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Biosynthesis and Characterization of Polyhydroxyalkanoate Copolyesters in *Ralstonia eutropha* PHB^{-}4 Harboring a Low-Substrate-Specificity PHA Synthase PhaC2$_{Ps}$ from *Pseudomonas stutzeri* 1317*

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Abstract A series of polyhydroxyalkanoate (PHA) copolymers consisting of short-chain-length (SCL) and medium-chain-length (MCL) 3-hydroxyalkanoate (3HA) monomers were synthesized in the recombinant *Ralstonia eutropha* PHB^{-}4 harboring a low-substrate-specificity PHA synthase PhaC2$_{Ps}$ from *Pseudomonas stutzeri* 1317. These polyesters, whose monomer compositions varied widely in chain length, were purified and characterized by acetone fractionation, nuclear magnetic resonance (NMR), gel-permeation chromatography (GPC), and differential scanning calorimetry (DSC). This was the first time that the physical properties of PHA copolymers polymerized by PhaC2$_{Ps}$ were characterized. The results indicated that the variation in MCL 3HA contents did not have an obvious influence on the molecular weights of these PHA copolymers but was effective in changing their physical properties. The variation in the thermal property of PHA copolymers with 3-hydroxyoctanoate (3HO) content was also investigated in this study.
Keywords polyhydroxybutyrate, PHA synthase, physical property, *Pseudomonas stutzeri*

1 INTRODUCTION

Polyhydroxyalkanoates (PHA) are produced by various bacteria as intracellular carbon and energy storage materials under nutrient-limitation conditions in the presence of excess carbon source[1]. They have received increasing attention from the scientific and industrial communities as biodegradable polyesters that can be used to replace the conventional petrochemical-based plastics[2—5]. Generally, PHA are classified into three groups based on the number of carbon atoms in the monomer units incorporated in 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.

The monomer composition of PHA has considerable effects on its physical properties[3,5,8]. As a typical SCL PHA, polyhydroxybutyrate (PHB) is a stiff crystalline material that has a high melting temperature. PHB is too brittle to be processed; therefore, its industrial applications are limited[3,5,9]. MCL PHA has a much lower crystallinity and higher elasticity compared with PHB. But its tensile strength is low and elongation to breaking point is high[7]. A number of studies showed 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 more pliable with increasing of the 3HHx fraction[10—13]. A copolymer of 3HB and longer chain 3HA with very high 3HB molar fraction, P(94% 3HB-co-3HA), has mechanical properties that are similar to those of low-density polyethylene (LDPE)[9]. It seems that SCL-MCL PHA has favorable mechanical properties that can range from hard crystalline to elastic properties, depending on the mole percentage of different monomers incorporated into the copolymers[6,8,9,12,14—18]. These superior properties of SCL-MCL PHA will likely widen the scope of application of PHA. Thus, regulation of the monomer composition in SCL-MCL PHA during the microbial production process is very important for the applications of PHA with desirable properties.

Since PHA synthases are the key enzymes for PHA biosynthesis, to a considerable extent, the substrate specificity of the PHA synthases determines the composition of the accumulated PHA *in vivo*. We recently reported a PHA synthase PhaC2$_{Ps}$ from *Pseudomonas stutzeri* strain 1317 that exhibited extraordinarily low substrate specificity and was 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 recombinant strain of *Ralstonia eutropha* PHB^{-}4 harboring plasmid pCJY08 containing PHA synthase gene *phaC2*$_{Ps}$ of *P. stutzeri* 1317 accumulated SCL-MCL PHA copolymers with various monomer compositions when grown on mixed carbon sources. The polyesters polymerized by PhaC2$_{Ps}$ were purified and investigated for their physical properties.

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2 MATERIALS AND METHODS

2.1 Bacterial strain and growth condition

R. eutropha PHB^{-}4 is a PHA-negative mutant of *R. eutropha* H16, 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 has been described in detail previously[20]. The recombinant *R. eutropha* PHB^{-}4 was grown at 30℃ in Luria-Bertani (LB) medium. To maintain the stability of the plasmid, kanamycin was added to the medium at a final concentration of 50mg·L^{-1}. All liquid cultures were incubated in conical flasks at 200r·min^{-1} (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 200r·min^{-1} and 30℃ in 500-ml conical flasks containing 100ml of medium. First, the cells were grown on LB medium for 24 h. Under this growth condition, no PHA accumulated in the cells. The biomass were harvested and transferred into a mineral salt (MS) medium[2] for another 48h of incubation to promote PHA accumulation. Sterilized octanoate was added to the MS medium along with 20g·L^{-1} gluconate, as described previously. For the maintenance of plasmid pCJY08 in *R. eutropha* PHB^{-}4, kanamycin was added to the medium at a final concentration of 50mg·L^{-1}.

2.3 Gas chromatography (GC) analysis of PHA in dry cells

Liquid cultures were harvested by centrifugation, the biomass were washed twice with distilled water followed by overnight lyophilization. The lyophilized cell material was subjected to methanolysis in the presence of 15% (by volume) 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 Auto-System XL GC-TurboMass, USA) was used to confirm the compositions of the produced polymers.

2.4 PHA isolation and acetone fractionation

Intracellular PHA polymers were isolated from lyophilized cells by hot chloroform extraction at 100℃ for 4h, filtered through a Whatman number 1 filter paper to remove the cellular debris, and 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 Determination of thermal properties of PHA polymers

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

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

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

2.7 Chemical structure analysis of PHA polymers

^{13}C-nuclear magnetic resonance (NMR) analysis was carried out on PHA samples. Thirty-five milligrams of each polymer was dissolved in 1ml 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.

3 RESULTS

3.1 Biosynthesis of SCL-MCL PHA by recombinant *R. eutropha* PHB^{-}4/pCJY08 from mixed carbon sources

In previous study, the recombinant *R. eutropha* PHB^{-}4 harboring plasmid pCJY08 produced PHA of both SCL and MCL monomers when grown in a mixture of gluconate and fatty acids[20]. The monomer composition and content of the synthesized PHA can be effectively modulated by the substrate composition in the culture medium. Therefore, different concentrations of octanoate were added to the MS medium containing 20g·L^{-1} gluconate to promote the accumulation of SCL-MCL PHA with various monomer contents. The results are summarized in Table 1.

It was shown that the 3-hydroxyoctanoate (3HO) content in the accumulated PHA was extensively regulated by modulating the octanoate concentration in the MS medium. The molar ratio of 3HB:MCL-3HA decreased as the concentration of octanoate increased from 0.5g·L^{-1} to 3.0g·L^{-1} (Table 1). When 3.0g·L^{-1} octanoate was added to the medium, both cell dry weight (CDW) and PHA content were reduced probably due to the toxicity of medium-chain-length fatty acids to *R. eutropha*. Shake flask results also showed that the cells grew very poor with barely detectible PHA synthesized *in vivo* if more than 3.0g·L^{-1} of octanoate was added to the MS medium (data not shown).

Table 1 PHA accumulation in recombinant *R. eutropha* PHB⁻4 harboring pCJY08 from mixed carbon sources

Sample No.	Concentration of octanoate, g·L^{-1}	DCW①, g·L^{-1}	PHA content②, %	PHA molar composition③, %				
				3HB	3HHx	3HO	3HD	3HDD
1	0	5.05±0.01	40.89±1.89	100	—④	—	—	—
2	0.5	3.78±0.14	35.69±0.63	92.4	1.4	4.1	1.2	0.9
3	1.0	3.80±0.09	32.76±1.34	81.6	2.2	11.8	2.5	1.9
4	1.5	3.52±0.06	33.70±1.45	73.3	3.4	20.8	2.5	—
5	2.0	3.32±0.07	34.73±1.44	62.6	3.9	31.0	1.1	1.4
6	2.5	3.45±0.23	33.51±2.73	54.9	5.7	35.2	2.2	2.0
7	3.0	2.87±0.16	22.86±2.31	37.6	6.8	52.3	1.8	1.5

Note: Cells were cultivated in two-stage cultivation at 30℃ and 200r·min^{-1} for 72h, as described in Section 2. A total of 20g·L^{-1} gluconate and varying concentrations of octanoate were added simultaneously as mixed carbon sources at the beginning of the second-stage cultivation. The intracellular PHA content and PHA composition were determined by gas chromatography. All the above-mentioned results were obtained from more than six experiments that were conducted in parallel.
① DCW: dry cell mass.
② PHA contents are given as mass percentage of CDW.
③ 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate.
④ —: not detected.

All SCL-MCL copolymers listed in Table 1 were recovered from the freeze-dried cells of the recombinant *R. eutropha* PHB⁻4 using the standard extraction method. Before further characterization, the polyesters were fractionated using hot acetone. A total of 300mg of various purified polyesters was completely dissolved in acetone at 40℃, respectively, indicating that the polyesters were copolymers.

3.2 Physical properties of SCL-MCL PHA copolymers with different monomer compositions

To investigate the compositions and sequence distributions of the copolyesters synthesized by the recombinant *R. eutropha* PHB⁻4/pCJY08, 125-MHz ^{13}C-NMR analysis was used. Fig.1 shows the 125-MHz ^{13}C-NMR spectrum of the copolymer accumulated on 0.5g·L^{-1} octanoate (sample No.2), along with the chemical shift assignments for each carbon resonance and an expanded spectrum of carbonyl resonance. The chemical shift assignments of all carbon resonances observed in this study were entirely consistent with those observed in previous publications[7,9,15,16,22,23]. The carbonyl carbon resonances (169.1—169.5) were clearly resolved into three peaks that were caused by the different diad sequences of connected 3HB and MCL 3HA units (Fig.1). The peaks at 169.12 and 169.42 were assignable to the carbonyl resonances in the 3HB*-3HB sequence and the 3HA*-3HA sequence, respectively. The peak at 169.28 corresponded to the carbonyl resonance in the 3HB*-3HA and 3HA*-3HB sequences of connected SCL and MCL units. Similar ^{13}C-NMR spectra were obtained for other copolyesters by ^{13}C-NMR analysis. The results further showed that all the PHA generated by the recombinant *R. eutropha* PHB⁻4/pCJY08 grown on mixed carbon sources were copolymers of 3HB and MCL 3HA monomers.

The molecular weights of the polymers that accumulated in the recombinant *R. eutropha* PHB⁻4/pCJY08 were determined by GPC analysis (Table 2). The weight-average molecular weight (M_w), number-average molecular weight (M_n) and polydispersity (M_w/M_n) were similar for the copolyesters despite the differences in their monomer compositions. These data were compared with the molecular weights of PHB homopolymer synthesized by the same strain grown on gluconate only. It was found that the molecular weights of SCL-MCL PHA were lower than those of PHB, but their polydispersity indices were comparable to those of PHB. This result indicated that the relative distributions of the polymers isolated from the recombinant *R. eutropha* PHB⁻4/pCJY08 were similar.

The thermal properties of the SCL-MCL PHA copolymers and the PHB homopolymer produced by the recombinant *R. eutropha* PHB⁻4/pCJY08 were studied using DSC analysis (Fig.2). The thermal properties for each polymer are summarized in Table 2. It was shown that the glass-transition temperatures (T_g) of the SCL-MCL PHA samples were lower than that of the PHB homopolymer for which the temperature was 3℃. The glass-transition temperatures of copolymers decreased from −1 to −24℃ as the 3HO content increased from 4.1% to 52.3% (Tables 1 and 2). The addition of MCL 3HA monomers to the PHA copolymers clearly lowered the melting temperatures (T_m) compared with that of the PHB homopolymer (Table 2). No melting peak was observed for sample 7 with 3HB molar composition of 37.6% (Fig.2), indicating that the copolyester was an amorphous copolymer. Furthermore, the enthalpy of fusion (ΔH_m) for copolymers was also altered by the addition of MCL monomers and it was obviously lower than that of PHB. These results showed that the variation in MCL 3HA content caused dramatic changes in the thermal properties of the copolymers.

4 DISCUSSION

Recently, SCL-MCL PHA copolymers have attracted considerable attention of both academicians

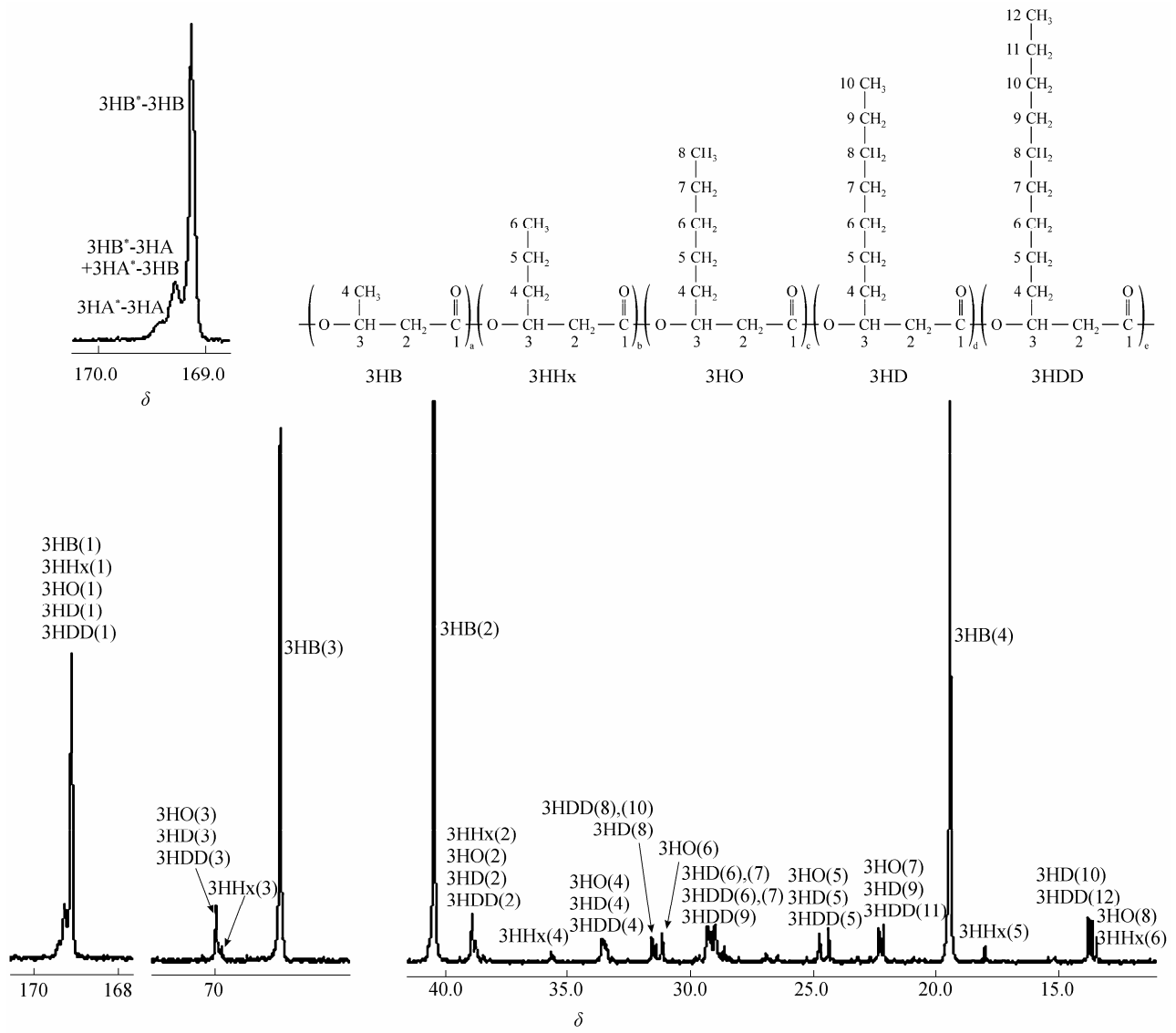

Figure 1 125-MHz ^{13}C-NMR spectrum of the copolymer synthesized by recombinant *R. eutropha* PHB^{-}4/pCJY08 on mixed carbon sources containing 20g·L^{-1} gluconate and 0.5g·L^{-1} octanoate
(3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate; 3HA, denotes MCL 3-hydroxyalkanoates in this figure)

Table 2 Molecular weights and thermal properties of SCL-MCL PHA copolymers produced by recombinant *R. eutropha* PHB^{-}4

Sample No.	PHA molar composition①, %		Molecular weight②			Thermal properties③		
	3HB (C4)	3HA (C6—C12)	$M_w\times10^{-4}$, Da	$M_n\times10^{-4}$, Da	M_w/M_n	T_g, ℃	T_m, ℃	ΔH_m, J·g^{-1}
1	100	0	162.3	134.5	1.22	3	175	58
2	92.4	7.6	19.5	12.4	1.58	−1	151	42
3	81.6	18.4	15.7	9.4	1.67	−2	141	25
4	73.3	26.7	12.4	8.1	1.54	−3	139	25
5	62.6	37.4	10.5	7.6	1.39	−6	138	8
6	54.9	45.1	11.7	7.5	1.55	−7	134	12
7	37.6	62.4	15.7	10.0	1.57	−24	—④	—

Note: The cultivation conditions are the same as those shown in Table 1.
① 3HB (C4), 3-hydroxybutyrate; 3HA (C6—C12), including 3-hydroxyhexanoate, 3-hydroxyoctanoate, 3-hydroxydecanoate and 3-hydroxydodecanoate.
② M_w, weight-average molecular weight; M_n, number-average molecular weight; M_w/M_n, polydispersity.
③ T_g, glass-transition temperature; T_m, melting temperature; ΔH_m, enthalpy of fusion.
④ —: not detected.

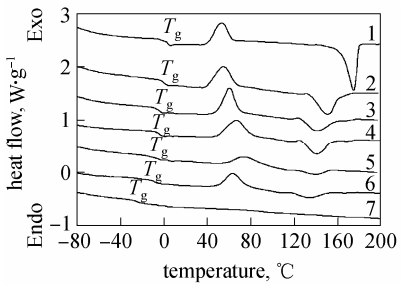

Figure 2 DSC thermograms of the polyesters isolated from recombinant *R. eutropha* PHB^{-}4 harboring plasmid pCJY08 (*phaC2*$_{Ps}$) grown at different octanoate concentrations

(1—7, sample No.1—7 that correspond to those shown in Tables 1 and 2; T_g, glass-transition temperature)

and industrialists because of their superior physical and mechanical properties compared with those of either SCL or MCL PHA[8,9]. Therefore, it is of special interest to produce SCL-MCL PHA copolymers with different MCL 3HA compositions for various commercial applications. In our previous studies, the PHA synthase PhaC2$_{Ps}$ cloned from *P. stutzeri* 1317 showed comparatively low substrate specificity and could catalyze the conversion of SCL and MCL 3HA-CoA substrates into PHA copolymer[19,20]. In this study, a series of SCL-MCL PHA with changeable monomer compositions were synthesized by the recombinant *R. eutropha* PHB^{-}4 harboring *phaC2*$_{Ps}$ gene when grown on the mixed carbon sources (Table 1).

In this study, all SCL-MCL PHA copolymers polymerized by PhaC2$_{Ps}$ were purified and further characterized by acetone fractionation, NMR spectroscopy, GPC, and DSC. It was indicated that all the materials were primarily composed of 3HB and 3HO units. The molecular weights of the copolymers were not influenced greatly by the variation in MCL 3HA contents but were obviously lower than that of the PHB homopolymer (Table 2). In the DSC thermogram, the glass-transition temperatures and melting temperatures of SCL-MCL PHA copolyesters were lower than those of PHB homopolymer, and both decreased with increase in the 3HO fraction (Fig.2). The results strongly suggested that the variation of monomer compositions in SCL-MCL PHA copolymers was effective in changing the physical properties.

Thus far, the biosynthesis of SCL-MCL PHA is still far from meeting the application requirements. There are only very few PHA synthases that have low substrate specificity and are likely to catalyze the polymerization of SCL and MCL 3HA-CoA[7,24—26]. Of all the synthases reported, PhaC1$_{61-3}$ from *Pseudomonas* sp. 61-3 and PhaC2$_{Ps}$ from *P. stutzeri* 1317 seemed to be the only two promising candidates for SCL-MCL PHA synthesis on further study and production. The comparative studies of PhaC1$_{61-3}$ and PhaC2$_{Ps}$ will certainly help to better understand PHA synthase specificity and SCL-MCL PHA properties.

PhaC1$_{61-3}$ from *Pseudomonas* sp. 61-3 has been most extensively studied[22,26,27]. To date, only the SCL-MCL copolyesters synthesized by PhaC1$_{61-3}$ were isolated and investigated for their physical properties. These studies mainly focused on the copolymers with a remarkably high 3HB fraction[6,9,12,16]. The results indicated that even a very low molar fraction of MCL 3HA in the copolymers had dramatic effects on the thermal properties and would provide the copolymers with superior physical properties compared with that of the PHB homopolymer.

In this study, the variation in the octanoate concentration in the MS medium led to the production of a series of SCL-MCL PHA with different MCL 3HA contents accumulated by the recombinant *R. eutropha* PHB^{-}4/pCJY08 harboring *phaC2*$_{Ps}$. This approach, used to alter the monomer compositions of PHA copolymers, was carried out easily compared with other methods reported previously[9]. The molecular weights of the copolymers in this study were similar to those of copolymers synthesized by PhaC1$_{61-3}$[9,14]. In the DSC thermogram, it was clearly observed that the addition of a very small amount of MCL 3HA to the SCL-MCL PHA copolymer dramatically reduced the glass-transition temperature, melting temperature and enthalpy of fusion compared with those of PHB (Fig.2). The phenomenon of the changes in thermal properties was also in agreement with similar studies on the copolymers synthesized by PhaC1$_{61-3}$[9,14,16]. However, the glass-transition temperature and melting temperature of the copolymers from PhaC2$_{Ps}$ were slightly higher than those of the copolymers from PhaC1$_{61-3}$ with very similar MCL 3HA content[9,14]. This difference can be accounted for by the following two explanations. First, the constitutions of the MCL 3HA fractions in these copolymers are different. Both 3HO and 3-hydroxydecanoate (3HD) were the main components in the MCL 3HA fractions of the copolymers from PhaC1$_{61-3}$. As for the copolymers from PhaC2$_{Ps}$, the MCL 3HA fraction mainly consisted of 3HO. The longer chain MCL 3HA unit seemed to be more effective in changing the thermal properties of SCL-MCL PHA copolymers compared with a shorter one. A similar phenomenon was also observed by Noda *et al*.[8]. Second, the diad fractions calculated from the molar fractions of 3HB and MCL 3HA units (determined by GC analysis) are not completely consistent with the observed values that were determined from relative peak areas of carbonyl carbon resonances in NMR spectra (Fig.1). It was suggested that the copolyesters synthesized by PhaC2$_{Ps}$ in this study might contain block polymers that affected the thermal properties.

Moreover, it was also helpful in the elucidation of the physical properties of SCL-MCL PHA, which contained more than 10% MCL 3HA and has been rarely studied before. It was shown that the thermal properties changed slowly when the molar content of MCL 3HA increased from 7.6% to 45.1% (Table 2). As the molar content of MCL 3HA increased further, the copolymers tended to become amorphous.

5 CONCLUSIONS

In this study, a very simple cultivation method was successfully developed to synthesize SCL-MCL

PHA copolymers with different MCL 3HA contents. The SCL-MCL PHA polymerized by PhaC2$_{Ps}$ were purified and characterized with respect to the physical properties for the first time. The results indicated that the variation in MCL 3HA contents did not have a significant influence on the molecular weights of the copolymers but was effective in changing their physical properties. In addition, the thermal property variation with MCL 3HA content in PHA copolymers was primarily investigated in this study.

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NOMENCLATURE

ΔH_m	enthalpy of fusion, J·g^{-1}
M_n	number-average molecular weight, Da
M_w	weight-average molecular weight, Da
M_w/M_n	polydispersity
T_g	glass-transition temperature, ℃
T_m	melting temperature, ℃

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