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Biosynthesis of high-molecular-weight poly(D-lactate)-containing block copolyesters using evolved sequence-regulating polyhydroxyalkanoate synthase PhaC$_{AR}$

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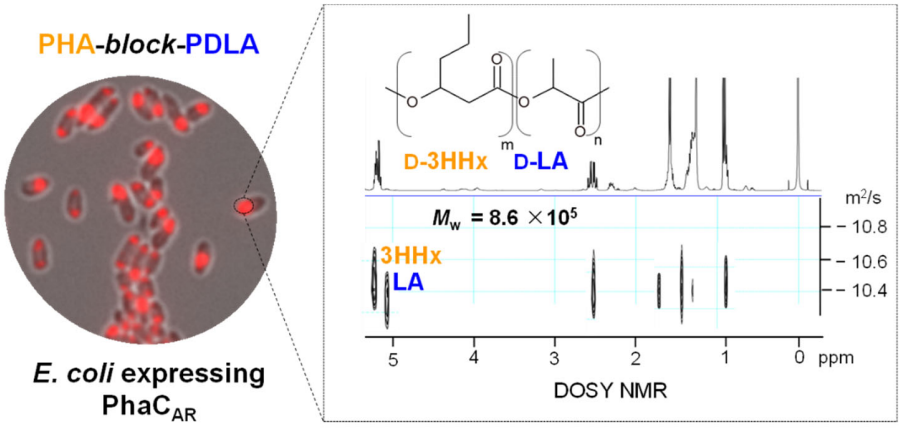
Abstract

Bacterial polyhydroxyalkanoate (PHA) synthase PhaC$_{AR}$ is a unique enzyme that can synthesize block copolymers. In this study, poly(D-lactate) (PDLA)-containing block copolymers were synthesized using PhaC$_{AR}$ and its mutated variants. Recombinant *Escherichia coli* harboring *phaC*$_{AR}$ and relevant genes were cultivated with supplementation of the corresponding monomer precursors. Consequently, PhaC$_{AR}$ synthesized poly(3-hydroxybutyrate)-*b*-2 mol% PDLA [P(3HB)-*b*-PDLA]. The incorporation of D-lactate (LA) enantiomer was confirmed by chiral gas chromatography. Previously identified beneficial mutations in PhaC$_{AR}$, N149D (ND) and F314H (FH), which increased activity toward a medium-chain-length substrate 3-hydroxyhexanoyl (3HHx)-CoA, improved the incorporation of LA units. The combined pairwise mutation NDFH synergistically increased the LA fraction to 21 mol% in P(3HB)-*b*-PDLA. Interestingly, a greater amount of LA units (51 mol%) was incorporated by copolymerization with 3HHx units, which yielded P(3HHx)-*b*-PDLA. The block copolymerization of 3HHx and D-LA units was confirmed by NMR analyses and solvent fractionation of polymers. The PDLA crystal in P(3HHx)-*b*-PDLA was detected using differential scanning calorimetry and wide angle X-ray diffraction. Its mass-average molecular weight was 8.6×10^5. Thus, block copolymerization utilized high-molecular-weight PDLA as a component of PHAs.



KEYWORDS: PHA synthase, *in vitro* evolution, sequence regulation, PDLA-like copolymers, block copolymer

Introduction

Polyhydroxyalkanoates (PHAs) are aliphatic polyesters produced by microbes, which are utilized as biobased and biodegradable plastics and recognized as an environmentally friendly alternative to petroleum-based and non-biodegradable plastics.[1, 2] Naturally occurring PHAs are composed of 3-hydroxyalkanoates (3HAs) with various side-chain lengths. The physical properties of PHAs can be regulated from rigid to pliable by randomly copolymerizing the monomer constituents with different side-chain lengths. For example, a random copolymer P(3-hydroxybutyrate-

co-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] is commercially manufactured and used as straw, cutlery, brush, *etc.*[3, 4] An important goal for further increasing the range of applications is to expand the physical properties of the polymer. Therefore, artificial PHAs with unusual structures have attracted considerable research interest. [5]

Block copolymerization is a potent method to create new materials with useful properties; thus, the biosynthesis of PHA block copolymer has been a hot issue. Based on our previous reports, PHA synthase $PhaC_{AR}$ has a sequence-regulating capacity, and it successively polymerizes multiple substrates, hydroxyacyl-CoAs, into block copolymers.[6, 7] This process proceeds spontaneously without any manipulations during polymer synthesis. The characterization of $PhaC_{AR}$ has demonstrated that the block structure is generated between P(3HA) and P(2-hydroxyalkanoate) [P(2HA)] segments.[7] Block copolymers exert characteristic properties by combining segments with distinct physical properties.[8] Therefore, the properties of P(3HA) and P(2HA) segments are key factors in the molecular design of PHA block copolymers.

This study aimed to utilize polylactate [PLA or poly(2-hydroxypropionate)] as a segment of PHA block copolymers. PLAs are utilized as biobased, biocompatible, non-toxic, and processible polymer materials in various applications.[9] They are also a potent segment of block copolymers because of their properties, such as being tough, and transparent. In addition, PLA, and PHAs are immiscible.[10] Immiscibility is necessary for forming microphase separation, which plays an important role in the physical properties of block copolymers.[8]

Furthermore, the function of PHA synthase is the most important factor to achieve the goal. $PhaC_{AR}$ has a broad substrate scope toward 2- to 6-hydroxyacyl-CoAs,[7] but ability to incorporate lactate (LA) units has not been reported. Previously, we performed the directed evolution of $PhaC_{AR}$ to reinforce the activity toward 3HHx-CoA and successfully identified two beneficial point mutations N149D and F314H that increased the 3HHx-incorporating capacity of the enzyme.[11] Here, we conceived the idea of using these mutants to synthesize LA-containing polymers. Notably, these mutations were effective in incorporating LA. Using the evolved $PhaC_{AR}$, a novel PHA block copolymer containing PLA as a segment was synthesized.

Experimental Section

Plasmid construction

Two beneficial mutations in $PhaC_{AR}$, namely, N149D (ND) and F314H (FH), were identified previously.[11] $pBSP_{Re}phaC_{AR}$N149DpctalkK and $pBSP_{Re}phaC_{AR}$F314HpctalkK were digested by *Nde*I and *Bgl*II, and the resulting 0.5 and 8.3 kb fragments, respectively, were ligated to yield $pBSP_{Re}phaC_{AR}$N149DF314HpctalkK (NDFH), which harbors a pairwise mutation in $PhaC_{AR}$.

Culture conditions, polymer extraction, and analysis

Recombinant *E. coli* JM109 harboring $pBSP_{Re}phaC_{AR}$pctalkK [11] and its derivatives were used for polymer production. The cells were grown on 1.5 mL of LB medium containing 100 μg/mL of ampicillin at 30 °C for 12 h for preculture. The seed culture was used to inoculate 100 mL of LB medium containing 100 μg/mL of ampicillin, 1.0 g/L of sodium (*R,S*)-3HHx, 2.5 g/L of sodium (*R,S*)-3HB, and/or 10.0 g/L of sodium D-LA [(*R*)-LA] in 500-mL shake flasks, which were cultivated with reciprocal shaking at 120 rpm and 30 °C for 48 h. LA-Na was prepared by neutralizing 99% D-lactic acid with sodium hydroxide (Musashino Chemical Laboratory, Ltd.). 3HHx-Na was prepared by hydrolyzing ethyl 3-hydroxyhexanoate as previously described.[12] All chemicals were purchased from Tokyo Chemical Industry (Japan), Junsei Chemical (Japan), or FUJIFILM Wako Pure Chemicals Corporation (Japan) unless otherwise stated. The polymers were extracted from lyophilized cells with chloroform for 48 h at 60 °C and purified by reprecipitation by adding an excess amount of methanol as previously described.[11] The purified polymers were subjected to ^{1}H NMR, ^{13}C NMR, and Diffusion ordered spectroscopy (DOSY)-NMR analyses. The molecular weight of the polymers was determined using size-exclusion chromatography (JASCO Corporation, Japan) equipped with two tandem Shodex K-806L columns, which is applicable to the molecular weight range of 3×10^{2}–2×10^{8} (particle size 10 μm, Shodex, Japan). Polystyrene standards were used for calibration.[13]

Chiral gas chromatography (GC)

P(49 mol% 3HHx-*co*-LA) was dissolved in 500 μL of chloroform to a LA concentration of 5 mg/mL and combined with 500 μL of 15 vol% sulfuric acid in ethanol. The mixture was heated at 100 °C for 2 h for ethanolysis. PDLA and PLLA were treated with the same procedure. The obtained ethyl esters were applied to GC equipped with a chiral capillary column Rt-bDEXsa (Fisher Scientific).

Capillary electrophoresis (CE)

The CE measurement of 3HHx in the medium was performed with a 7100 CE instrument (Agilent Technologies) equipped with a UV detector. An uncoated/ fused silica capillary (50 μm i.d., 40 cm effective length) was used. Background electrolyte (BGE) (Otsuka Electronics Co., Ltd., Japan) was used. At the beginning of each working day, the capillary was rinsed with 1 N NaOH solution (flush 10 min), ultrapure water (flush 10 min), and BGE buffer (flush 20 min). Before each run, the capillary was conditioned with 20 min. The sample injection was performed 50 mbar

for 4 sec. The positive polarity mode at 30 kV was applied for 20 min to separate the analyte. The supernatant of the harvested culture medium was diluted 10 times with ultrapure water and applied into this apparatus.

Solvent fractionation

Approximately 20 mg of the purified polymer was dissolved in 1 mL of tetrahydrofuran in a glass tube with a screw cap by heating at 100°C for 10 min. The solution was subsequently combined with 8 mL of cyclohexane and incubated at 4°C for 1 h. Cyclohexane-soluble and -insoluble fractions were separated by passing through a PTFE membrane filter with a pore size of 0.2 μm. Each fraction was applied to ^{1}H NMR. A blend of P(3HHx) and PLLA was tested using the same conditions as the control.

Differential scanning calorimetry (DSC) analysis

The thermal properties of the solvent-cast films (approximately 2 – 3 mg) were examined using a DSC3+STAR^{e} system (Mettler Toledo). The DSC measurements were conducted as described previously with modification.[14] The thermograms were obtained by performing two heating cycles. In the first cooling cycle, the sample was cooling from 25 °C to −30 °C at a rate of 50 °C/min and continuing cooling from −30 °C to −50 °C at a rate of 20 °C/min. It was then held at −50 °C for 2 min, and the sample was heated from −50 °C to 210 °C at a rate of 20 °C/min and maintained at this temperature for 2 min, rapidly cooled to −30 °C at the rate of 50 °C/min, continuing cooling from −30 °C to −50 °C at 20 °C/min, holding at −30 °C in 5 min. In the second heating cycle, the sample was heated from −50 °C to 210 °C at a rate of 20 °C/min, followed by a 2-min hold at 210 °C.

Wide angle X-ray diffraction analysis

Wide angle X-ray diffraction (WAXD) was performed at the BL-6A of the Photon Factory (Tsukuba, Japan) using a synchrotron X-ray radiation (λ = 1.50 Å). The film sample was directly subjected to the WAXD experiments at 25°C. The X-ray diffraction data were collected for 60 sec using a Pilatus 100k detector, and the obtained 2D diffraction profiles were circularly averaged to yield the 1D profiles. The scattering vector ($q = (4\pi/\lambda) \sin(\theta/2)$, where the θ is scattering angle) was calibrated based on the diffractions from silver behenate. The PDLA homopolymer used as the reference sample was prepared by following our previous paper[15] using 1,4-benzenedimethanol as the initiator and was annealed at 110 °C for 3 h prior to the WAXD measurement.

Results

Incorporation of LA units into the polymers synthesized using PhaC$_{AR}$ and its derivatives

Recombinant *E. coli* JM109 cells expressing each of the parent PhaC$_{AR}$, ND, FH, and NDFH mutants were cultured on LB medium with supplementation of 3HB, LA, or combination. The metabolic pathways for polymer production are shown in Figure 1. Consequently, the parent PhaC$_{AR}$, and its mutants produced comparable amounts of P(3HB) (Table 1, entries 1–4). No PLA homopolymer production was observed under all conditions tested (entries 5–8). By contrast, when 3HB, and LA were co-supplied, the polymers containing 3HB and LA were produced (entries 9–12). In particular, NDFH could highly incorporate LA units (21 mol%). Therefore, the combination of ND, and FH mutations showed a synergistic effect on LA incorporation, although these mutations were originally identified on the basis of the selection criterion of the elevation of 3HHx incorporation.[11]

Thus, in this study, the synthesis of a copolymer comprising 3HHx and LA was examined (Table 2). Under the copolymer synthesis conditions, FH and NDFH produced polymers containing 3HHx and LA (entries 19 and 20). The LA fractions (46 mol% and 51 mol%) were higher than those in the case of 3HB/LA (2 mol%–21 mol% LA). These results indicate that NDFH can highly incorporate 3HHx and LA units. For P(3HHx) production, FH and NDFH produced the same amount of polymer (entries 15 and 16). Under these conditions, the residual 3HHx-Na in the media (entries 15 and 16) at 24 h after inoculation were 0.65 and 0.67 g/L, respectively (Figure S1). Thus, utilization of (*R,S*)-3HHx for cell growth was little or none.

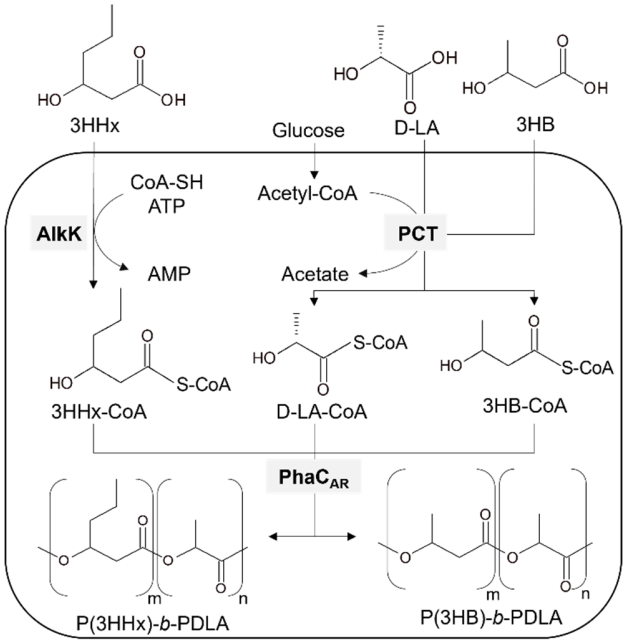


Figure 1. Metabolic pathways for the synthesis of P(3HB)-*b*-PDLA and P(3HHx)-*b*-PDLA in *E. coli*. PCT, propionyl-CoA transferase. AlkK, CoA ligase.

Table 1. Polymer production containing 3HB and LA in *E. coli* expressing PhaC$_{AR}$ and its mutants

Entry	PhaC$_{AR}$ mutants	Precursor (g/L)		Cell dry weight (g/L)	Polymer production (g/L)	Monomer composition (mol%)	
		3HB	LA			3HB	LA
1	Parent	2.5	-	3.9 ± 0.4	0.6 ± 0.02	100	0
2	ND	2.5	-	3.9 ± 0.1	0.5 ± 0.01	100	0
3	FH	2.5	-	4.2 ± 0.1	0.6 ± 0.02	100	0
4	NDFH	2.5	-	4.3 ± 0.2	0.6 ± 0.03	100	0
5	Parent	-	10	2.0 ± 0.1	nd	-	-
6	ND	-	10	2.0 ± 0.1	nd	-	-
7	FH	-	10	2.1 ± 0.1	nd	-	-
8	NDFH	-	10	2.5 ± 0.2	nd	-	-
9	Parent	2.5	10	3.8 ± 0.8	0.6 ± 0.1	98	2
10	ND	2.5	10	3.9 ± 0.5	0.6 ± 0.1	96	4
11	FH	2.5	10	4.3 ± 0.3	0.7 ± 0.1	91	9

12	NDFH	2.5	10	3.7 ± 0.1	0.8 ± 0.1	78	21

nd: not detected. The full NMR spectra are shown in Figure S2. Data are the average ± standard deviation of three trials. Precursor concentrations are indicated as sodium salt equivalent.

Table 2. Polymer production containing 3HHx and LA in *E. coli* expressing PhaCAR and its mutants

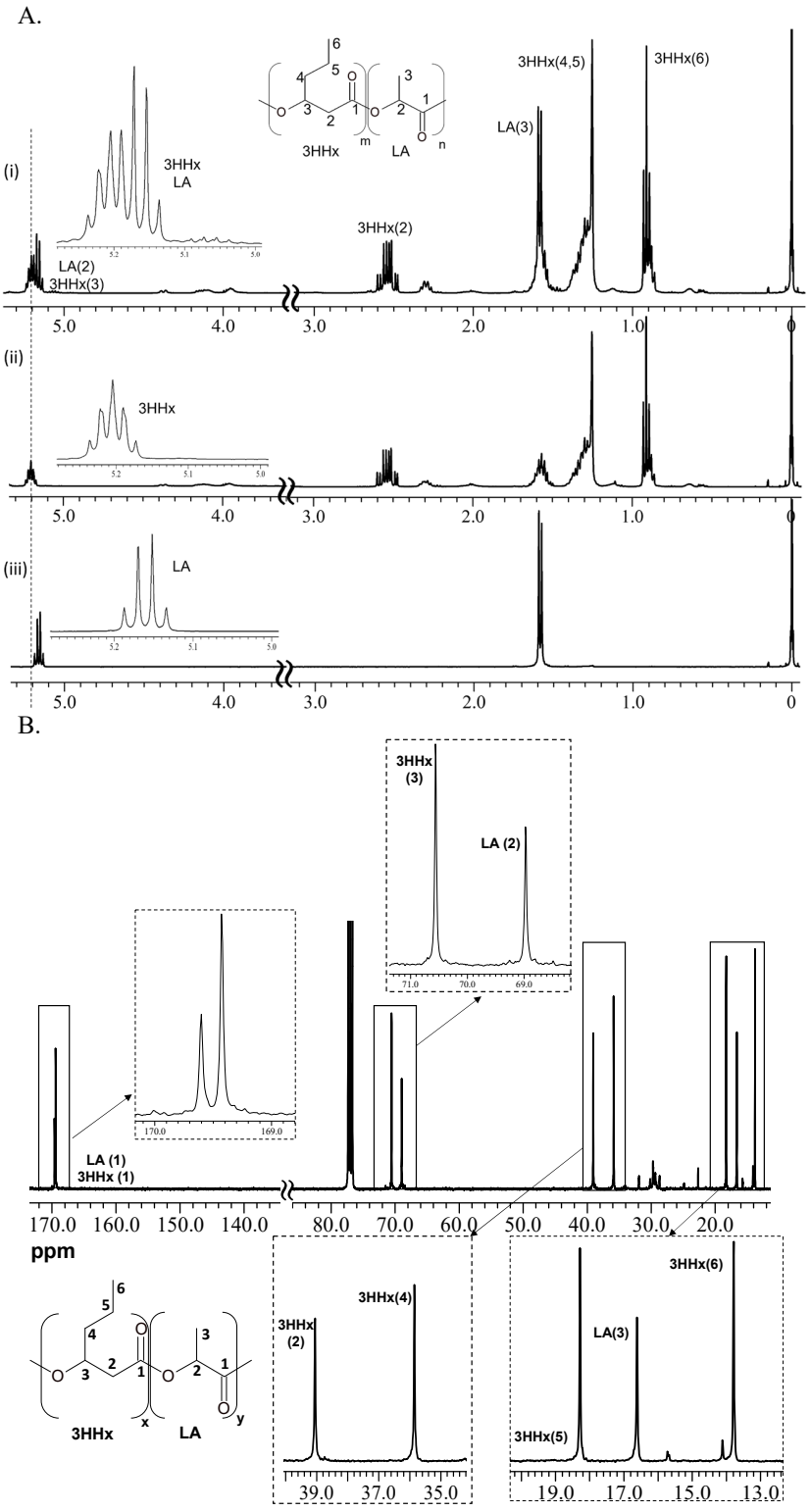
Entry	PhaCAR mutants	Precursor (g/L)		Cell dry weight (g/L)	Polymer production (g/L)	Monomer composition (mol%)	
		3HHx	LA			3HHx	LA
13	Parent	1.0	-	1.9 ± 0.1	nd	-	-
14	ND	1.0	-	2.0 ± 0.0	trace	100	0
15	FH	1.0	-	2.6 ± 0.1	0.22 ± 0.01	100	0
16	NDFH	1.0	-	2.6 ± 0.1	0.22 ± 0.01	100	0
17	Parent	1.0	10	2.2 ± 0.0	nd	-	-
18	ND	1.0	10	2.2 ± 0.0	nd	-	-
19	FH	1.0	10	2.5 ± 0.0	0.29 ± 0.02	54	46
20	NDFH	1.0	10	2.6 ± 0.0	0.33 ± 0.02	49	51

nd: not detected. The full NMR spectra are shown in Figure S3. Data are the average ± standard deviation of three trials. Precursor concentrations are indicated as sodium salt equivalent.

Monomer sequence analysis of P(3HHx-*co*-LA)

The polymer containing 3HHx and LA synthesized using FH was applied to ^{1}H and ^{13}C NMR analyses (Figures 2A and B). The resonance of the methine protons of the 3HHx and LA units is the fingerprint region to determine dyad and triad sequences. In addition, the resonance of the sample at 5.1–5.3 ppm was identical to that of P(3HHx) and PLA at 5.15–5.26 ppm and 5.12–5.20 ppm, respectively (Figure 2A [ii] and [iii]),[11, 13] indicating that the polymer contained P(3HHx) and PLA homopolymers as major components. The weak resonance at 5.06 ppm was ascribed to the LA*-3HHx linkage as previous report,[16] indicating that the polymer contained a small portion of the randomly polymerized structure. The 3HHx(3) resonance in LA-3HHx* dyad was presumably observed at slightly lower field than the 3HHx-3HHx* homo dyad based on the analogy with P(LA-*co*-3HB),[17] and thus, the signal was overlapped with the signal of the homo dyad.

^{13}C NMR resonances of the sample were identical to those of P(3HHx) and PLA.[13] In addition, the peaks around 169.42–169.72 ppm were split into two distinguished peaks, which were ascribed to dyad sequences of 3HHx*-3HHx and LA*-LA.[16] Heterodyads of 3HHx and LA units were not detectable in ^{13}C NMR. Collectively, the obtained polymer was either a block copolymer of P(3HHx) and PLA or their blend.


A.
(i)
(ii)
(iii)
3HHx
LA
LA(2)
3HHx(3)
3HHx(2)
3HHx(4,5)
3HHx(6)
LA(3)
B.
3HHx (3)
LA (2)
LA (1)
3HHx (1)
ppm
3HHx(4)
3HHx (2)
3HHx(6)
LA(3)
3HHx(5)

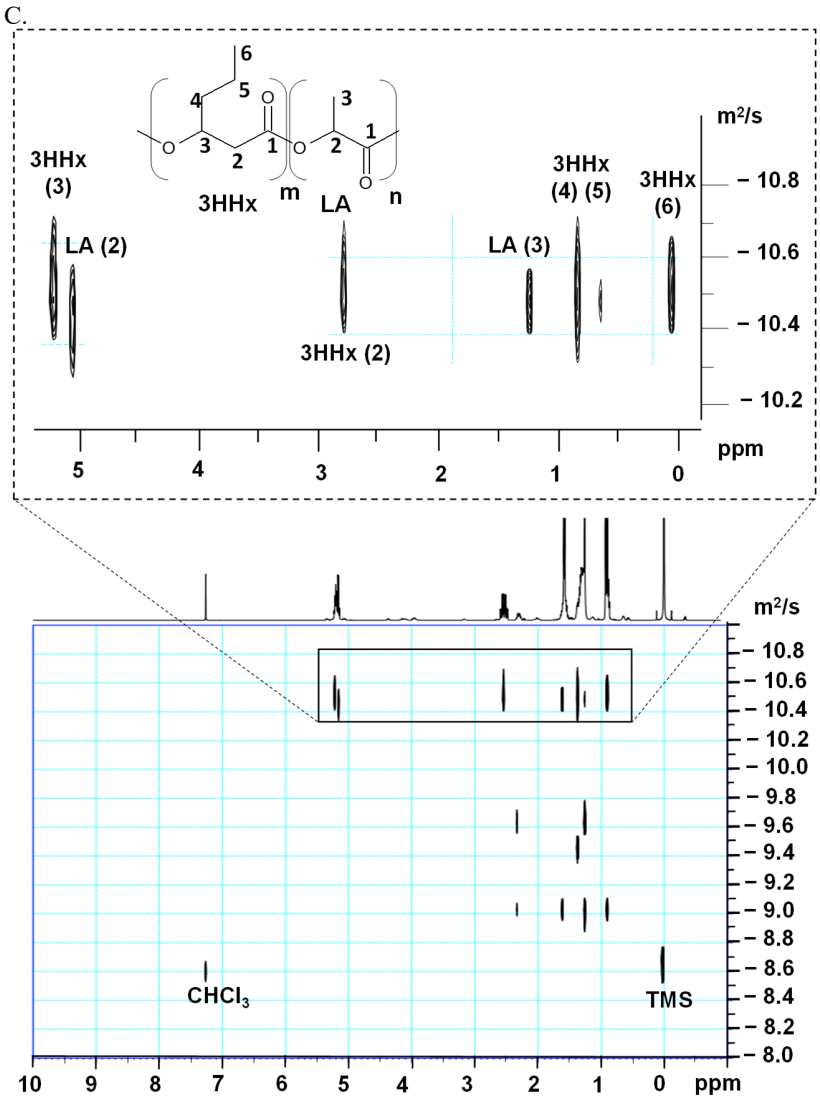


Figure 2. [1]H NMR (A), [13]C NMR (B), and DOSY-NMR (C) spectra of polymers synthesized by recombinant *E. coli* JM109 expressing FH. A(i): Binary polymer containing 3HHx and LA, A(ii): P(3HHx), A(iii): PLLA. The full NMR spectra are shown in Figures S5 and S6.

Enantiomer analysis of LA units

The enantiomer of LA units in the polymer synthesized by NDFH (entry 20) was determined using chiral GC. The ethanolysis product of the polymer sample exhibited a peak corresponding to the D-LA standard (Figure 3), indicating that the polymer was composed of D-LA. Thus, the polymer contained a PDLA segment. Given that PCT recognizes D,L-LA as substrates,[18] the result was due to strict enantiospecificity of NDFH.

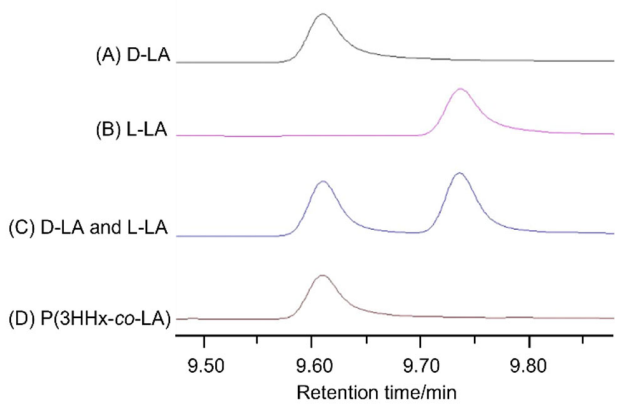


Figure 3. Enantiomeric analysis of the LA units in P(3HHx-*co*-LA) synthesized using NDFH by chiral GC. (A), (B), and (C): L-LA and D-LA standards, (D) P(3HHx-*co*-LA).

Solvent fractionation

Solvent fractionation was performed to identify whether the obtained polymer was a polymer blend or block copolymer of P(3HHx) and PLA. P(3HHx) is soluble in cyclohexane, whereas PLAs are insoluble. Given their distinct solubility, the polymer samples were separated into cyclohexane-soluble and -insoluble fractions (Table 3). When a polymer blend of P(3HHx) and PLA was applied to fractionation, the cyclohexane-soluble fraction did not contain PLA. By contrast, when the copolymers produced by FH and NDFH were tested, PLA was detected in the cyclohexane-soluble fraction, indicating the presence of a covalent linkage between P(3HHx) and PLA segments. Therefore, the copolymers produced by FH and NDFH mutants were a block copolymer P(3HHx)-*b*-PDLA.

Table 3. Solvent fractionation of polymers comprising 3HHx and LA

Polymers	Fractions	Monomer composition (mol%)		Recovery (mol%)
		3HHx	LA	
Polymer blend of P(3HHx) and PLLA	Original blend	50	50	100
	Soluble fraction	100	0	47
	Insoluble fraction	31	69	51
Copolymer synthesized by FH	Original copolymer	52	48	100
	Soluble fraction	81	19	31
	Insoluble fraction	32	68	56
Copolymer synthesized by NDFH	Original copolymer	48	52	100
	Soluble fraction	78	22	36
	Insoluble fraction	42	58	51

Full NMR spectra are shown in Figure S4.

Molecular weight and thermal properties analysis

The weight-average molecular weight of P(3HHx) synthesized using FH and NDFH was 2.3×10^6 and 1.3×10^6 (M_w), respectively (Table 4), indicating that FH achieved a higher molecular weight than NDFH. The polymer containing 3HHx and LA had lower molecular weights (3.5×10^5 and 8.6×10^5 (M_w), respectively) than P(3HHx). The polymer was eluted as a unimodal peak by SEC (Figure S7), supporting that the sample was a block copolymer rather than blend. In fact, the DOSY NMR of the polymer, which indicated the similar diffusion coefficients of LA and 3HHx units, agreed with the interpretation (Figures 2C).

The DSC analysis of P(3HHx)-*b*-PDLA exhibited a melting peak at 144 °C ascribed to PDLA crystal. The presence of PDLA crystal phase in P(3HHx)-*b*-PDLA was also supported by WAXD analysis. As shown in Figure 4A, the P(3HHx)-*b*-PDLA film annealed at 110 °C clearly showed four diffraction peaks at q = 10.57, 11.96, 13.63, 15.99 nm^{-1}. Those peaks coincided with the diffraction of (011), (110)/(200), (113)/(203), and (211) reflections of α-form PDLA crystal (Figure 4B).[19] The melting temperature of PDLA segment in the block copolymer was lower than that of PDLA homopolymer (170–180 °C). The phenomenon was presumably due to the small crystal size and was also observed for other block copolymers.[14] The polymer exhibited two T_gs at –15 and 38 °C, which were ascribed to P(3HHx) and PDLA amorphous phases, respectively. The T_g of PDLA segment (38 °C) was lower than that of PDLA (60 °C), suggesting that the amorphous phases are partly mixed state.

Table 4. Molecular weight and thermal properties of polymers

Entry	Sample	Molecular weight			Thermal properties		
		M_w (×10^5)	M_n (×10^5)	*Đ*M	T_g (°C)	T_m (°C)	ΔH (J/g)
15	P(3HHx)	23	5.1	4.6	−28*	44	19.9
16	P(3HHx)	13	3.8	3.5	nt	nt	
19	P(54mol% 3HHx-*co*-LA)	3.5	1.9	1.7	nt	nt	
20	P(49mol% 3HHx-*co*-LA)	8.6	3.7	2.3	−15, 38	144	21.3

M_w, mass-average molecular weight; M_n, number-average molecular weight; ĐM = M_w/M_n; T_g: glass-transition temperature; T_m: melting temperature. *determined from the 2nd heating scan. nt: not tested. Thermograms are shown in Figure S8.

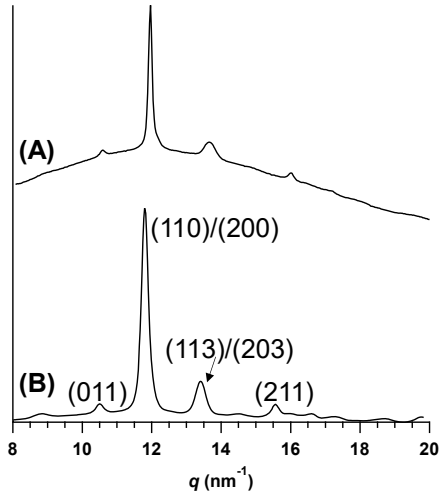


Figure 4. WAXD profiles of P(3HHx)-*b*-PDLA film (A) and PDLA homopolymer powder (B). Both samples were annealed at 110 °C for 3 h.

Discussion

The first LA-incorporating PHA synthase was reported in 2008, which was an engineered bacterial PHA synthase, $PhaC1_{Ps}STQK$.[20] The enzyme is a class II PHA synthase derived from *Pseudomonas* sp. 61-3 containing Ser325Thr/Gln481Lys mutations and randomly copolymerized LA-CoA and 3HB-CoA. However, $PhaC1_{Ps}STQK$ had a limited range of LA fraction in the copolymer. An inverse relationship was observed between the LA fraction and polymer production of random copolymers of LA and 3HAs.[20, 21] In particular, the production and molecular weight of biosynthesized PDLA homopolymer (and PDLA-like copolymer) was severely limited to low values (molecular weight was 10^3 order of magnitude). Previously, *in vitro* analysis of $PhaC1_{Ps}STQK$ using LA-CoA demonstrated that the synthesis of a PDLA homopolymer stopped at the initial stage of the reaction, at which the molecular weight is approximately 2,000.[22] The anomalous behavior might be due to the high glass transition temperature of PLA (60°C).

In this study, P(3HHx)-*b*-PDLA with an M_w of 10^5 order of magnitude was successfully synthesized using FH and NDFH. The number of PDLA segment(s) in the block copolymer remained undetermined. However, *in vitro* analysis of $PhaC_{AR}$ revealed its two-step reaction corresponding to each segment synthesis.[23] In addition, PDLA crystals were detected in P(3HHx)-*b*-PDLA (Figure 4). Therefore, P(3HHx)-*b*-PDLA is unlikely to be a multiple block copolymer composed of many segments. Therefore, given the molar fraction of LA (nearly 50 mol%), the molecular weight of the PDLA segment was estimated to be 10^4–10^5 order of magnitude, which was considerably greater than the abovementioned upper limit. The generation of the high-molecular-weight PDLA segment was also supported by DOSY NMR analysis (Figure 2C).

NDFH with enhanced 3HHx-incorporating ability incorporated a greater amount of LA units than the parent $PhaC_{AR}$ did. At present, the correlation between the activities toward two substrates, 3HHx-CoA and LA-CoA, remains unknown. However, it should be noted that the PDLA homopolymer was not obtained using NDFH (Table 1, entry 8). Therefore, the synthesis of P(3HB) and P(3HHx) segments is an enabling factor of the synthesis of a PDLA chain. According to the comparison between entries 12 and 20, the P(3HHx) segment seemed to have a greater promoting effect on the PDLA segment synthesis than the P(3HB) segment. Therefore, the presence of P(3HHx) segment rather than enhanced activity toward 3HHx-CoA may promote the synthesis of PDLA segment. *In vitro* analysis of the block copolymer synthesis is necessary to elucidate the role of P(3HHx) segment in the synthesis of the PDLA segment.

For the biosynthesis of PHA block copolymers using $PhaC_{AR}$, 3HA, and 2HA units it is required to combine 3HA and 2HA units.[7] P(2HB) and P(glycolate-*ran*-3HB) have been reported as 2HA-based segments.[7, 24] In this study, PDLA was considered as an option in the molecular design of PHA block copolymers. Given the broad substrate scope of NDFH, the finding expanded the structural variety of PHA block copolymers. The PDLA region in the copolymer can serve as a hard segment. This finding is contrary to the previously reported block copolymer P(3HB)-*b*-P(2HB), in which P(2HB) serves as a soft segment. The physical properties of P(3HHx)-*b*-PDLA will be addressed in our future work.

The PHA production in the present system utilizes extracellularly supplemented 3HHx as a precursor. The use of precursors facilitates the construction of metabolic pathway and its regulation. On the other hand, 3HHx-CoA can be supplied via β-oxidation, *de novo* fatty acid biosynthesis, and unidentified pathways.[25] In addition, an artificial pathway partly using reverse β-oxidation reportedly supplied 3HHx-CoA.[26] The combination of NDFH and such pathways can be used to synthesize block copolymers from non-related carbon sources. The strategy is effective for improving productivity of the polymers.

Conclusions

In this work, the high-molecular-weight PDLA-containing block copolymers, P(3HB)-b-PDLA and P(3HHx)-b-PDLA were successfully synthesized using evolved $PhaC_{AR}$s. NDFH has a particularly high capacity incorporating LA units. The molecular weight of the PDLA segments was estimated to be 10^4–10^5 order of magnitude. The covalent linkages of P(3HHx) and PDLA segments were verified by solvent fractionation. P(3HHx) segment may facilitate the enzymatic synthesis of PDLA.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. H.T.P.: investigation; writing-original draft. S.F.: methodology; investigation. K.I.: investigation. H.T.: supervision; reviewing and editing. T.I. and T.S.: investigation. K.M.: conceptualization; supervision; writing-reviewing and editing.

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Notes

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ABBREVIATIONS

AlkK, CoA ligase; CoA, coenzyme A; D-LA, D-lactate; GC, gas chromatography; NMR, nuclear magnetic resonance; PCT, propionyl-CoA transferase; PHA, polyhydroxyalkanoate; PhaC, PHA synthase; 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; PDLA, poly(D-lactate); P(3HB), poly(3-hydroxybutyrate); P(3HHx), poly(3-hydroxyhexanoate)

SUPPORTING INFORMATION

This Supporting Information is available free of charge at:

Additional specific data: Residual 3HHx concentration in the medium; ^{1}H NMR, ^{13}C NMR, DOSY NMR; Size exclusion chromatography trace; and DSC thermograms

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SYNOPSIS

New synthetic method of isotactic PDLA segment derived from renewable resources using engineered polyester synthase.