactopyranoside (IPTG). Therefore, tiglic acid and glucose were fed into the system three times to maintain the concentrations at low levels. After 4 h of cultivation, 1.25 g/L glucose, 2 g/L tiglic acid, and 1 mM IPTG were added. To enhance the biosynthesis of the P (3H2MB) homopolymer, 1.25 g/L glucose and 2 g/L tiglic acid were fed to the bacteria at 28 and 52 h. Cultivation was continued for 72 h after the first addition of tiglic acid and glucose. After cultivation, the cells were harvested by centrifugation and lyophilized. PHA was extracted from the lyophilized cells by immersion in CHCl₃ at 60 °C for 6 h and then at room temperature for 3 days. The polymer CHCl₃ solution was poured into hexane, and the precipitated polymer was collected by filtration using filter paper. The polymers were purified by reprecipitation with methanol and hexane.

Characterization of P(3H2MB)

Nuclear magnetic resonance (NMR) analysis

The compositions and chemical structure of the PHAs in this study were determined by NMR (Biospin AVANCE

III 400A, Bruker, Billerica, MA, USA). The polymers were dissolved in CDCl₃, and the composition was determined from integration of the peaks in 400 MHz ¹H NMR spectra. The peak assignment was confirmed by HH-COSY. The isotacticity of the polymer was evaluated by 100 MHz ¹³C NMR.

Gel permeation chromatography

The molecular weights of the PHAs were determined using a 10A GPC system (Shimadzu, Kyoto, Japan). The polymers were dissolved in CHCl₃ and separated by two K-806M-joined columns (Shodex, Tokyo, Japan) at 40 °C.

Differential scanning calorimetry (DSC)

DSC was conducted using a Pyris 1 instrument (Perkin-Elmer, Waltham, MA, USA) equipped with a liquid nitrogen cooling system under a helium gas atmosphere. To determine the melting temperature (*T_m*), cold crystallization temperature (*T_{cc}*), *T_g*, and enthalpy of fusion (ΔH_m), the polymers were first heated to 220 °C at a rate of 20 °C/min, cooled steeply (at a rate of ~300 °C/min) to -50 °C and then heated again at a rate of 20 °C/min. *T_m*, *T_{cc}*, *T_g*, and ΔH_m were determined from the second heating scan of the DSC thermogram. To determine the crystallization temperature (*T_c*) from the cooling step, the cooling rate was set at 20 °C/min.

The crystallization kinetics were analyzed by isothermal crystallization analysis using DSC. The polymers were heated at 220 °C and then quench-cooled to the isothermal crystallization temperature (*T_{iso}*), the exothermal heat flow was measured, and the half-crystallization time (*t*_{1/2}), Avrami constant *k*, and Avrami index *n* were determined.

To evaluate the thermal stability of P(3H2MB), cyclic DSC was conducted. First, the polymer was heated to 220 °C at a heating rate of 20 °C/min and then cooled to 0 °C at a cooling rate of 20 °C/min. The polymer was then heated again to 220 °C at a heating rate of 20 °C/min. The polymer was melted and recrystallized repeatedly four times, and the changes in the melting point and molecular weight were measured. The measurements were performed under a helium atmosphere.

Polarized optical microscopy (POM)

The morphology and growth of the spherulites were observed with an optical microscope (BX51, Olympus, Tokyo, Japan) equipped with crossed polarizers and a Linkam hot stage. The solvent cast film was first heated to 220 °C at 80 °C/min on the hot stage and held at that temperature for 1 min. The sample was then quenched to *T_{iso}* at a rate of 80 °C/min. The sample was crystallized isothermally at a given *T_{iso}* to monitor the growth of spherulites as a function of time. Polarized optical microscopic photographs were taken every 5 s, and the

radial growth rate of the spherulites was calculated from the slope of the spherulite radius versus time plot.

The primary nucleation rates (I) of P(3HB) and P(3H2MB) were evaluated from the spherulite growth rates (G) and Avrami constants (k) using the equation below:

$$I = \frac{3k}{\pi G^3}$$

Here, k was obtained from isothermal crystallization analysis by DSC when the Avrami index (n) was ~ 4 and the crystallization proceeded homogeneously and three-dimensionally.

Mechanical properties

Stress-strain tests of the CHCl_3 solution cast films were performed at room temperature using a Shimadzu EZ test machine. P(3H2MB) and P(3HB) were dissolved in CHCl_3 and cast in a Petri dish. After evaporating the CHCl_3 , the cast films were conditioned for 3 weeks at ambient temperature until the secondary crystallization reached equilibrium. The sample was $\sim 100 \mu\text{m}$ thick and cut into dimensions of $5 \text{ mm} \times 20 \text{ mm}$. To fix the film and confirm a firm grip by the machine, paper and double-sided tape were inserted between the film and the grip surface. The gauge length was set to 10 mm. The strain rate was set at 10 mm/min; the data shown are the averages of at least three measurements.

Evaluation of the biodegradation of 3H2MB-rich polymer

The ability of microorganisms from the environment to hydrolyze the 3H2MB polymer was evaluated by analyzing the formation of a clear zone around the colonies on agar plates supplemented with a polymer suspension as the sole carbon source²¹. The assay plates contained medium with suspended polymer and 1.5 g/L agar. The medium with polymer contained 11.6 g/L $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 4.6 g/L KH_2PO_4 , 1.0 g/L NH_4Cl , 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g/L CaCl_2 , 0.06 g/L FeCl_3 , and 2.0 g/L polyester powder. For efficient and high-throughput screening of microorganisms, a low-molecular-weight 3H2MB-rich polymer was synthesized from the P(3H2MB-co-3HB) copolymer and used as the polyester powder. For this process, a copolymer (3H2MB $\sim 90 \text{ mol\%}$, $M_w = 1.2 \times 10^5$, $M_w/M_n = 2.4$) biosynthesized by recombinant *E. coli* LSBJ cells that were fed 1 g/L glucose, 2 g/L tiglic acid, and 0.02 g/L (2*E*)-but-2-enoic acid (crotonic acid) as a 3HB precursor (at 4 and 28 h of cultivation) was added to increase the copolymer yield by dissolution in CHCl_3 and reaction with 15% (v/v) sulfuric acid/methanol at 100°C for 1 h. The polymer was precipitated in methanol, and a fine powder of 3H2MB-rich polymer (3H2MB $\sim 92 \text{ mol\%}$, $M_n \sim 7000$) was obtained.

Environmental microbial consortia were obtained from soil in Nagatsuta, Yokohama, Japan. To selectively propagate

3H2MB-polymer-degrading bacteria, the microbial consortia were first cultivated in polymer-suspended liquid medium containing 3H2MB-rich polymer as the sole carbon source. The flasks containing the microbial consortia were shaken at 30°C for 2–7 days. The cultured microbial consortia were transferred to agar plates with suspended polymer. After incubation at 30°C for several days, some colonies that formed a clear zone were selected as 3H2MB-polymer-degrading strains.

Results

Biosynthesis of P(3H2MB) homopolymer

To produce a P(3H2MB) homopolymer, additional genetic modification of our original bacterial chassis was necessary. This required the use of the *E. coli* strain LSBJ [*fadR601*, *atoC*(Con), $\Delta fadB$, $\Delta fadJ$] as a chassis. Previous studies have demonstrated that *E. coli* LSBJ produces various types of PHA homopolymers and copolymers with defined repeating unit compositions^{16,22,23}. Additionally, to activate the monomer and polymerize the P(3H2MB) homopolymer, three additional enzymes were used, namely, PCT from *M. elsdenii*, which has been used successfully to activate lactyl-CoA for in vivo polymerization, and (*R*)-specific enoyl-CoA hydratase (PhaJ_{Ac}) and PHA synthase (PhaC_{Ac}) from *A. caviae*, which have been used to successfully produce PHA polymers (Fig. 1A)¹⁷. Tiglic acid was added as the precursor of the 3H2MB monomer. Tiglic acid is converted to tiglyl-CoA by PCT, hydrated to 3H2MB-CoA by PhaJ_{Ac} , and then polymerized to form P(3H2MB) by PhaC_{Ac} . Glucose was fed to support cell growth. By deleting the *fadB* and *fadJ* genes, it was possible to prevent the decomposition of tiglic acid during β -oxidation. The P(3H2MB) homopolymer was biosynthesized using an optimized substrate feeding protocol for this strain, and 0.22 g/L of P(3H2MB) homopolymer was obtained from 1.05 g/L dry cell weight. Approximately 4% of the supplemented tiglic acid was converted to P(3H2MB).

Characterization of P(3H2MB) films

To utilize PHA materials as substitutes for traditional petroleum-based plastics, it is necessary to define the physical properties and confirm the adaptability of the materials to industrial processing. Thus, we performed an in-depth characterization of these properties for P(3H2MB). The P(3H2MB) homopolymer was extracted from freeze-dried cells, and solvent cast films were prepared (Fig. 1B). The films were generally transparent, with slight opacity. The molecular weight of the P(3H2MB) homopolymer, as measured by GPC, was $M_n = 15.1 \times 10^4$ with $M_w/M_n = 2.77$ (Table 1).

Tensile tests showed that the P(3H2MB) cast films were highly ductile compared to P(3HB) cast films (Table 1). The elongation at break (520%) was significantly higher than that of P(3HB) (12%) and comparable to that of iPP

Table 1 Comparison of thermal and mechanical properties of polymers.

Polymer	M_n ($\times 10^4$)	M_w/M_n	T_g ($^{\circ}\text{C}$)	T_m ($^{\circ}\text{C}$)	T_m° ($^{\circ}\text{C}$) ^a	ΔH_m (J/g)	ΔH_m° (J/g) ^b	T_{d5} ($^{\circ}\text{C}$) ^c	$T_{d_{\max}}$ ($^{\circ}\text{C}$) ^c	Young's modulus (MPa)	Tensile strength (MPa)	Elongation at break (%)
P(3HB)	22.8	2.30	4	176	196	79	129	257	292	1420 $\pm$ 80	58 $\pm$ 7	12 $\pm$ 0
P(3H2MB)	15.1	2.77	15	197	220	47	88	278	304	583 $\pm$ 64	37 $\pm$ 2	520 $\pm$ 117
iPP	9.7	3.5	-10 ^d	166	208 ^d	71	148 ^d	—	—	1700 ^d	38 ^d	400 ^d
PLLA	10.9	1.7	59	174	207 ^e	41	93.7 ^e	—	—	2600 ^e	57 ^e	3 ^e

^aEquilibrium melting point determined by Hoffman–Weeks methods.^bEquilibrium fusion enthalpy determined for 100% crystallinity, calculated from DSC and XRD data.^c T_{d5} : temperature at 5% weight loss, $T_{d_{\max}}$: temperature at the highest weight loss rate during thermal decomposition, measured using TG-DTA.^dData obtained from refs. ^{24,25}.^eData obtained from refs. ^{26,40}.

(400%)^{24,25} (Fig. 1C). Although P(3H2MB) exhibited a considerably higher elongation to break than P(3HB) and PLLA, its Young's modulus was lower²⁶. The brittleness of bioplastics is regarded as one of the major obstacles to their practical use; thus, the high ductility of P(3H2MB) represents its significant advantage over currently available bioplastics.

Stereoregularity of P(3H2MB) biosynthesized from tiglic acid

NMR analysis was used to accurately characterize the molecular structure of the P(3H2MB) homopolymer (Fig. 1D and E). ¹³C NMR analysis indicated that the homopolymer was highly isotactic, consisting of a single enantiomer. The methyl ester of 3H2MB obtained by

methanolysis of the P(3H2MB) homopolymer (Supplementary Fig. 1) exhibited a negative specific optical rotation ($[\alpha]_{20}^D = -11.0^\circ$, $c = 1.0$ in CDCl_3). Among the four enantiomers of 3H2MB, the enantiomer that was polymerized by PHA synthase (3*R* enantiomer) and whose methyl ester has negative specific optical rotation corresponded to only (2*R*,3*R*)-3H2MB based on the results of a previous study²⁷. Therefore, the biosynthesized isotactic homopolymer was P[(2*R*,3*R*)-3H2MB], which is consistent with the stereochemistry of the monomer supplied by the enzyme Pha_{Ac}²⁸.

Melting behavior of P(3H2MB)

The thermal properties of the P(3H2MB) homopolymer were characterized by DSC. The melting temperature (T_m) of the P(3H2MB) homopolymer (197 °C) was higher than that of P(3HB) (176 °C) (Fig. 2A). According to Hoffman–Weeks analysis (Supplementary Fig. 2), the equilibrium melting temperature (T_m°) was determined to be 220 °C, ~24 °C higher than that of P(3HB) (196 °C)²⁹. The T_m° observed for P(3H2MB) is the highest among those of all the biosynthesized PHAs reported to date⁷. Even when P(3H2MB) was quenched at a cooling rate higher than 300 °C/min from the melt at 220 °C, crystallization was completed during quenching, which resulted in an undetectable cold crystallization temperature (T_{cc}) and an undetectable T_g in the second heating thermogram. This phenomenon indicates that P(3H2MB) crystallizes very rapidly compared to P(3HB) and other PHAs. To determine the T_g of P(3H2MB), the sample was quenched from the melt at 220 °C by immediate immersion in liquid nitrogen. The T_g of P(3H2MB) was observed at 15 °C (Supplementary Fig. 3), which is 11 °C higher than that of P(3HB) (Fig. 2A). In the case of PHAs, as the carbon number of the β -alkyl group increases, T_g and T_m decrease. However, when a methyl group was introduced on the α -carbon, the T_g and T_m anomalously increased. In a previous study^{14,15}, PHA containing 3H2MB as a comonomer exhibited a lower T_g than P(3HB) and was amorphous. These thermal properties may be derived from copolymerization with other monomers, such as 3HV or 3H2MV. The fusion enthalpy of P(3H2MB) observed in the heating scan was smaller ($\Delta H_m = 47 \text{ J/g}$) than that of the P(3HB) homopolymer ($\Delta H_m = 79 \text{ J/g}$). However, the degrees of crystallinity of P(3H2MB) and P(3HB) determined by X-ray diffraction (XRD) were 63% and 65%, respectively, which are comparable (Supplementary Fig. 4). The higher flexibility of P(3H2MB) compared to that of P(3HB), based on Young's modulus, may be derived from the lower fusion enthalpy of the former. In other words, fewer interactions between molecular chains in the crystal result in the greater flexibility of P(3H2MB). The difference between the T_m and the crystallization temperature (T_c) detected in the

cooling scan is defined as ΔT . Generally, ΔT is considered an important index for polymer processing, and a smaller ΔT indicates faster crystallization behavior³⁰. The ΔT values of the P(3HB) and P(3H2MB) homopolymers were measured and compared with that of iPP (Fig. 2B). For P(3H2MB), ΔT (54 °C) was small compared to those of P(3HB) (111 °C) and PLLA (76 °C) and was closer to that exhibited by commercially available iPP (48 °C).

Crystallization behavior of P(3H2MB)

The isothermal crystallization behavior of P(3H2MB) was investigated using DSC. The apparent exothermal transition corresponding to crystallization of P(3H2MB) could be detected in thermograms recorded at isothermal crystallization temperatures (T_{iso}) ranging from 140 to 170 °C. At T_{iso} values below 140 °C, crystallization started before the sample temperature reached T_{iso} . The shortest half-crystallization time ($t_{1/2}$) of P(3H2MB) might have been observed at ~100 °C, which lies midway between the T_g and T_m . The shortest $t_{1/2}$ of P(3HB) was observed at ~60 °C. The T_{iso} vs. $t_{1/2}$ curve of P(3H2MB) shifted by ~30–40 °C toward higher temperatures compared to that of P(3HB). Thus, the crystallization behaviors of P(3H2MB) at 140 °C and P(3HB) at 100 °C, including primary nucleation and spherulite growth, were compared. The shortest $t_{1/2}$ of P(3H2MB) was much shorter than the shortest $t_{1/2}$ for P(3HB) (Fig. 2C). Whether P(3H2MB) exhibits a shorter $t_{1/2}$ than iPP is unclear. However, P(3H2MB) exhibited a much shorter $t_{1/2}$ than PLLA.

To further understand the fast crystallization behavior of P(3H2MB), the growth of the P(3H2MB) crystals was monitored by POM (Fig. 2D). The P(3H2MB) and P(3HB) polymers that were melted at 220 °C for 1 min were quenched to T_{iso} , after which homogeneous and sporadic crystallization was observed. The spherulites appeared in the molten state. Because the spherulites of P(3H2MB) appeared at temperatures above 140 °C, crystallization of P(3H2MB) started before the sample temperature reached T_{iso} when T_{iso} was below 140 °C. In contrast, spherulites of P(3HB) did not appear before the sample temperature reached T_{iso} . The radial growth rate of spherulites (G) was determined from the temporal increase in the spherulite size at a given T_{iso} . The G value of P(3H2MB) was determined to be 0.91 $\mu\text{m/s}$ at $T_{iso} = 140^\circ\text{C}$, and the spherulites of P(3HB) grew at a rate of 3.4 $\mu\text{m/s}$ at $T_{iso} = 100^\circ\text{C}$. The spherulite morphology of P(3H2MB) was much finer than that of P(3HB), even in the 30 °C higher T_{iso} region (Fig. 2D).

Here, the primary nucleation rates (I) for P(3HB) and P(3H2MB) were calculated from POM observations and DSC measurements at the initial stage of crystallization, where crystallization proceeded homogeneously and three-dimensionally (Avrami index $n = 4$). The obtained

Fig. 2 Melting and crystallization behavior of P(3H2MB) homopolymer. **A** DSC thermograms of P(3HB) and P(3H2MB) during the first heating scan from 50 to 220 °C and the second heating scan from −50 to 220 °C after rapid quenching from the melt in the first heating scan. **B** DSC thermograms of polymers during successive heating and cooling scans recorded at 20 °C/min. The crystallization rates of these polymers were evaluated by the index ΔT ($=T_m - T_c$). **C** Half-crystallization times ($t_{1/2}$) of P(3H2MB), P(3HB), PLLA, and iPP at various isothermal crystallization temperatures (T_{iso}). **D** Polarized optical microscope images of P(3HB) and P(3H2MB) at different T_{iso} values.

I values for the two polymers are listed in Table 2, together with the spherulite growth rate (G) and Avrami constant (k). Consistent with the number of spherulites, I was much larger for P(3H2MB) than for P(3HB), despite the higher T_{iso} of P(3H2MB). The I value of P(3H2MB) at 140 °C was much higher than that of P(3HB) at 100 °C. Crystallization behavior is dependent on both primary nucleation and spherulite growth. The present results indicate that P(3H2MB) exhibited faster primary nucleation than P(3HB). Generally, increasing the number density of spherulites is preferred because various properties, such as the transparency, impact resistance, and flexibility of the polymer, tend to be improved in the presence of more spherulites. In many cases, a nucleating agent is applied to promote primary nucleation and improve these properties. However, the present results indicate that P(3H2MB) not only has superior thermal properties but also forms an exceptional spherulitic morphology without the addition of a nucleation agent.

Table 2 Kinetic parameters for polymer crystallization.

Polymer	T_{iso} (°C)	n (−)	k (min^{-n})	G ($\mu\text{m/s}$)	I (event/ mm^3/s)
P(3H2MB)	140	3.9	1.1×10^3	0.91	6.3×10^2
P(3H2MB)	150	4.1	1.2×10^2	0.76	5.4×10^2
P(3HB)	70	3.6	71	2.6	14
P(3HB)	100	3.8	6.7	3.4	3.1

T_{iso} isothermal crystallization temperature, n Avrami index, k Avrami constant, G spherulite growth rate, I primary nucleation rate.

Thermal stability of P(3H2MB)

One of the main drawbacks for the use of P(3HB) as a biodegradable alternative to common petroleum plastics is that the thermal decomposition temperature is close to its T_m , resulting in decomposition of the material during thermal processing. Despite the superior T_m and crystallization behavior of P(3H2MB), P(3H2MB) must be

thermally stable to be applicable. Therefore, the thermal decomposition of P(3H2MB) was investigated by thermal gravimetric-differential thermal analysis-mass spectrometry (TG-DTA-MS). The endothermal peaks and accompanying weight change revealed that P(3H2MB) degraded at a significantly higher temperature than P(3HB) (Table 1). The temperature range during which the largest loss of mass occurred was 292 °C for P(3HB), which was associated with the formation of a molecular degradation product with a parent molecular mass (m/z) of 86 (Supplementary Fig. 5). P(3H2MB) generated a molecular degradation product with a parent molecular mass (m/z) of 100 at 310 °C (Supplementary Fig. 6). Based on the mass spectra, P(3H2MB) and P(3HB) generated 2-methylbut-2-enoic acid (tiglic acid) and 2-butenic acid (crotonic acid), respectively, as the main thermal degradation products. This result indicates that P(3H2MB) degrades via the *cis*-elimination process, similar to the proposed pathway of elimination for P(3HB) (Fig. 3A)³¹. For both P(3HB) and P(3H2MB), no solid residue was detected after thermal degradation at 500 °C. The higher T_{d_max} observed for P(3H2MB) may be attributed to the presence of the α -methyl group, which interferes with α -deprotonation (Fig. 3B). In the *cis*-elimination process,

the reaction proceeds via a planar six-membered cyclic transition state³². In this transition state, the α -methyl group and the β -methyl group are eclipsed, resulting in an energy barrier that is higher than that for P(3HB). Moreover, reducing the number of α -protons may also restrict *cis*-elimination. According to this model, P[(2*R*, 3*R*)-3H2MB] is expected to generate (*E*)-2-methylbut-2-enoic acid (tiglic acid), and P[(2*S*, 3*R*)-3H2MB] is expected to generate (*Z*)-2-methylbut-2-enoic acid (angelic acid). The generation of tiglic acid by thermal degradation of P(3H2MB) means that the feedstock of P(3H2MB) can be recovered from P(3H2MB), which may facilitate chemical recycling of P(3H2MB)³³. In typical industrial manufacturing processes, plastics are melted and recrystallized repeatedly for molding into the final products (by pelletizing, compounding, molding, and recycling processes). Such repetitive processes can degrade the polymer, resulting in a reduction in the molecular weight and loss of thermal stability. Therefore, it is important to understand how the resilience of these materials is subject to changes in the thermal conditions to identify adequate substitutes for petroleum-based plastics. The changes in the T_m and molecular weight of P(3H2MB) during the cyclic melting and recrystallization processes were

Fig. 4 Biodegradation of 3H2MB-rich polymer. **A** Biodegradation test by the clear zone method in mineral salt medium agar plates containing low-molecular-weight 3H2MB-rich polymer [P(92 mol% 3H2MB-co-3HB), $M_n \sim 7000$] using microorganisms isolated from on-campus soil (Yokohama City). **B** The microorganisms indicated by the arrow in panel (**A**) were spread on a new plate.

evaluated by DSC and GPC. P(3H2MB) displayed superior thermal resilience with a nearly unchanged T_m during four repeat cycles of thermal processing (Fig. 3C). For P(3H2MB), the rate of reduction in the molecular weight over time also decreased, demonstrating higher stability for thermal processing (Fig. 3D). Generally, the thermal degradation process proceeds in the molten state. Thus, thermal degradation could be prevented by faster crystallization and a shorter melting time. A smaller difference between the melting point and crystallization temperature (smaller ΔT) results in less persistence of the molten state. A smaller crystallization enthalpy is also advantageous for molding in the cooling process. These results indicate that the physical properties of P(3H2MB) can alleviate the main drawbacks (e.g., slow crystallization behavior and thermal instability) that have prevented widespread adoption of PHAs as biodegradable thermoplastic substitutes for conventional petroleum-based plastics.

Origin of fast crystallization behavior

According to thermal dynamic theory, the equation below describes the relationship between the melting temperature and fusion enthalpy in general.

$$T_m^0 = \Delta H_m^0 / \Delta S_m^0$$

Here, T_m^0 , ΔH_m^0 , and ΔS_m^0 represent the equilibrium melting point, equilibrium fusion enthalpy, and equilibrium fusion entropy, respectively. The melting temperature of P(3H2MB) was significantly higher than those of the other PHAs. On the other hand, the fusion enthalpy of P(3H2MB) was relatively small compared to that of P(3HB). Therefore, it is expected that P(3H2MB) has a smaller fusion entropy than P(3HB).

The most common crystalline structure of P(3HB) is an α -form with a 2_1 -helix conformation (Supplementary Fig. 7)^{34,35}. According to quantum chemical calculations, the conformation of the repeating unit in the P(3HB) α -form crystal (conformation E) is stable for the 5th position among the calculated conformation models, and the conformation that forms the 3_1 -helix (conformation A) is the most stable (Supplementary Table 1, Supplementary Fig. 8), which is consistent with results of previous studies^{34,36}. However, for P(3H2MB) with the α -methyl group in the *R*-configuration, the most stable conformation was identical to that of the P(3HB) α -form crystal (conformation E, Supplementary Table 1, Supplementary Fig. 8). Thermal dynamic theory calculations suggested that the P(3H2MB) chains preferentially adopt the 2_1 -helix-like conformation, even in the molten state, and that the melting/crystallization transition is accompanied by a small conformational change. Consequently, P(3H2MB) may exhibit small entropic changes during melting and rapid formation of crystalline structures during cooling.

Biodegradability of 3H2MB-rich polymer

Because the aim is to address the issues associated with microplastics generated from traditional plastic materials, the alternative materials must biodegrade in the environment. PHA copolymers consisting of 3HB and α -methylated repeating units such as 3H2MB or 3H2MV can be produced by naturally occurring bacteria found in the activated sludge of sewage plants, and these materials are biodegradable¹¹. However, whether PHA with a high ratio of 3H2MB repeating units could be biodegraded by bacteria in the environment is unknown, and there is uncertainty as to whether this could be accomplished because of the stereo-configuration of the 3H2MB repeating unit. To verify the biodegradability of the 3H2MB-rich polymer, we

assessed the microbial degradation of the material using a clear zone agar-plate assay with various microbial environmental samples^{37,38}. For efficient and high-throughput screening of the microorganisms, a low-molecular-weight 3H2MB-rich polymer (92 mol% 3H2MB) was synthesized from the P(3H2MB-co-3HB) copolymer and used as a polyester powder. The test revealed that some fungi and bacteria were capable of degrading the 3H2MB-rich polymer (Fig. 4). This result indicates that there are microorganisms that can degrade the 3H2MB-3H2MB linkage or at least the 3H2MB-3HB linkage. The isolated microorganisms also degraded the P(3HB) polymer. The identification of these microorganisms is currently in progress. The use of these microbes that can degrade P[(2R,3R)-3H2MB] in the environment at ambient temperature provides advantages over the use of iPP and PLLA.

Discussion

Currently, iPP is widely used for various products, such as commodity, packaging, fiber, and automotive products. The prevalence of iPP and other nonbiodegradable plastics in these products exacerbates the global problems posed by microplastics. A solution to these problems is to develop biodegradable plastics that can adequately substitute for iPP and nonbiodegradable plastics. For biodegradable plastics to be used in the same manner as iPP, thermal stability above the T_m and good crystallization behavior must be achieved. As demonstrated in this study, P(3H2MB) had the highest T_m among the biosynthesized PHAs and was less susceptible to thermal decomposition and exhibited a faster crystallization behavior than P(3HB) and PLLA. Furthermore, P(3H2MB) should be biodegradable to some extent in nature. In this study, P(3H2MB) was biosynthesized using tiglic acid as a precursor; tiglyl-CoA can also be provided intracellularly because it is an intermediate of the isoleucine degradation pathway³⁹, indicating that further metabolic engineering may improve the production of this compound from cheap biobased feedstocks. Tiglic acid can also be biosynthesized via the Claisen condensation pathway by thiolase and dehydration by dehydratase. Moreover, depending on the stereoselectivity of β -ketoacyl-CoA reductase, P[(2R, 3R)-3H2MB] could be biosynthesized de novo without going through tiglic acid. In summary, these characteristics point to P(3H2MB) as the most promising candidate for high-performance biodegradable plastics from a renewable source that can replace nonbiodegradable plastics. Further development of P(3H2MB) will contribute to creating sustainable products and lessen the impact of microplastics on the global environment.

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Conflict of interest

The authors declare no competing interests.

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