Polymer Degradation and Stability 95 (2010) 1340–1344

Contents lists available at ScienceDirect

Polymer Degradation and Stability

journal homepage: www.elsevier.com/locate/polydegstab

Establishment of a metabolic pathway to introduce the 3-hydroxyhexanoate unit into LA-based polyesters via a reverse reaction of β-oxidation in *Escherichia coli* LS5218

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ARTICLE INFO

Article history:
Received 13 December 2009
Received in revised form
22 January 2010
Accepted 22 January 2010
Available online 2 February 2010

Keywords:
Poly(LA-*co*-3HB-*co*-3HHx)
LA-based polyester
PhaC1$_{Ps}$(ST/QK)
Microbial cell factory
Metabolic engineering
β-oxidation pathway

ABSTRACT

New lactate (LA)-based terpolymers, P[LA-*co*-3-hydroxybutyrate (3HB)-*co*-3-hydroxyhexananoate (3HHx)]s, were produced in recombinant *Escherichia coli* LS5218 harboring three genes encoding LA-polymerizing enzyme (LPE), propionyl-coenzyme A (CoA) transferase (PCT) and (*R*)-specific enoyl-CoA hydratase (PhaJ4). When the recombinant LS5218 was grown on glucose with the feeding of butyrate, 3HB-CoA and 3HHx-CoA were supplied, probably via reverse reactions of the β-oxidation pathway and PhaJ4. LPE copolymerized the two monomers 3HB-CoA and 3HHx-CoA with LA-CoA, which was generated by PCT, to yield the terpolymers. Gas chromatography analysis revealed that the terpolymers consisted of 2.7–34 mol% LA, 38–81 mol% 3HB and 17–33 mol% 3HHx units, which can be varied depending on the butyrate concentration fed in the medium. In addition, ^{1}H-^{13}C COSY NMR analysis provided evidence for a linkage between LA and 3HHx units in the polymer.

1. Introduction

The discovery of a "lactate (LA)-polymerizing enzyme (LPE)", which is an engineered polyhydroxyalkanoate (PHA) synthase to recognize the coenzyme A (CoA) ester of LA (LA-CoA) as a substrate, enabled construction of a one-step fermentation process for LA-based polyesters in recombinant bacteria with artificial metabolic pathways [1,2]. The process originates from bacterial PHA biosynthesis, in which hydroxyacyl-CoAs, most typically 3-hydroxybutyryl-CoA (3HB-CoA), are polymerized into polyesters by PHA synthase [3]. The microbial process enabled considerable change in the production of LA-based polymers compared with the conventional processes, which include fermentative production of LA and successive chemical polymerization of cyclic lactides using heavy metal catalysts [4].

The first LPE was the Ser325Thr/Gln481Lys mutant of PHA synthase 1 from *Pseudomonas* sp. 61-3 [PhaC1$_{Ps}$(ST/QK)] [5,6], which synthesizes P(LA-*co*-3HB) copolymers from two corresponding monomers, LA-CoA and 3HB-CoA, in recombinant *Escherichia coli*. Propionyl-CoA transferase (PCT) was used for converting LA to LA-CoA, while 3HB-CoA was supplied by the well-known 3HB-CoA supplying enzymes, β-ketothiolase (PhaA) and NADPH-dependent acetoacetyl-CoA reductase (PhaB). In our first report on P(LA-*co*-3HB) production, the LA fraction in the copolymer was 6 mol% [1]. Thus, in the following studies, we undertook the enrichment of LA in the copolymer for the purpose of complete regulation of monomer composition, i.e. from P(3HB) to poly(lactic acid) (PLA). For this purpose, reinforcement of the LA supply in the cell has been attempted by means of anaerobic culture conditions [7] and the use of the *E. coli* LA-overproducing mutant JW0885 [8]. These approaches successfully achieved the production of LA-enriched copolymers with up to 47 mol% LA [7].

From the viewpoint of material properties, considerable interest has been focused on novel LA-based copolyesters formed in combination with other PHA monomeric constituents. Although PLAs have certain advantageous properties, such as good processability and transparency [9], they are insufficient to meet the needs of all applications. LA-based copolyesters are expected to improve the lack of elasticity of PLA by copolymerizing medium-chain-length (MCL, C_6–C_{14}) 3-hydroxyalkanoate (3HA) units with varied fractions. In this context, LA-based copolyesters should have significant potential to expand the range of applications of the polymers, and copolymer variety is very important for exploring such desirable properties.

The purpose of this study is to synthesize LA-based polyesters comprised of MCL monomers. The MCL monomers are known to contribute to the elasticity and flexibility of polyesters when they

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doi:10.1016/j.polymdegradstab.2010.01.029

are copolymerized as a minor fraction with the 3HB unit [10]. As the initial step, we established a metabolic pathway for co-supply of both LA and MCL monomers in recombinant *E. coli*, which was achieved with the help of the interesting finding that butyrate can serve as a precursor of the 3-hydroxyhexanoate (3HHx) monomer. Here, the biosynthesis and structural analysis of a novel LA-based terpolymer, P(LA-*co*-3HB-*co*-3HHx), are reported.

2. Experimental

2.1. Plasmids

pTV118NpctC1STQKAB [1], which bears the *phaC1*$_{Ps}$(STQK) gene from *Pseudomonas* sp. 61-3 [11], the *phaA* and *phaB* genes from *Ralstonia eutropha* (also known as *Cupriavidus necator*) [12] and the *pct* gene from *Megasphaera elsdenii* [13], was modified for production of LA-based polyester comprising of MCL monomers as follows. First, a *Pst*I-*Afl*II fragment of pGEM″C1ABJ4 [11] including *phaA*, *phaB*, and (*R*)-specific enoyl-CoA hydratase (*phaJ4*) [14] genes was inserted into pTV118NpctC1STQKAB, which was digested with the same restriction enzymes, to yield pTVpctC1(ST/QK)ABJ4 bearing the *pct*, *phaC1*$_{Ps}$STQK, *phaAB*, and *phaJ4* genes. Subsequently, pTVpctC1(ST/QK)ABJ4 was digested with *Pst*I and *Nru*I, and self-ligated after blunting treatment to eliminate the *phaAB* genes. The resultant vector was referred as pTVpctC1(ST/QK)J4.

2.2. Bacterial strain and culture condition

E. coli LS5218 [*FadR*, *atoC*(Con)] [15] was used as a host for productions of LA-based polyesters. *E. coli* JM109 [16] was used as a negative control strain. *E. coli* LS5218 harboring pTVpctC1(ST/QK)ABJ4 or pTVpctC1(ST/QK)J4 were transferred into 100 ml Luria-Bertani (LB) medium containing 2% glucose, 10 mM calcium pantothenate, 100 μg/mL ampicilin and variable amounts of either sodium butyrate, sodium hexanoate (0.1, 0.25, 0.5, 0.75, 1, 1.25, and 1.5 mg/mL), or 3 mg/mL sodium dodecanoate in a 500 ml shake flask. Cells were cultivated under aerobic conditions at 30 °C for 48 h on a reciprocal shaker at 120 rpm.

2.3. Polymer extraction and analyses

The intracellular polymer was extracted with chloroform and purified by precipitation with methanol as described previously [8]. Polymer content was calculated based on the weight of extracted polymer and the dry cell weight. These polymers were subjected to further analyses.

The monomer composition of the extracted polymers were determined by gas chromatography (GC) on a Shimadzu GC-2010 system equipped with Neutra Bond-1 capillary column (30 m by 0.25 mm) and a flame ionization detector as described previously [8,17]. P(3HB-*co*-3-hydroxyvalerate-*co*-3HHx) and PLA (Toyota, Japan) were used as standards. Molecular weights of extracted polymers were determined by gel permeation chromatography (GPC) (Shimadzu, Japan) equipped with TSKgel Super HZM-H (Tosoh, Japan) using polystyrene standards (Waters, USA) to calibrate [1]. The ^{1}H-NMR spectrum of the polymer was obtained using a Bruker MSL400 spectrometer (400 MHz) and the chemical shifts are reported in ppm with tetramethylsilane as an internal reference. The ^{1}H-^{13}C COSY NMR spectrum was recorded using a JEOL JNM-A400II instrument (400MHz).

3. Results

3.1. Production of P(LA-co-3HB-co-3HHx)s in E. coli LS5218 with the feeding of butyrate

The MCL monomers of PHA are known to be supplied from fatty acids and/or lipids via the β-oxidation pathway and (*R*)-specific enoyl-CoA hydratase (PhaJ4) [18,19]. The mutant strain *E. coli* LS5218, which constitutively expresses the genes involved in fatty acid uptake and the β-oxidation pathway, is commonly used for the production of MCL PHA from fatty acids. Therefore, we first examined the production of P(LA-*co*-3HA) by cultivating *E. coli* LS5218 harboring pTVpctC1(ST/QK)ABJ4 on glucose, with a feeding of dodecanoate. However, the cells produced only 18 wt% of P(14 mol% LA-*co*-3HB) and no MCL monomers were detected, suggesting that cells did not utilize dodecanoate in the presence of more preferred carbon source, glucose. However, we previously succeeded in synthesizing LA-based polyesters consisting of the 3HV unit in *E. coli* by feeding propionate together with glucose [8]. This result suggested that *E. coli* could take in smaller fatty acids along with glucose. Therefore, we attempted to feed smaller fatty acids, butyrate and hexanoate, which serve as precursors of the monomers, 3HB-CoA and 3HHx-CoA, through β-oxidation pathway.

The *E. coli* LS5218 harboring pTVpctC1(ST/QK)J4 were grown on glucose with a feeding of hexanoate or butyrate. When hexanoate was added, cell growth was significantly inhibited and no polymer accumulated (data not shown), suggesting that hexanoate had negative effects for both cell growth and polymer synthesis. In contrast, when butyrate was fed to the medium, cells grew normally and accumulated polymers. Unexpectedly, GC analysis revealed that the polymers consisted of the 3HHx unit along with the LA and 3HB units. The changes in monomer composition of the polymers upon the addition of various concentrations of sodium butyrate are summarized in Table 1. The terpolymers P(LA-*co*-3HB-*co*-3HHx)s were produced when 0.25–1.5 mg/mL sodium butyrate was added. The highest LA fraction (34 mol%) was obtained at the sodium butyrate concentration of 1.75 mg/mL. No LA was incorporated

Table 1
Production of LA-based polyesters in recombinant *E. coli* LS5218 harboring pTVpctC1(ST/QK)J4 with feeding of butyrate.

Sodium Butyrate Conc. (mg/mL)	Cell Dry Weight (g/L)	Polymer Content (wt%)[a]	Monomer Composition (mol%)[b]			Molecular Weights[c]		
			LA	3HB	3HHx	M_n ($\times 10^3$)	M_w ($\times 10^3$)	M_w/M_n
0	4.6 ± 0.1	2.0 ± 0.6	0	100 ± 0	0	24	34	1.4
0.25	4.5 ± 0.1	2.2 ± 0.3	2.7 ± 0.3	81 ± 11	17 ± 12	15	26	1.8
0.5	4.5 ± 0.4	3.8 ± 0.5	12 ± 4	58 ± 6	30 ± 8	12	20	1.7
0.75	4.3 ± 0.3	1.6 ± 0.5	34 ± 8	38 ± 4	28 ± 4	8.7	14	1.6
1	4.6 ± 0.1	0.9 ± 0.5	29 ± 1	40 ± 1	31 ± 2	8.1	13	1.6
1.25	4.6 ± 0.1	0.6 ± 0.3	13 ± 1	49 ± 2	38 ± 1	8.3	13	1.6
1.5	4.7 ± 0.1	0.6 ± 0.1	17 ± 1	50 ± 2	33 ± 2	8.3	13	1.6

[a] Polymer content was determined based on dry cell weight and the weight of the extracted polymer.
[b] Monomer composition was determined by GC analysis. LA, lactate; 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate. Polymer content and monomer composition are presented as the average of three independent experiments.
[c] M_n, number-average molecular weight; M_w, weight-average molecular weight; M_w/M_n, polydispersity index.