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NOTE

Preparative synthesis of Poly[(*R*)-3-hydroxybutyrate] monomer for enzymatic cell-free polymerization

Satoshi Tomizawa, Masako Yoshioka, Kazunori Ushimaru and Takeharu Tsuge

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INTRODUCTION

Polyhydroxyalkanoates (PHAs) are produced as a carbon and energy storage material in various bacteria, and have received much attention as biodegradable and biocompatible plastics. PHAs can be produced from renewable carbon sources such as sugars, fatty acids and plant oils by fermentation.[1] Poly[(*R*)-3-hydroxybutyrate], P(3HB), is the most widely studied and best characterized PHA. The physical properties of P(3HB) are similar to those of isotactic polypropylene.

PHA synthase (PhaC) is an enzyme that catalyzes a PHA polymerization reaction. To form P(3HB), PhaC requires an *R*-enantiomer 3-hydroxybutyryl coenzyme A ((*R*)-3HB-CoA) as a substrate and polymerizes the acyl moiety of (*R*)-3HB-CoA. In 1995, Gerngross and Martin[2] demonstrated the first enzymatic cell-free synthesis of P(3HB) by using purified PhaC and (*R*)-3HB-CoA. Subsequently, other groups have evolved the cell-free synthesis in terms of monomer supply and polymer yield.[3–7] Cell-free synthesis is useful not only for polymer production but also for the functional analysis of PhaC. However, technical difficulties in the preparation of (*R*)-3HB-CoA monomer still limit the ability of cell-free synthesis. Conventional preparation methods require the purification of (*R*)-3HB-CoA because the synthesis reactions are performed in organic solvents and use large quantities of starting chemicals relative to CoA.[8,9] These solvents and excess chemicals seriously inhibit enzyme activity when the as-prepared monomers are used for cell-free synthesis. Thus, it is desirable to develop a method for the preparation of (*R*)-3HB-CoA that does not use organic solvents and large quantities of starting chemicals.

Apart from the cell-free synthesis, chemical recycling of waste plastics should be promoted from the viewpoint of sustainability. By thermal degradation, P(3HB) can be converted into crotonic acid with a high yield.[10,11] Furthermore, the intermolecular dehydration of two crotonic acids generates crotonic anhydride. Recently, it has been shown that crotonic anhydride is generated as a minor product from P(3HB) by thermal degradation.[12] It is, thus, worth utilizing crotonic acid and its anhydride as recovered feedstock.

In this study, we focused on the synthesis of (*R*)-3HB-CoA using crotonic anhydride and CoA as the starting materials in an aqueous solution (Figure 1a). By mixing crotonic anhydride and CoA at a molar ratio of 1:1, crotonyl-CoA was efficiently generated by an abiotic ester-exchange reaction. The crotonyl-CoA was then *R*-specifically hydrated using (*R*)-specific enoyl-CoA hydratase (PhaJ) to generate (*R*)-3HB-CoA. This method was compared with (*R,S*)-3HB-CoA synthesis, using β-butyrolactone and CoA as the starting materials (Figure 1b) in terms of the reaction kinetics. Here, we demonstrate that the as-prepared (*R*)-3HB-CoA by the method described here can be used for cell-free synthesis and achieves a very high P(3HB) molecular weight.

EXPERIMENTAL PROCEDURE

Materials

Crotonic anhydride and β-butyrolactone were purchased from Sigma (St Louis, MO, USA), and CoA lithium salt was purchased from Wako Pure Chemical Industries (Osaka, Japan). The two enzymes used in this study, PhaJ from *Aeromonas caviae* FA440 and His-tagged PhaC from *Ralstonia eutropha* H16, were prepared as described previously.[13,14] Their enzyme purities were confirmed using SDS–polyacrylamide gel electrophoresis.

Free CoA assay

To determine the concentration of free CoA in the final reaction solution, Ellman's reagent, 5,5′-dithiobis(2-nitrobenoic acid) (DTNB, Kanto Chemicals, Tokyo), was used. The DTNB solution was mixed with an aliquot of the reaction solution to give 1 mM DTNB concentration, and the absorbance was measured at 412 nm. The molar absorbance coefficient $\varepsilon_{412} = 13.6 \times 10^3\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$ was used to determine the concentration of free CoA.[15]

Enzyme activity assay

The hydratase activity of PhaJ was determined by monitoring the absorbance at 263 nm caused by the double bond of crotonyl-CoA.[13] An activity assay of PhaC was performed by measuring the free CoA released from (*R*)-3HB-CoA during P(3HB) polymerization, using DTNB. One unit of enzyme activity (U) was defined as the amount of enzyme required to catalyze a 1-μmol substrate

Department of Innovative and Engineered Materials, Tokyo Institute of Technology, Yokohama, Japan
Correspondence: Dr T Tsuge, Department of Innovative and Engineered Materials, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8502, Japan.
E-mail: tsuge.t.aa@m.titech.ac.jp
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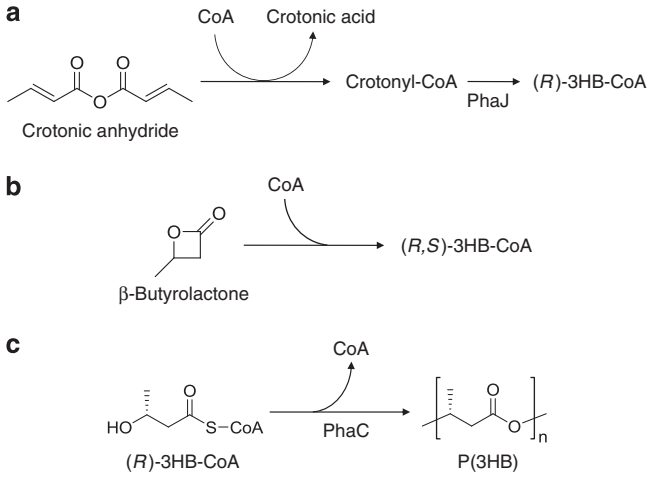

Figure 1 (**a**) Ester-exchange reaction between CoA and crotonic anhydride, followed by *R*-specific hydration by (*R*)-specific enoyl-CoA hydratase (PhaJ) to yield *R*-enantiomer 3-hydroxybutyryl coenzyme A ((*R*)-3HB-CoA). (**b**) Ester-exchange reaction between CoA and β-butyrolactone, yielding racemic (*R,S*)-3HB-CoA. (**c**) Polymerization reaction catalyzed by PHA synthase (PhaC).

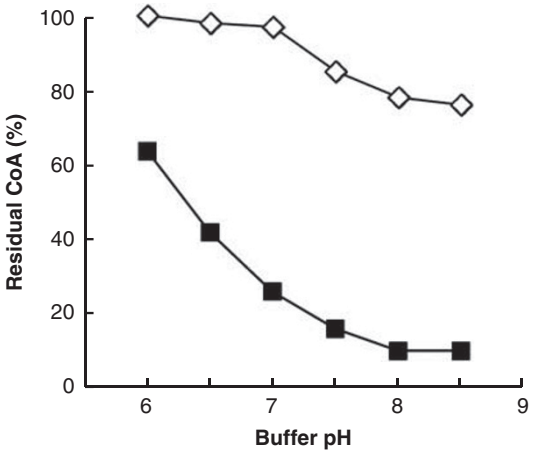

Figure 2 Effect of pH on ester-exchange reaction between CoA and crotonic anhydride (■) or β-butyrolactone (◇). Residual CoA concentrations after 10 min of reaction performed in 100 mM potassium phosphate buffer at 30 °C are shown.

decrease in 1 min. The protein concentration was determined by a fluorescence-based method using a Qubit Protein Assay Kit (Invitrogen, Carlsbad, CA, USA), with bovine serum albumin as the standard.

Abiotic ester-exchange reaction

The ester-exchange reaction between CoA and crotonic anhydride or β-butyrolactone was investigated in potassium phosphate buffer at 30 °C with different pH (6.5–8.0) and buffer concentrations (20, 100, 500 mM). The initial CoA concentration was set at 5 mM. The molar ratio of CoA to crotonic anhydride in the reaction solution was 1:1 whereas the molar ratio of CoA to β-butyrolactone was 1:10. This difference was caused by the low reaction efficiency between β-butyrolactone and CoA.

Cell-free synthesis of P(3HB)

The synthesis of P(3HB) from crotonic anhydride was carried out at 30 °C with a total volume of 5 ml. The initial reaction mixture contained 5 mM CoA lithium salt and 5 mM crotonic anhydride in 20 mM phosphate buffer (pH 8.0) to generate crotonyl-CoA by an ester-exchange reaction. Later, PhaJ (1 U) and PhaC (1 U) were added to the reaction mixture at the same time to initiate hydration and polymerization reactions, respectively. The polymerization progress was followed by the free CoA released by using DTNB. In addition, the P(3HB) structure formation during the reaction was followed semiquantitatively by measuring the turbidity at 600 nm.

High performance liquid chromatography analysis

The CoA derivatives were analyzed by high performance liquid chromatography using a 10A LC-VP system (Shimadzu, Kyoto, Japan) equipped with an ultraviolet–visible detector.[6] Effluents were detected at 254 nm because of the ultraviolet absorption by the adenyl moiety of CoA.

Molecular weight analysis

The P(3HB) synthesized in the cell-free system was collected using centrifugation. The dried P(3HB) pellet was dissolved in chloroform and purified by precipitation with methanol. The number-average molecular weight (M_n), weight-average molecular weight (M_w) and molecular weight distribution (M_w/M_n) were determined by gel permeation chromatography (GPC) at 40 °C, using a Shimadzu 10A GPC system equipped with a Shimadzu 10A refractive index detector. Low polydispersity polystyrene standards were used to make the molecular weight calibration curve. In addition, the absolute molecular weight ($M_{w(\mathrm{MALLS})}$) of the P(3HB) was determined using the above GPC system equipped with a Dawn Heleos 8+ multi-angle laser light scattering (MALLS) photometer (Wyatt Technology, CA, USA).

RESULTS AND DISCUSSION

Figure 1a shows the (*R*)-3HB-CoA synthetic route using crotonic anhydride and CoA as the starting materials. The first step of this scheme is the abiotic synthesis of crotonyl-CoA by an ester-exchange reaction between the crotonic anhydride and thiol group of CoA. The resulting crotonyl-CoA is then *R*-specifically hydrated by the action of PhaJ, yielding (*R*)-3HB-CoA. $\mathrm{PhaC_{Re}}$ polymerizes the acyl moiety of (*R*)-3HB-CoA by releasing free CoA, as shown in Figure 1c. All of the reactions could be carried out in an aqueous solution under moderate reaction conditions. As a comparative study, (*R,S*)-3HB-CoA synthesis was also carried out using β-butyrolactone and CoA (Figure 1b).[9]

The effect of the pH on the ester-exchange reaction was investigated. Figure 2 shows the amount of residual CoA after the ester-exchange reaction for 10 min at each pH. Both the crotonic anhydride and β-butyrolactone showed increasing reaction yields at higher pH values. However, it should be considered that PhaC and PhaJ favor a neutral pH in the range of 6.5–8.0.[16] Thus, it is better to adjust the pH of the buffer solution to 8.0 to directly use the reaction solution for subsequent P(3HB) polymerization.

To monitor the progression of the ester-exchange reaction at pH 8.0 with different buffer concentrations, the time-dependent change in the CoA concentration was measured using a DTNB assay. By mixing crotonic anhydride and CoA in an aqueous solution, the ester-exchange reaction immediately proceeded as shown in Figure 3. In 20 mM of phosphate buffer, 90% of the CoA was converted into crotonyl-CoA within 30 min of incubation. Further incubation up to 60 min slightly increased the yield of crotonyl-CoA (data not shown). A more rapid reaction was observed at high buffer concentrations. In 500 mM of phosphate buffer, >90% of the CoA was converted within only 1 min of incubation. The ester-exchange reaction between the crotonic anhydride and CoA was much faster than that between the β-butyrolactone and CoA.

Figure 4 shows the P(3HB) polymerization reaction conducted at different buffer concentrations. A low buffer concentration (low ionic strength) was preferable for the polymerization by PhaC, which is in

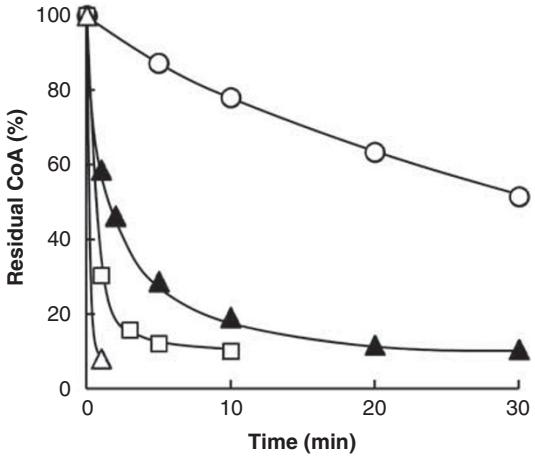

Figure 3 Time-dependent changes in CoA concentration in ester-exchange reaction at different buffer concentrations. Ester-exchange reaction between CoA and crotonic anhydride in potassium phosphate buffer at 20 mM (▲), 100 mM (□) and 500 mM (△). Ester-exchange reaction between CoA and β-butyrolactone in 100 mM potassium phosphate buffer (○).

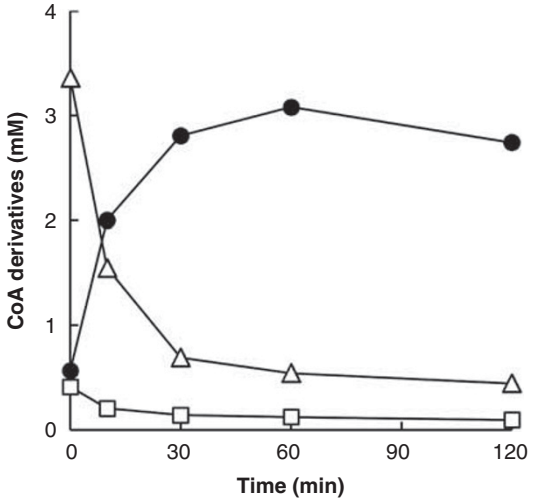

Figure 5 High performance liquid chromatography (HPLC) analysis of CoA-derivative concentrations during poly[(*R*)-3-hydroxybutyrate] (P(3HB)) polymerization, using as-prepared *R*-enantiomer 3-hydroxybutyryl coenzyme A ((*R*)-3HB-CoA) from crotonic anhydride and CoA: (●) free CoA, (□) crotonyl-CoA and (△) 3HB-CoA.

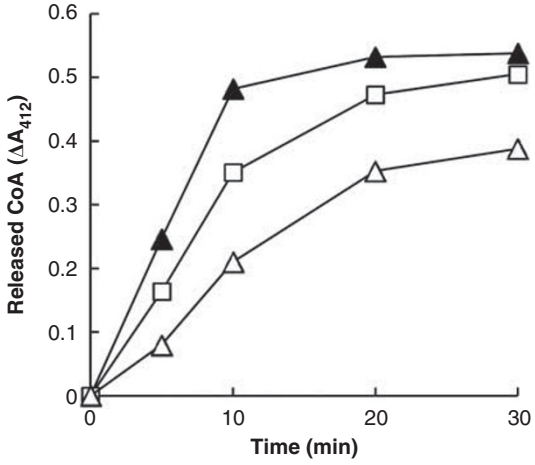

Figure 4 Released CoA monitoring during poly[(*R*)-3-hydroxybutyrate] (P(3HB)) polymerization at 20 mM (▲), 100 mM (□) and 500 mM (△) potassium phosphate buffers.

good agreement with a previous report.[16] Although the ester-exchange reaction was relatively slow at a low buffer concentration, 20 mM buffer was used for further experiments by primarily considering the PhaC activity.

To synthesize P(3HB) from crotonyl-CoA, two enzymes, PhaJ and PhaC, were added to the reaction mixture at 30 min after initiating the ester-exchange reaction. The concentrations of CoA and its derivatives after the addition of these enzymes were determined using high performance liquid chromatography and are shown in Figure 5. The concentration of crotonyl-CoA decreased to 0.4 mM immediately after PhaJ was added. Thus, (*R*)-3HB-CoA was already abundant (3.4 mM) at 0 min. In association with the P(3HB) polymerization, the CoA concentration increased to the maximum of 3.1 mM after 60 min. After 120 min of incubation, the concentrations of crotonyl-CoA, 3HB-CoA and CoA in this reaction mixture were 0.1, 0.4 and 2.7 mM, respectively. On the basis of the concentrations of the CoA derivatives, the yield of each reaction was estimated as follows: hydration reaction by PhaJ, 0.82 (mol mol^{-1}); polymerization reaction by PhaC, 0.78 (mol mol^{-1}). Accordingly, considering the ester-exchange reaction yield of 0.9 (mol mol^{-1}), the overall reaction yield from crotonic anhydride to P(3HB) was calculated to be 0.58 (mol mol^{-1}).

The molecular weight of the synthesized P(3HB) was determined by GPC, using polystyrene standards. The M_n, M_w and polydispersity index (M_w/M_n) were 400×10^4 g mol^{-1}, 640×10^4 g mol^{-1} and 1.6, respectively. Furthermore, to ensure accurate molecular weight, $M_{w(\mathrm{MALLS})}$ of P(3HB) was determined by GPC-MALLS to be 580×10^4 g mol^{-1}. Usually, bacterially synthesized P(3HB) shows an M_w value in the range of (30–150) $\times 10^4$ g mol^{-1}. Therefore, the P(3HB) synthesized in this study had a very high molecular weight. The monomer prepared here proved to be useful for the synthesis of P(3HB) with a high molecular weight. Such a high-molecular-weight P(3HB) can be processed into strong fibers and films more easily than the usual P(3HB).[17] Thus, this result suggests the feasibility of upgrade recycle from usual P(3HB) to high-molecular-weight P(3HB) by combining thermal degradation and enzymatic cell-free synthesis.

To increase the P(3HB) yield by recycling the released CoA in the reaction system, we tried to add crotonic anhydride to the reaction solution during the polymerization reaction. An high performance liquid chromatography analysis revealed that the free CoA released by PhaC successfully reacted with the crotonic anhydride, and was then converted to (*R*)-3HB-CoA by PhaJ (data not shown). However, the progression of the polymerization was not observed because the crotonic anhydride seriously inhibited the PhaC activity. It should be noted that, in the above preparative method, the crotonic anhydride has to be completely reacted before being used for the cell-free synthesis.

CONCLUSIONS

In this study, we developed a method to prepare (*R*)-3HB-CoA for cell-free P(3HB) synthesis. By mixing crotonic anhydride and CoA in an aqueous solution, crotonyl-CoA was generated via an ester-exchange reaction. This reaction was much faster than that between β-butyrolactone and CoA. A higher pH and higher buffer

concentration enabled an efficient ester-exchange reaction, whereas PhaC preferred a low buffer concentration and neutral pH. The as-prepared (*R*)-3HB-CoA with low buffer concentration at pH 8.0 was subjected to cell-free P(3HB) synthesis. A molecular weight analysis revealed that the synthesized P(3HB) was of a very high molecular weight. Thus, we demonstrated that the as-prepared (*R*)-3HB-CoA was applicable to cell-free synthesis without any further treatment.

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