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Biosynthesis of Poly(3-Hydroxybutyrate-*co*-3-Hydroxyhexanoate) with a High 3-Hydroxyhexanoate Fraction and Low Molecular Weight for Polymer Blending

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Abstract. Polyhydroxyalkanoates (PHAs) are aliphatic polyesters that are biosynthesized and accumulate in bacterial cells. Among PHAs, poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) s [P(3HB-*co*-3HHx)s] are known to be practical PHA copolymers with an appropriate degree of flexibility. In the biosynthesis of PHA, ethanol induces chain transfer (CT) reaction, resulting in the biosynthesis of low-molecular-weight PHA. In this study, P(3HB-*co*-3HHx)s were biosynthesized using recombinant *Escherichia coli* by feeding fatty acid(s) and ethanol as the 3HHx precursor and CT agent, respectively, to obtain polymers with high 3HHx fractions and low molecular weights. Two biosynthesized P(3HB-*co*-3HHx)s, whose 3HHx fractions were 49 and 86 mol% and weight average molecular weights were 1.5×10^4 and 2.8×10^4, respectively, were blended with P(3HB-*co*-5 mol% 3HHx) (PHBH5) as the base material. The blending properties were investigated to explore how the thermal properties can be modified. Thermal analysis by differential scanning calorimetry (DSC) showed that blends prepared by solution casting were obviously immiscible when 30 wt.% or more of biosynthesized polymer was added, even if its molecular weight was low. In the blended polymers, a lower crystallization effect compared to neat PHBH5 was observed without changing the melting temperature. However, crystallization was promoted in the blended material under specific blending conditions. Thus, blending is a simple method for producing polymer materials with altered thermal and microstructural properties.

Keywords: recombinant *E. coli*, poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate), low molecular weight, polymer blending, polyhydroxyalkanoate, miscibility, crystallization.

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Биосинтез поли(3-гидроксибутирата-со-3-гидроксигексаноата) с высоким содержанием 3-гидроксигексаноата и низкой молекулярной массой для смешения полимеров

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Аннотация. Полигидроксиалканоаты (ПГА) представляют собой алифатические полиэфиры, которые синтезируются и накапливаются в бактериальных клетках. Среди множества ПГА большой интерес представляет сополимер поли(3-гидроксибутират-со-3-гидроксигексаноат) [П(3ГБ-со-3ГГ)], характеризующийся высокой эластичностью. В реакциях биосинтеза ПГА этанол индуцирует реакцию переноса цепи, в результате чего происходит биосинтез низкомолекулярных ПГА. В этом исследовании для получения низкомолекулярного сополимера П(3ГБ-со-3ГГ) с высоким содержанием мономеров 3ГГ рекомбинантный штамм *Escherichia coli* культивировали с подачей в среду жирных кислот и этанола в качестве предшественников мономеров 3ГГ и агента переноса цепи соответственно. Получены два образца П(3ГБ-со-3ГГ) с содержанием мономеров 3ГГ 49 и 86 мол.% и средневесовой молекулярной массой $1{,}5 \times 10^4$ и $2{,}8 \times 10^4$ соответственно. Были исследованы термические свойства смесей полученных сополимеров с П(3ГБ-со-5 мол.% 3ГГ) (PHBH5). Термический анализ с помощью дифференциальной сканирующей калориметрии показал, что смеси, приготовленные литьем из раствора, очевидно, были несмешиваемыми при добавлении биосинтезированного полимера 30 мас.% или более, даже при его низкой молекулярной массе. В смешанных полимерах наблюдался более низкий эффект кристаллизации по сравнению с чистым PHBH5 без изменения температуры плавления. Однако некоторые условия смешивания способствовали ускорению кристаллизации смешанного материала. Таким образом, смешивание представляет собой простой метод получения полимерных материалов с измененными термическими и микроструктурными свойствами.

Ключевые слова: рекомбинантная *E. coli*, поли (3-гидроксибутират-со-3-гидроксигексаноат), низкая молекулярная масса, смешение полимеров, полигидроксиалканоат, смешиваемость, кристаллизация.

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Introduction

The use of petroleum-based polymer materials in daily life has led to various environmental problems. Hence, the development of eco-friendly materials has become a topic of major interest. Polyhydroxyalkanoates (PHAs) are a family of polyesters produced by many prokaryotes as energy storage molecules under conditions of stress or unbalanced nutrient supply (Tsuge, 2002; Tsuge et al., 2015). These microbial polyesters have attracted global interest because they are completely biodegradable and environmentally benign and have thermal properties comparable to those of fossil-based plastics. Among the various PHAs, poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] has numerous potential applications, particularly in nanomedicine (Kılıçay et al., 2011) and tissue engineering (Qu et al., 2006; Zhang et al., 2018). Since P(3HB-*co*-3HHx) contains 3-hydroxyhexanoate (3HHx), a medium-chain monomer, the copolymer exhibits flexibility unlike highly crystalline poly(3-hydroxybutyrate) [P(3HB)] and poly(3HB-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] (Doi et al., 1995; Volova et al., 2021). However, the 3HHx fraction in currently available P(3HB-*co*-3HHx) is insufficient, which limits its potential applications. Therefore, to expand the applications of P(3HB-*co*-3HHx), further research is required on diversifying the 3HHx fraction and altering its microchemical structure (Xu et al., 2018).

The properties of PHA have been improved by copolymerization (Cespedes et al., 2018), increasing the molecular weight (Kahar et al., 2005), chain chemical modification (Levine et al., 2016), polymer processing (Iwata et al., 2004), and blending (Basnett et al., 2013). Among these, blending, which involves mixing two kinds of polymers with different microstructures, is the simplest way to produce polymer materials with new properties. The blend performance can be easily modified by adjusting the mixing ratio and mixing state. There are many reports on the blending of PHAs with other bioplastics (Luo et al., 2009; Martelli et al., 2012; Zembouai et al., 2014; Botta et al., 2015). These polymers can be processed by solution casting or melt pressing (Nerkar et al., 2014; Zembouai et al., 2014), thereby enabling the modification of physical properties at low cost. P(3HB-*co*-3HHx) blends have been studied extensively in recent years owing to their ability to reduce crystallinity (Cai et al., 2012).

Escherichia coli is a bacterium that does not biosynthesize PHA naturally; however, expressing PHA biosynthetic genes from other bacteria confers PHA-producing-ability to *E. coli*.

Generally, PHA synthesized by recombinant *E. coli* has a higher molecular weight than that synthesized by natural PHA producers. This is probably because, unlike natural bacteria, *E. coli* does not have PHA depolymerase. On the other hand, the molecular weight of biosynthesized PHA can be lowered by using the chain transfer (CT) reaction, in which PHA synthase transfers the elongating polymer chain to an alcoholic compound that acts as a CT agent (Hiroe et al., 2013; Tsuge, 2016). Therefore, the higher the frequency of CT reactions, the lower the molecular weight of the biosynthesized PHA. The frequency of CT reactions can be controlled by adjusting the concentration of alcoholic compounds added to the culture medium. Low-molecular-weight polymers can be effectively utilized in polymer blends because they exhibit relatively high miscibility with other polymers (Blumm, Owen, 1995; Ohkoshi et al., 2000).

In this paper, we report the biosynthesis of P(3HB-*co*-3HHx) with a high 3HHx fraction and low molecular weight using recombinant *E. coli* by feeding fatty acid(s) and ethanol as the 3HHx precursor and CT agent, respectively. This study aimed to investigate how the thermal properties can be modified by blending biosynthesized P(3HB-*co*-3HHx)s using P(3HB-*co*-5 mol% 3HHx) (PHBH5) as the base material.

Materials and methods

Materials

Commercial grade PHBH5 was provided by Kaneka Corporation (Osaka, Japan), while P(3HB-*co*-3HHx) copolymers with higher 3HHx fractions and lower molecular weights were synthesized using recombinant *E. coli* strains.

Biosynthesis of P(3HB-co-3HHx) using recombinant E. coli strains

Recombinant *E. coli* strains were cultivated in 100 mL M9 medium (Sambrook, Russell, 2011) on a reciprocal shaker (130 strokes min^{-1}) in a 500-mL flask at 37 °C for 72 h. When required, kanamycin (50 mg/L) was added to the medium.

E. coli strain LS5218 [*fadR601*, *atoC512*(Const)] (Spratt et al., 1981) harboring the plasmid pBBR1*phaPCJ*$_{Ac}$*AB*$_{Re}$ (Ushimaru et al., 2015), which carried the genes *phaP*$_{Ac}$(D4N) (*Aeromonas caviae* phasin gene with D4N mutation), *phaC*$_{Ac}$(NSDG) (*A. caviae* PHA synthase gene with N149S and D171G mutations), *phaJ*$_{Ac}$ (*A. caviae R*-specific enoyl-CoA hydratase gene), *phaA*$_{Re}$ (*Ralstonia eutropha* 3-ketothiolase gene), and *phaB*$_{Re}$ (*R. eutropha* acetoacetyl-CoA reductase gene) (Fig. 1), was cultured in M9 medium supplemented with dodecanoic acid (2.5 g/L) and 0.4 vol.% Brij35. Ethanol (5 g/L) was also added as a CT agent to produce a low-molecular-weight polymer. The sample obtained under these conditions is referred to as sPHBH49.

The *E. coli* strain LSBJ [*fadB:: Cm*, ΔfadJ, *atoC512* (Const), *fadR601*] (Tappel et al., 2012; Mohd Fadzil et al., 2018) harboring pBBR1*phaPCJ*$_{Ac}$*AB*$_{Re}$ (Ushimaru et al., 2015) was cultured in M9 medium supplemented with yeast extract (2.5 g/L) and ethanol (10 g/L). Glucose (2.5 g/L) and two fatty acids, namely hexanoic acid (0.4 g/L) and butyric acid (0.1 g/L), were fed to the medium three times every 24 h (0, 24, and 48 h after cultivation was started). The sample obtained under these conditions is referred to as sPHBH86.

Polymer extraction

The bacterial cultures were harvested by centrifugation. The harvested cells were washed with hexane followed by distilled water twice. The cell pellets were then lyophilized for 72 h. Subsequently, the dried cells were placed in chloroform to extract intracellular PHA from the cells. After filtration, the PHA polymers were recovered from the filtrate by precipitation with hexane, and dried in vacuum dryer for 3 days to ensure complete solvent removal.

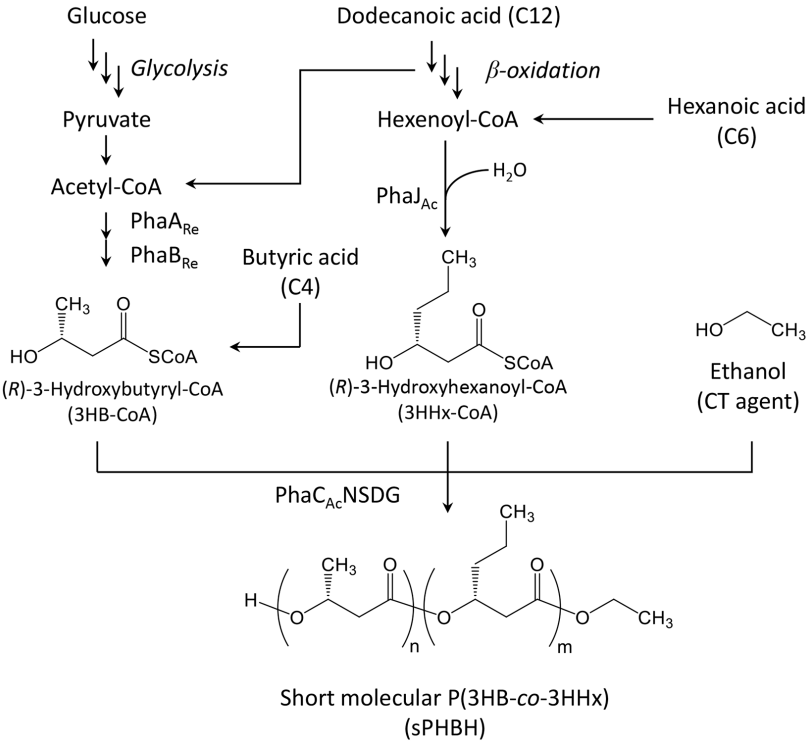

Fig. 1. The main biosynthetic pathway constructed in recombinant *E. coli* and enzymes involved in the synthesis of ethanol-capped P(3HB-*co*-3HHx). PhaA$_{Re}$, *Ralstonia eutropha* 3-ketothiolase; PhaB$_{Re}$, *R. eutropha* acetoacetyl-CoA reductase; PhaC$_{Ac}$NSDG, N149S and D171G (NSDG)-mutated *Aeromonas caviae* PHA synthase; PhaJ$_{Ac}$, *A. caviae* (*R*)-specific enoyl-CoA hydratase

Preparation of blended materials

Blended samples were prepared by mixing the base polymer PHBH5 with 10, 20, 30, and 50 wt.% of biosynthesized P(3HB-*co*-3HHx) samples in chloroform solution for 3 days. The blended films were obtained by evaporating the solvent at room temperature for 3 days. Then, the materials were dried in a vacuum dryer for 1 day to completely evaporate the remaining solvent.

Gas chromatography (GC) analysis

The content of biosynthesized P(3HB-*co*-3HHx) in the cells was determined using a Shimadzu 2014S GC system with a flame ionization detector. Intracellular PHA in the freeze-dried cells was subjected to methanolysis by adding 15 vol.% sulfuric acid in methanol prior to analysis, as described previously (Huang et al., 2018).

Nuclear magnetic resonance (NMR) analysis

Monomer fraction estimation and structural analysis of the P(3HB-*co*-3HHx) copolyesters were carried out using ^{1}H-NMR and ^{13}C-NMR, respectively. Samples (50 mg) were dissolved in chloroform-*d*, and tetramethylsilane (TMS) was used as an internal reference. NMR spectra were recorded using a Bruker Biospin AVANCE III spectrometer (Furutate et al., 2021).

Gel permeation chromatography (GPC) analysis

The molecular weights of P(3HB-*co*-3HHx) samples were characterized using a Shimadzu 10A GPC system with a 10A refractive index detector

equipped with two Shodex K-806M joint columns. Chloroform was used as the eluent at a flow rate of 0.8 mL/min, and calibration was performed using polystyrene standards with low polydispersity.

Differential scanning calorimetry (DSC) analysis

Thermal analysis of the P(3HB-*co*-3HHx) and blended samples was carried out using a Perkin Elmer Pyris 1 system under nitrogen flow. The samples (6–10 mg) were encapsulated in an aluminum pan and heated to 200 °C at 20 °C/min, quenched to –50 °C, and heated again at 20 °C/min. The glass transition temperature (T_g) was determined as the midpoint of the heat capacity change in the second heating scan of the DSC thermogram (Furutate et al., 2021).

Results

Biosynthesis of P(3HB-co-3HHx)s with ethanol feeding

Previously, we demonstrated the biosynthesis of P(3HB-*co*-3HHx) with 40 mol% 3HHx from dodecanoic acid (C12 fatty acid) using recombinant *E. coli* LS5218 harboring pBBR1*phaPCJ*$_{Ac}$*AB*$_{Re}$ (Table 1) (Ushimaru et al., 2015). This polymer had a weight average molecular weight (M_w) and polydispersity of 1.8×10^6 and 4.8, respectively. In this study, to lower the molecular weight of biosynthesized P(3HB-*co*-3HHx), the culture medium was supplemented with ethanol (5 g/L) to induce CT reaction. As a result, the dry cell weight and PHA content reached 1.1±0.1 g/L and 58±1 wt.%, respectively after 72 h of cultivation (Table 1), and P(3HB-*co*-3HHx) with a 3HHx fraction of 49 mol% and a M_w of 1.5×10^4 (Table 2) was obtained. The M_w of the polymer obtained using an ethanol-supplemented culture was two orders of magnitude lower than that of the polymer obtained using a non-ethanol-supplemented culture (Ushimaru et al., 2015). In addition, the M_w was one order of magnitude lower than that of the PHBH5 base material (Table 2). This low-molecular-weight P(3HB-*co*-49 mol% 3HHx) is referred to as sPHBH49.

To further increase the 3HHx fraction, instead of using dodecanoic acid, the culture medium was supplemented with hexanoic acid, a C6 fatty acid, as a direct precursor for 3HHx. A small amount of butyric acid, a C4 fatty acid, was also added to allow the incorporation of small amounts of 3HB into the polymer (Fig. 1). Additionally, ethanol (10 g/L) was added to the culture medium to lower the molecular weight of the biosynthesized PHA. After 72 h of cultivation, the dry cell weight and PHA content reached 1.7±0.1 g/L and 25±1 wt.%, respectively (Table 1). The obtained P(3HB-*co*-3HHx) exhibited a high 3HHx fraction (86 mol%) and relatively low molecular weight (M_w, 2.8×10^4) (Table 2); it is referred to as sPHBH86.

Characterization of P(3HB-co-3HHx)s by NMR

From the ^{1}H- and ^{13}C-NMR spectra, the molecular structures of the P(3HB-*co*-3HHx) samples were investigated. From the ^{1}H-NMR spectra, the copolymer compositions were determined as listed in Tables 1 and 2. As in the ^{13}C-NMR spectra, the presence of the 3HHx comonomer caused a carbonyl peak to split into multiplets. The carbonyl resonances around 168–170 ppm were identified as belonging to 3HB*-3HB, 3HB*-3HHx and 3HHx*-3HB, and 3HHx*-3HHx, which are structures possibly existing in the copolymer chain (Abe et al., 1994). The randomness of the copolymer samples, described by parameter *D*, was calculated as follows (Kamiya et al., 1989):

$$D = (F_{3HHx\text{-}3HHx}F_{3HB\text{-}3HB})/(F_{3HHx\text{-}3HB}F_{3HB\text{-}3HHx}) \quad (1)$$

where $F_{3HHx\text{-}3HHx}$, $F_{3HB\text{-}3HB}$, $F_{3HHx\text{-}3HB}$, and $F_{3HB\text{-}3HHx}$ represent the fractions of 3HHx-3HHx, 3HB-3HB,

3HHx-3HB, and 3HB-3HHx diads, respectively. The diad sequence distributions derived from the carbonyl carbon were used to calculate *D* values, shown in Table 3.

Altogether, the observed values were consistently comparable with the calculated Bernoullian statistics applicable to statistically random copolymerization. Based on the observed *D* values, it is suggested that PHBH5 is a slightly blocky copolymer, while sPHBH49 and sPHBH86 resemble random copolymers.

Blends of sPHBH49 and the base material

Using sPHBH49, polymer blending was conducted with PHBH5 as the base material. Figure 2A shows the DSC results of the PHBH5 blends and the neat sPHBH49 sample. Neat sPHBH49 showed a weak T_g peak at –12 °C, which is in good agreement with the reported T_g (–9 °C) for the closest 3HHx fraction (53 mol%) copolymer (Feng et al., 2003). On the other hand, sPHBH49 did not show a T_m peak, suggesting that it is an amorphous polymer. As for the PHBH5 blends, the T_m peaks were observed at 142–144 °C and were almost unchanged regardless of the blending ratio. Meanwhile, the enthalpy of fusion (area of the melting peak) tended to decrease as the sPHBH49 content increased. Interestingly, the cold crystallization temperature (T_{cc}) of PHBH5 in the second heating scan decreased from 63 °C to 56 °C with the addition of 10 wt.% sPHBH49, and the crystallization peak became sharper. This implies that crystallization of the blended sample became easier and faster,

Table 1. PHA biosynthesis by recombinant *E. coli*

E. coli strain	Fatty acid added	Ethanol (g/L)	Dry cell wt. (g/L)	PHA content (wt.%)	PHA composition (mol%)		Reference
					3HB	3HHx	
LS5218	Dodecanoic acid (C12)	-	1.2±0.1	62±4	60	40	Ushimaru et al., 2015
LS5218	Dodecanoic acid (C12)	5	1.1±0.1	58±1	51	49	This study
LSBJ	Hexanoic acid (C6), butyric acid (C4)	10	1.7±0.1	25±1	14	86	This study

Cells were cultured in 100 mL M9 medium containing fatty acid(s) at 37 °C for 72 h. For the culture of LSBJ strain, glucose was supplemented to support cell growth. The PHA content and composition were determined by GC and ^{1}H-NMR, respectively, in this study. 3HB, 3-hydroxyburyrate; 3HHx, 3-hydroxyhexanoate. Results are expressed as mean ± standard deviation (n = 3).

Table 2. Thermal properties and molecular weights of PHA samples used in this study

Polymer sample	Sample name	Thermal property [a]		Molecular weight		Source
		T_g (°C)	T_m (°C)	M_w (×10^4)	M_w/M_n	
P(3HB-*co*-5 mol% 3HHx)	PHBH5	–3.3	143	51	2.4	Kaneka Co., Osaka, Japan
P(3HB-*co*-49 mol% 3HHx)	sPHBH49	–12	-[b]	1.5	1.5	This study
P(3HB-*co*-86 mol% 3HHx)	sPHBH86	–13	-[b]	2.8	1.7	

T_g, glass transition temperature; T_m, melting temperature; M_w, weight average molecular weight; M_n, number average molecular weight.
[a]Determined from DSC second heating scan.
[b] Not detectable.

Table 3. Diad sequence distributions and *D* values of P(3HB-*co*-3HHx) samples

Sample name	PHA composition (mol%)		Diad sequence distribution				*D* value
	3HB	3HHx		3HB*-3HB	3HB*-3HHx + 3HHx*-3HB	3HHx*-3HHx	
PHBH5	95	5	(observed)	0.89	0.10	<0.01	3.56
			(calculated)	0.90	0.10	<0.01	-
sPHBH49	51	49	(observed)	0.32	0.49	0.19	1.01
			(calculated)	0.26	0.50	0.24	-
sPHBH86	14	86	(observed)	0.25	0.45	0.30	1.53
			(calculated)	0.02	0.24	0.74	-

PHA compositions were determined by ^{1}H-NMR. 3HB, 3-hydroxyburyrate; 3HHx, 3-hydroxyhexanoate. The observed relative intensities were determined from the relative peak areas of the carbonyl carbon resonances in the ^{13}C-NMR spectra. The calculated values were obtained using Bernoullian statistics.

probably because of the promotion of primary nucleation. When more sPHBH49 was added, T_{cc} shifted to the higher temperature side and the crystallization peak became broader, implying that crystallization became more difficult. With the addition of up to 30 wt.% sPHBH49, a single T_g was observed in the –2 to –4 °C range. These blends appeared to be miscible in the amorphous phase. However, when more sPHBH49 was added, two T_g derived from each polymer were detected. Thus, the blended state was partially immiscible.

Blends of sPHBH86 and the base material

Using sPHBH86, polymer blending was also conducted with PHBH5 as the base material. Figure 2B shows the second scan DSC thermograms of the PHBH5 blends and the neat sPHBH86 sample. Neat sPHBH86 showed a weak T_g peak at –13 °C but did not show a T_m peak, suggesting that it is an amorphous polymer. For the blends with 30 wt.% or more sPHBH86, two distinct T_g peaks appeared. Thus, it is apparent that PHBH5 and sPHBH86 are immiscible. Despite this, crystallization occurred in all blended samples regardless of the blend ratio without changing T_m. The crystallization of PHBH5 occurred even after blending with amorphous PHBH86, but crystallization in the presence of sPHBH86 was harder than that in the presence of sPHBH49.

Discussion

For the P(3HB-*co*-3HHx) blends, the two polymers are reported to be miscible in the amorphous phase when the difference in the 3HHx fraction between these polymers is less than 20 mol%, but are immiscible when the difference is larger than 30 mol% (Feng et al., 2003). On the other hand, as mentioned earlier, low-molecular-weight polymers are known to exhibit relatively high miscibility with other polymers (Blumm, Owen, 1995; Ohkoshi et al., 2000). Therefore, to modify the material properties with a small amount of additive, P(3HB-*co*-3HHx) with a low molecular weight and a high 3HHx fraction may be effective for polymer blending. This study investigated how the thermal properties can be changed by blending polymers with a low molecular weight and high 3HHx fraction P(3HB-*co*-3HHx), which were biosynthesized in this study.

The crystallization behavior of the P(3HB-*co*-3HHx) blends was evaluated using DSC. The shift and unification of T_g is an

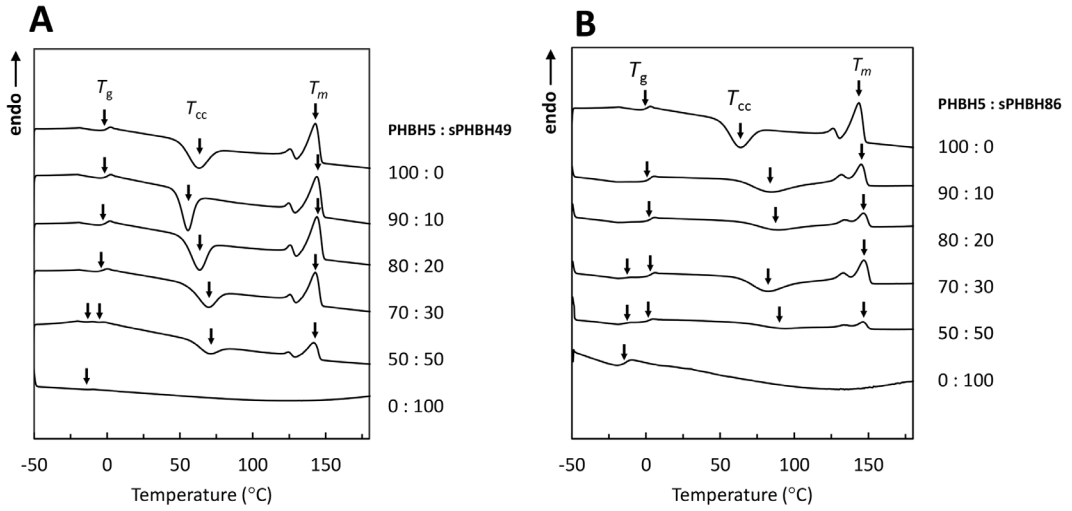

Fig. 2. DSC thermograms (second heating scan) of (A) PHBH5, sPHBH49, and its blends and (B) PHBH5, sPHBH86, and its blends. The samples were heated to 200 °C at 20 °C/min, quenched to –50 °C, and heated again at 20 °C/min during the second scan

important indicator for understanding whether the blended polymers are completely miscible with each other in the amorphous phase. When 30 wt.% or more of sPHBH49/86 was added to PHBH5, two distinct T_g peaks appeared in the second scan thermograms (Fig. 2). These blends were immiscible because of the large gap in their copolymer compositions, as previously demonstrated by Feng et al. (2003), even though one of them is a low-molecular-weight polymer. Initially, we expected miscibility between PHBH5 and the low-molecular-weight polymers; however, poor miscibility was observed with P(3HB-*co*-3HHx) biosynthesized using the CT reaction.

DSC analysis revealed that crystallization occurred on the lower temperature side in the 10 wt.% sPHBH49 blends. This implies that crystallization of the blended sample was promoted, probably because of easier primary nucleation. Low-molecular-weight polymers tend to form crystal nuclei, and it has also been reported that low-molecular-weight P(3HB) has a nucleating effect (Dong et al., 2010; Tsuge et al., 2017). However, it is surprising that even a non-crystalline polymer like sPHBH49 showed a nucleating effect in the PHBH5 blend. Further research is required to elucidate the mechanism of crystallization.

Conclusions

This study demonstrated that the crystallinity of blends of P(3HB-*co*-3HHx) could be tuned without changing the melting temperature. Additionally, the crystallization of the blended samples became easier and faster with the addition of 10 wt.% sPHBH49. Such a simple blending method provides an alternative way to produce materials with better performance.

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