A microbial factory for lactate-based polyesters using a lactate-polymerizing enzyme

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Abstract

Poly(lactic acid) (PLA) is synthesized as a representative bio-based polyester by the chemo-bio process on the basis of metal catalyst-mediated chemical polymerization of lactate (LA) supplied by microbial fermentation. To establish the one-step microbial process for synthesis of LA-based polyesters, we explored whether polyhydroxyalkanoate (PHA) synthase would exhibit polymerizing activity toward a LA-coenzyme A (CoA), based on the fact that PHA monomeric constituents, especially 3-hydroxybutyrate (3HB), are structurally analogous to LA. An engineered PHA synthase was discovered as a candidate by a two-phase in vitro polymerization system previously developed. An LA-CoA producing Escherichia coli strain with a CoA transferase gene was constructed, and the generation of LA-CoA was demonstrated by capillary electrophoresis/MS analysis. Next, when the engineered PHA synthase gene was introduced into the resultant recombinant strain, we confirmed the one-step biosynthesis of the LA-incorporated copolyester, P(6 mol% LA-co-94 mol% 3HB), with a number-average molecular weight of 1.9 × 105, as revealed by gel permeation chromatography, gas chromatography/MS, and NMR.

Introduction

The current polymer materials in common use are nearly all derived from petrochemical sources, and the industry is a significant contributor to greenhouse gas emissions, particularly during the processes of production and incineration of plastics. At present, the development of nonpetrochemical sources for plastics has focused on renewable resources, such as sugars, plant oils, and even CO2 to replace diminishing supplies of fossil fuel. Poly(lactic acid) (PLA) is a representative bio-based polyester, which is chemically synthesized by ring-opening polymerization of a cyclic diester (lactide) of lactic acid (LA), produced by microbial fermentation (the left portion in Fig. 1) (1, 2). By introducing variations in molecular weight and crystallinity, PLA is turned into highly valuable materials for biomedical, food, and general-purpose applications, as described in numerous patents. Thus, PLA combines inexpensive large-scale fermentation with chemical processing capacity to produce a value-added polymer product. However, as the chemo-process of PLA can be carried out via hazardous metal catalysts with high reaction velocities, it often leaves chemical residues that are subject to health and safety concerns. The paradigm shift from the chemo-process to the bio-process for PLA production is thus preferable to overcome this problem.

The complete biosynthesis of PLA is an enormous challenge for both academic research and industry. For this purpose, a “LA-polymerizing enzyme,” which can function as an alternative to a metal catalyst, would be desired to establish the bio-process, as shown in Fig. 1. The simplest strategy would be the discovery of a PLA-producing micro-organism, but this approach has not succeeded yet. Thus, we focused on the microbial biosynthetic system for polyhydroxyalkanoates (PHAs), which are natural polyesters, as energy storage materials. PHAs are also biodegradable and biocompatible bio-based polyester (3, 4). We attempted to convert the present chemo-process of PLA to the one-step bio-process based on the well-established metabolic strategy for the synthesis of PHAs. PHA synthase functions as a key enzyme for polymerization of various monomers, depending on its substrate specificity. In this study, we presumed that if a PHA synthase capable of LA-polymerization were to be obtained, the recombinant microbe transformed with this PHA synthase gene would synthesize LA-based polyester. Namely, it would imply the recruitment from PHA synthase to a LA-polymerizing enzyme. This hypothesis, based on the substrate specificity of the enzyme, arises from the fact that monomeric constituents of PHA share the common chemical structure, hydroxy acid, with 2-hydroxypropionate (the same as LA).

An attempt to construct a microbial production system for an LA-based polyester has been triggered by the discovery of an engineered PHA synthase with acquired LA-polymerizing activity, as revealed by an in vitro polymerization system (5). Based on this finding, we have established a recombinant Escherichia coli strain for LA-based polyester. PLA has been synthesized by the chemo-bio process. Copolymerization of LA with other monomer units has also been performed by basically the same chemo-synthetic procedures. In bio-process with microbes, LA monomer substrate is recognized as a CoA form (LA-CoA) by LA-polymerizing enzyme that is recruited from microbial PHA synthase. A candidate PHA synthase with acquired LA-polymerizing activity was selected based on the water-organic solvent two-phase in vitro polymerization system (5).

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coli that allows the synthesis of LA-based polyester by introducing the gene encoding PHA synthase discovered here. The bio-process serves a versatile platform as a “microbial factory” for the one-step production of the LA-based polyester from renewable biomass (see Fig. 1).

Results
Selection of a PHA Synthase with LA Polymerization Activity. PHA synthase is thought to synthesize polyester via continuous trans-esterification of coenzyme A (CoA) esters of typically 3-hydroxyalkanoates (3HAs), including 3-hydroxybutyrate (3HB) (6). Thus, the most promising precursor for PLA biosynthesis is the CoA ester of lactate, lactyl-CoA (LA-CoA). To explore the PHA synthase capable of polymerizing the LA moiety in LA-CoA, we used a modified water-organic solvent two-phase in vitro system developed previously (5). In examining LA-polymerizing activity, an in vitro system is advantageous, because the targeted monomer substrates can be controllably generated without the aid of monomer-supplying metabolic pathways for LA-CoA and 3HB-CoA. The polymerizing activity of PHA synthase was primarily judged by the generation of the polymer-like precipitation.

The classification of the PHA synthase family was the basis for the selection of PHA synthase as a candidate of LA-polymerizing enzyme. Four major classes of PHA synthases can be distinguished with respect to the primary structures deduced from these sequences, the substrate specificities of the enzymes, and the subunit composition (7). In this study, we examined four representative PHA synthases belonging to the individual classes; synthases derived from Ralstonia eutropha (class I), Pseudomonas sp. 61–3 (class II), Synechocystis sp. PCC6803 (class III), and Bacillus sp. INT005 (class IV), and three engineered PHA synthases (PhaC1) from Pseudomonas sp. 61–3. The engineered PHA synthases were two single mutants [Ser325Thr (ST) and Gln481Lys (OK)] and one double mutant carrying these mutations (STQK). The two positions (325 and 481) were closely related to the activity or substrate specificity of the enzyme (8, 9). These mutants were selected from the huge mutant library of PhaC1 that has been created through evolutionary engineering directed to the reinforcement of 3HB incorporation ability into the PHA polymer chain (10, 11).

The PHA synthases selected for in vitro assay intrinsically polymerize 3HB-CoA into P(3HB). When LA-CoA was generated, no clear polymer-like precipitation occurred for any of the enzymes, suggesting that the PHA synthases hardly catalyzed any polymerization of LA-CoA. Thus, we next set out to generate 3HB-CoA together with LA-CoA. This experiment was set up for facilitating polymerization of LA-CoA by adding the favorable substrate 3HB-CoA to consequently synthesize the copolymer, based on the case of wild-type PhaC1 from Pseudomonas sp. 61–3 that the coexistence of favorable substrate(s), 3HA-CoA, leads to the enhanced polymerization of less favorable substrate, 3HB-CoA (12). When LA-CoA and 3HB-CoA were supplied, the mutant PhaC1STQK clearly exhibited polymer-like precipitation. Gas chromatography/MS (GC/MS) analysis revealed that the precipitant consisted of 36 mol% of the LA unit [supporting information (SI) Fig. S1]. The result strongly suggested that PhaC1STQK polymerized LA-CoA in the presence of 3HB-CoA. By contrast, the other enzymes did not synthesize any polymer. Therefore, we selected PhaC1STQK as a promising candidate of LA-polymerizing enzyme for further investigation.

Construction of a Recombinant E. coli Strain Generating LA-CoA. The finding of an engineered PHA synthase (PhaC1STQK) with the capacity to polymerize LA-CoA as a substrate prompted us to create a microbial production system of LA-based polymers, such as PLA and P(LA-co-3HB). Another essential component for microbial production of LA-based polymers is an intracellular supplier of LA-CoA. We used propionyl-CoA transferase (PCT) as a LA-CoA supplier because PCT catalyzes the generation of LA-CoA from lactate (acceptor) and acetyl-CoA (donor) (13). The ability of PCT to synthesize LA-CoA was examined using recombinant E. coli harboring the pct gene. By capillary electrophoresis/MS (CE/MS) analysis using the prepared recombinant cells, we found a peak, whose retention time and molecular masses, 838 and 419 (corresponds to bivalent ion), were identical to those of the LA-CoA standard (Fig. 2A).

Microbial Production of LA-Based Polyester Using PhaC1STQK. Based on the metabolic pathways for generation of the monomer substrates, LA-CoA and 3HB-CoA (Fig. 2B), we constructed two plasmids, pTV118N pctC1(STQK) (for PLA) and pTV118N pctC1AB(STQK) (for copolymer), respectively. The phaAB genes encode β-ketothiolase (PhaA) and acetacetyl-

Fig. 2. Construction of microbial production system for LA-based polyester. (A) CE/MS analysis of LA-CoA generated in recombinant Escherichia coli harboring the pct gene. The ions of m/z = 838 and 419 correspond to monovalent and bivalent ions of LA-CoA, respectively. pTV118N, control vector; pTV118N pct, expression vector for the pct gene. (B) LA-based polyester synthetic pathway in recombinant E. coli. The letters in boxes indicate the enzyme. LDH, lactate dehydrogenase; PCT, propionyl-CoA transferase; PhaA, β-ketothiolase; PhaB, NADPH-dependent acetacetyl-CoA reductase; PhaC, PHA synthase. (C) Construction of the plasmids used in this study. PRe and TRe denote respectively the promoter and terminator regions of phbCAB operon from Ralstonia eutropha. PRe denotes the lac promoter. The phaC1(STQK) gene encodes the Ser325Thr/Gln481Lys mutant of PHA synthase from Pseudomonas sp. 61–3. The phaA and phaB genes encode β-ketothiolase (PhaA) and NADPH-dependent acetacetyl-CoA reductase (PhaB) derived from R. eutropha, respectively. The pct gene encodes a propionyl-CoA transferase from Megasphaera elsdenii.
CoA reductase (PhaB), both of which are the most typical supplying pairs for 3HB-CoA generation from acetyl-CoA (see Fig. 2B). We next extracted polymer from recombinant E. coli strains harboring each plasmid and analyzed the extracts using GC/MS. The strain harboring pTV118NpctC1(STQK) did not produce detectable polymer under the conditions used. However, the recombinant harboring pTV118NpctC1AB(STQK), in which the phaAB genes were coexpressed, produced 19 wt% of polymer. GC/MS analysis revealed the presence of 6 mol% of the LA unit in the polymer and the 3HB unit as a main component (Fig. S2), suggesting that the combination of PhaC1STQK and PCT together with PhaAB does produce the P(LA-co-3HB) copolymer.

Characterization of LA-Based Polyester Produced in the Recombinant Strain. To further confirm that the LA unit was incorporated into the polymer chain, the extracted polymer was applied to preparative gel permeation chromatography (GPC), and the high-molecular-weight polymer fraction was collected. The molecular weights of the fractionated sample were determined to be $M_n = 1.9 \times 10^5$ and $M_w = 4.8 \times 10^5$, respectively, using analytical GPC (Table 1). The fractionated sample was subjected to GC/MS and NMR analyses. The mass spectrum of the peak at 6.5 min was identical to that of the ethyl-LA standard, indicating that the fraction contains the LA unit (Fig. 3A). Furthermore, $^{13}$C-NMR analysis revealed that the polymer had typical peaks of 3HB unit in P(3HB) and LA unit in PLA (Fig. 3B). However, in $^1$H-NMR analysis, small peaks appeared at 1.5 and 5.0 ppm, which slightly differed from those of PLA (1.6 and 5.1 ppm) (Fig. 3C). The high-field shifts of the peaks are presumably because of the presence of the LA unit within the backbone of P(3HB). Indeed, 2d-NMR revealed cross signals between the carbons of LA and the LA-like peaks of protons (Fig. 3D). Thus, the proton peaks

**Table 1. Properties of bio-based polyesters**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Monomer composition (mol%)</th>
<th>Molecular weight</th>
<th>Thermal properties $^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LA</td>
<td>3HB</td>
<td>$M_n (10^5)$</td>
</tr>
<tr>
<td>P(LA-co-3HB)</td>
<td>6</td>
<td>94</td>
<td>1.9</td>
</tr>
<tr>
<td>P(3HB)</td>
<td>0</td>
<td>100</td>
<td>2.9</td>
</tr>
<tr>
<td>PLA</td>
<td>100</td>
<td>0</td>
<td>nd</td>
</tr>
</tbody>
</table>

$^*$Monomer composition was determined by $^1$H-NMR. LA, lactate; 3HB, 3-hydroxybutyrate. $M_n$, number-average molecular weight; $M_w$, weight-average molecular weight; $M_w/M_n$, polydispersity index; nd, not determined.

$^1$Thermal properties are measured by differential scanning calorimetry. $T_g$, glass transition temperature; $T_m$, melting temperature.

![Fig. 3.](image-url)
at 1.5 and 5.0 ppm were assigned to LA, but were differentiated from PLA. LA molar fraction in the copolymer was 6 mol% based on the $^{1}$H-NMR.

Based on the number-average molecular weight (1.9 × 10$^{6}$) and LA molar fraction (6 mol%), the number of LA units was estimated to be much higher than that of the case in which the LA unit is incorporated as a terminator into the end of the polymer chains. Thus, the LA unit should be incorporated into the internal region of the polymer chain. In addition, the peak positions of $^{1}$H-NMR corresponding to the LA unit were not identical to those of PLA. This suggests that most of the LA units are distributed separately from each other and connected with the 3HB unit. Furthermore, the P(LA-co-3HB) has lower melting temperature ($T_m$) than P(3HB) that is probably because of the lowered crystallinity caused by random copolymerization of LA and 3HB units (see Table 1). In fact, the decrease in $T_m$ agrees with the result that chemically synthesized P(LA-co-3HB) random copolymers have lower $T_m$ compared with P(3HB) (14). Therefore, the P(LA-co-3HB) would be synthesized in a random manner rather than a blend or block copolymerization of PLA and P(3HB). From these results, we concluded that the polymer produced by recombinant strain was P(6 mol% LA-co-94 mol% 3HB) with a possible random sequence.

Discussion

In this study, the substitution of metal catalysts with an LA-polymerizing enzyme was a key methodology to convert the chemo-process of LA-based polyesters into a microbial bio-process (see Fig. 1). The desired LA-polymerizing enzyme has been recruited from PHA synthase, which is an essentially participating in biosynthetic pathway in the microbial polyester production system. The general structural formula of PHAs is presented as [−O-CHR-(CH$_2$)$_x$-CO$_2$H]. R basically presents hydrogen or alkyl chains of up to C$_7$ in length, and x can range from 1 to 3 or more. These variations in the length and composition of the side and main chains are the basis for the diversity of the PHA family and the material properties. When R is a methyl group and x = 1, the polyester is P(3HB). When R is a methyl group and x = 0 (equals to LA), the polymeric product is PLA. In the naturally occurring micro-organisms, LA has not been identified to date as a monomeric constituent in the PHAs. This fact suggests the absence or very scarce distribution of PLA synthases.

To date, the substrate specificity of the PHA synthases has been mainly discussed based on the alkyl chain length of the side chain built at the 3 position of hydroxy acids (7, 10, 11). On the other hand, the LA-reactivity of PHA synthase should be related to the substrate specificity toward the main chain length or conformation of hydroxyl groups. Wild-type PhaC1 from Pseudomonas sp. 61–3 has especially broad side-chain substrate specificity and its mutant (STQK) has enhanced activity toward LA (12). This concept is applicable for creating LA-based polyesters with various monomeric compositions, as well as the PLA from renewable carbon sources. In the future, this prototype system will be advanced by improvement of the LA-CoA supplying pathway. The flux of LA-CoA in vivo should be a limiting factor for the production of LA-enriched copolymer, because in vitro the system produced P(LA-co-3HB) with higher LA fraction. The intracellular generation level of LA-CoA should be affected by two factors: generation level of LA itself and the LA-CoA converting efficiency of PCT. Because LA is known to efficiently produce under the anaerobic condition, fine tuning of such a condition would optimize LA production. In addition, the total activity of PCT could be increased by enhancing expression level and enzyme engineering of PCT.

Materials and Methods

Selection of PHA Synthases by Using a Water-Oligosaccharide Two-Phase in Vitro Polymerization System. Thiophenyl (R)-3HB-((R)-3HB-TP) and thiophenyl (R)-(R)-LA-((R)-LA-TP) were synthesized as described in ref. S. (R)-(R)-3HB-TP and (R)-LA-TP, precursors of CA derivatives, were dissolved in 5 ml of hexane (organic solvent phase) to give 5 mM, respectively. The water phase contained 100-mM sodium phosphate (pH 7.5) and 1-mM CoA. We combined the two solutions prepared above to make the two-phase system. The polymerization was started by adding PHA synthase to the water phase, and the mixture was incubated at 30°C for 72 h. Seven PHA synthases were examined as mentioned in Results. All synthases were purified using a His-tag system (16). Candidate of PHA synthase with LA polymerization activity was chosen based on the amount of the generated polymer-like precipitant.

Plasmids. The pct gene from Megasphaera elsdenii (17) was amplified from genomic DNA by PCR using following primers: 5′-ATGAGAAATAGTAAATCATCATC-3′ and 5′-TTATTTTTTTGTCCACCCAGCCTGT-3′. A 1.6-kb fragment of pct gene was inserted into pTV118N (Takara, Japan) using TOPO PCR cloning kit (Invitrogen). Subsequently, the Ser325Thr/Gln481Lys mutated PHA synthase gene from Pseudomonas sp. 61–3 was inserted into pTV118N using EcoRI and PstI as described in ref. 5. The pTV118N plasmid was transformed into P. putida KT2440 cells by electroporation and amplified in E. coli JM109 (20) harboring the resultant plasmid was grown on LB medium at 37°C for 3 h. The final 10-mM isopropyl thio-galactopyranoside was added to induce the expression and the mixture was incubated at 30°C for 6 h. The cells were collected by centrifugation and transferred to M9 medium containing 1.5% glucose, 10-mM MgSO$_4$, and 10-mM calcium pantothenate, and cultivated at 37°C for 18 h. The harvested
cells were washed with water twice and extracted with methanol. Next 1.6 ml of chloroform was added to the same amount of methanol extract, and 640 μl of water was added and mixed. The solution was centrifuged and upper phase was applied to ultrafiltration (50 kDa) to remove contaminants with higher molecular weight. The path-through fraction was lyophilized and applied to GC/MS analysis as described in ref. 22. The ions of m/z = 838 and 419, which corresponds to monovalent of LA-CoA and its bivalent ion, respectively, were monitored.

**Polymer Extraction from Recombinant Cells.** Recombinant E. coli JM109 harboring pTV118NpctC1A(STQK) and pTV118NpctC1A(STQK) were grown on 100-ml LB medium containing 2% glucose, 10 mM calcium pantothenate, and 50 μg/mL ampicillin at 30°C for 72 h. The harvested cells were lyophilized and polymer was extracted with 5-ml chloroform at 100°C for 3 h. Cell debris was removed by passing through a PTFE filter. Then, 100-mL methanol was added to precipitate polymer. Solubility of the polymer was very similar to that of PHB or PLA: that is, soluble in chloroform and dichloromethane but insoluble in methanol and hexane. The precipitant was dried in vacuo to measure the weight of polymer, and polymer content was calculated based on cell dry weight. The polymer was subjected to further analyses.

**GC/MS, GPC, and NMR Analyses.** Of the polymer-like precipitate, 12.5 mg recovered from E. coli harboring pTV118NpctC1A(STQK) was dissolved in 250 μl of chloroform (50 μg/mL) and the solution was applied to GC/MS analysis as described in ref. 22. The recombinant E. coli harboring pTV118NpctC1A(STQK) did not obviously produce polymer, thus all chloroform soluble fraction was dried up, dissolved in 250-μl chloroform, and applied to GC/MS analysis. This precipitant was also applied to preparative GPC LC2901 (JAI, Japan) equipped with JAI/GEl-1H column (JAI, Japan). The high molecular weight fraction was collected and dried up to yield a white film. The fractionated polymer was further applied to analytical GPC (Shimadzu) equipped with TSKgel Super HZM-H (Tosoh). The molecular weight was estimated using polystyrene standard (Waters). Of the fractionated film, 12.5 mg was dissolved in 250 μl of chloroform (50 μg/mL), and the solution was applied to GC/MS analysis again. The fractionated film was dissolved in CDCl3 and analyzed by 1H-NMR and 13C-NMR spectroscopy.

**Differential Scanning Calorimetry Analysis.** Differential scanning calorimetry data were recorded in the temperatures ranging from −90 to 210°C on a Perkin-Elmer Pyris 1 instrument equipped with a cooling accessory under a nitrogen flow rate of 20 ml/min. The solvent-cast films (10 mg) were encapsulated in an aluminum pans and heated from −50 to 210°C at 20°C/min (first heating scan). The melt samples then followed by rapid quenching at −90°C, and maintained at −90°C for 5 min. They were heated from −90 to 210°C at 20°C/min (second heating scan). The glass-transition temperature (Tg) was taken as midpoint of the heat capacity change. The melting temperature (Tm) was determined from positions of the endothermic peaks. P(3HB) was produced using recombinant E. coli and PLA was purchased from Toyobo (Japan).

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**References.**

Supporting Information

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Fig. S1. GC/MS analysis of LA-based polyester produced by in vitro system. Ethyl-LA, ethyl lactate; Ethyl-3HB, ethyl 3-hydroxybutyrate.
Fig. S2. GC/MS analysis of LA-based polyester produced from the recombinant *E. coli* harboring the plasmid pTV118NpctC1AB(STQK). Ethyl-LA, ethyl lactate; Ethyl-3HB, ethyl 3-hydroxybutyrate.