Chemoenzymatic Synthesis of Poly(3-hydroxybutyrate) in a Water-Organic Solvent Two-Phase System

Kenji Tajima,* Yasuharu Satoh, Koji Nakazawa, Hatsumi Tannai, Tomoki Erata, and Masanobu Munekata

Division of Molecular Chemistry, Graduate School of Engineering, Hokkaido University, Sapporo 060-8628, Japan

Motoaki Kamachi

Showa Denko K.K., Specialty Chemicals Division, Chemical Sector, Kawasaki 210-0867, Japan

Robert W. Lenz

Department of Polymer Science and Engineering, University of Massachusetts, Amherst, Massachusetts 01003

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ABSTRACT: We succeeded in developing of a new method for in vitro poly(3-hydroxybutyrate) (P(3HB)) synthesis with coenzyme A (CoA) recycling. Thiophenyl (*R*)-3-hydroxybutyrate was used as a precursor for the monomer, (*R*)-3-hydroxybutylCoA ((*R*)-3HBCoA). The (*R*)-3HBCoA was synthesized by the ester exchange reaction between thiophenyl (*R*)-3-hydroxybutyrate and CoA. Although the polymerization reaction in the buffer (water phase) was performed, the reaction did not occurred. The results of a PHA synthase assay in the presence of thiophenol suggested that the reaction was inhibited by a small amount of thiophenol. To avoid the inhibition of PHA synthase by thiophenol, a water−organic solvent two-phase reaction system was developed for the polymerization reaction. The water−organic solvent two-phase reaction system has been used previously for the bioconversion of substrates with low solubility in water; it was used to remove thiophenol released during the ester exchange reaction from the water phase. Thiophenyl (*R*)-3-hydroxybutyrate and PHA synthase were dissolved in the organic solvent and water phases, respectively. Ester exchange between thiophenol and CoA occurs at the interface of water and the organic solvent phase, and the (*R*)-3HB 3-hydroxybutylCoA synthesized was polymerized sequentially by PhaC. The organic solvents with high a logP_{ow} of more than 3.9 such as hexane, octane, decane, or dodecane were found to be suitable for the organic solvent phase in the two-phase system. The polymerization reaction mixture showed signs of turbidity in 2 h, after which white precipitates formed. The structural analyses by ^{1}H NMR spectroscopy indicated that the product was P(3HB). The weight-averaged molecular weight and molecular weight distribution of P(3HB) synthesized were found to be 1.6×10^6 and 2.9, respectively. The maximum yield of P(3HB) based on thiophenyl (*R*)-3-hydroxybutyrate was 47%.

Introduction

Many bacteria are able to accumulate polyhydroxyalcanoate (PHA) as an energy resource.[1] PHAs have physical and chemical properties[2] similar to those of synthetic polymers and are also biodegradable.[3,4] Recently, overexpression of PHA synthases from various microorganisms in *Escherichia coli* (*E. coli*) has been shown to produce sufficient amounts of pure enzyme in soluble form.[5−16] Although several in vitro PHA synthesis systems have been reported to date,[10,11,16] they have been limited to an analytical scale due to various difficulties such as the poor availability of hydroxyalkanoylCoA, the method of CoA recycling, the method for regeneration of ATP, an energy-supplying substance, and so on.

In this paper, we describe poly(3-hydroxybutrate) (P(3HB)) synthesis from thiophenyl (*R*)-3-hydroxybutyrate by using a water−organic solvent two-phase reaction system. In the experiment, thiophenyl (*R*)-3-hydroxybutyrate and PHA synthase (PhaC) from *Ralstonia eutropha* were dissolved in the organic solvent and water phase, respectively, and a monomer was synthesized by the ester exchange between thiophenol and CoA on the interface of the two phases. To our knowledge, this is the first report of the enzymatic synthesis of a high-molecular weight polymer using the two-phase reaction system.

* To whom correspondence should be addressed. E-mail: ktajima@eng.hokudai.ac.jp. Telephone and Fax: +81-11-706-7816.

Experimental Section

Synthesis of Thiophenyl (*R*)-3-Hydroxybutyrate, Monomer Precursor. The synthesis of thiophenyl (*R*)-3-hydroxybutyrate (**5**) is shown in Scheme 1. The synthesis was performed by the method described by Yuan et al.[17]

Preparation of PHA Synthase (PhaC) from *R. eutropha*. Recombinant *E. coli* BL21(DE3) harboring both pREP-4 and pQEREC was used for producing PhaC. Purification and assay of PhaC were performed by the protocol described by Satoh et al.[16] The specific activity of the purified enzyme was 27.2 U/mg-protein, and it was stored at −80 °C.

Poly(ethylene glycol) (PEG) 20000, for which the respective molecular weight was 20000, was purchased from Wako Pure Chemical Industries, Ltd. The enzyme/PEG complex was prepared by mixing the PhaC with PEG in 0.1 M succinic anhydride-sodium tetraborate decahydrate buffer (pH 7.0).[18] Two hundred milligrams of PEG20000 in the buffer (1 mL) was gradually added to 1 mL of the buffer containing 2 mg/mL PhaC. One hundred microliter portions of the obtained mixture were poured into 1.5-mL micro tubes. The PhaC/PEG complex was lyophilized and then stored at −80 °C. The specific activity of the enzyme was 36.0 U/mg-protein.

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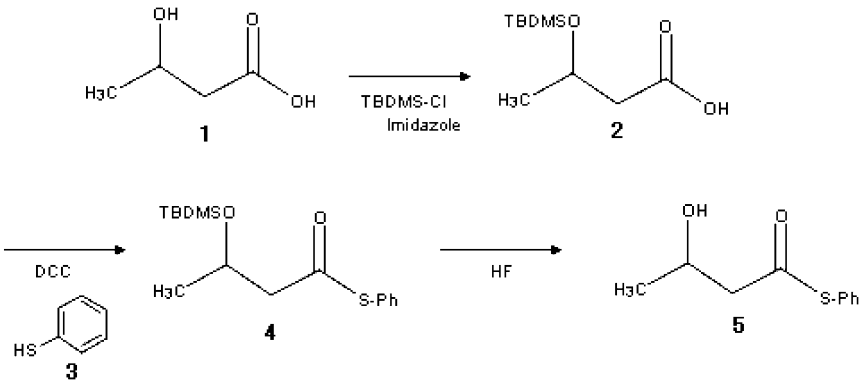
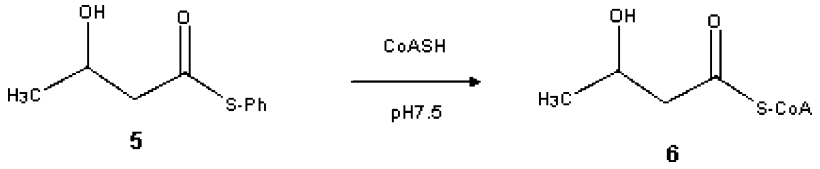
Scheme 1



Scheme 2



Chemoenzymatic Synthesis of P(3HB) in a Water—Organic Solvent Two-Phase Reaction System. For polymerization, a water−organic solvent two-phase reaction system was used. The water phase (5 mL) contained 100 mM sodium phosphate (pH 7.5) and 1.0 mM CoA. Thiophenyl (*R*)-3-hydroxybutyrate (**5**), a monomer precursor, was dissolved in 5 mL of each organic solvent to give 10 mM solutions. The water phase was poured into a test tube with a screw cap, and the organic solvent containing the monomer precursor was then poured into the tube. The polymerization reaction was started by the addition of PhaC (3.6 U) to the water phase. The polymerization reaction was carried out at 30 °C in static for 24 h.

Measurements. The ^{1}H NMR spectrum of P(3HB) was obtained at 400 MHz for protons using a Bruker MSL400 spectrometer. The ^{1}H-decoupled NMR spectrum was obtained at a 90° pulse with a 4 ms, 3000 Hz spectral width, and a 4 s repetition rate. The molecular weights of PHB were determined by GPC using tandem GPCK-807L columns (8.0 mm I. D. × 300 mm; Showa Denko K.K.) using chloroform as an eluent, and calibration was performed using standard polystyrene samples.

Results and Discussion

The ester exchange reaction between the thiophenyl group and CoA is necessary for the polymerization reaction to progress, because PhaC recognizes (*R*)-3-hydroxybutyrylCoA ((*R*)-3HBCoA) (**6**) as a substrate. It has been reported that the ester exchange progresses under weak alkaline conditions (see Scheme 2),[15] and the optimal pH of PhaC is around 7.0.[9] From these results, 7.5 was selected as the pH value of the reaction buffer. After the ester exchange was confirmed in sodium phosphate buffer (pH 7.5) containing CoA, the polymerization reaction in the buffer (one phase) was attempted. In general, formation of a white precipitate is usually observed as the polymerization reaction progresses for in vitro P(3HB) synthesis,[5,7,10,11,16] but precipitate formation was not observed in the reaction mixture. To determine why the reaction did not precede, the activity of PhaC in the presence of thiophenol (**3**) was measured. The specific activity of PhaC was drastically decreased by the addition of a small amount of thiophenol (Figure 1), suggesting that it is necessary to remove the thiophenol released by the ester exchange reaction from the reaction mixture. We therefore applied a water−organic solvent two-phase reaction system to the in vitro P(3HB) synthesis using thiophenyl (*R*)-3-hydroxybutyrate (**5**) as a precursor. The water−organic solvent two-phase reaction system has been used for bioconversion of substrates with low solubility in water.[19,20] In this system, substrates and enzymes are dissolved in organic solvent and water phase, respectively. In our reaction system, thiophenyl (*R*)-3-hydroxybutyrate, the precursor, was dissolved in the organic solvent phase (hexane). In general, some organic solvents act as protein denaturation reagents, and it was thought that PhaC could be denatured by a small amount of the organic solvent which dissolved into the water phase. Therefore, an attempt was made to confer organic solvent tolerance to PhaC. We prepared the PhaC/PEG complex by lyophilizing the PhaC solution containing PEG (see Experimental Section). To confirm that the PhaC had indeed achieved organic solvent tolerance by formation of the complex, the assay of PhaC was performed after treatment with several organic solvents. It was subsequently confirmed that PhaC had a high tolerance for hexane and chloroform (data not shown). As a result, the PhaC/PEG complex was used in the subsequent experiments. In addition, the formation of PEG complex induced a 1.4-fold increase in the PhaC-specific activity and enhanced the stability of PhaC. High specific activity and stability is one of the

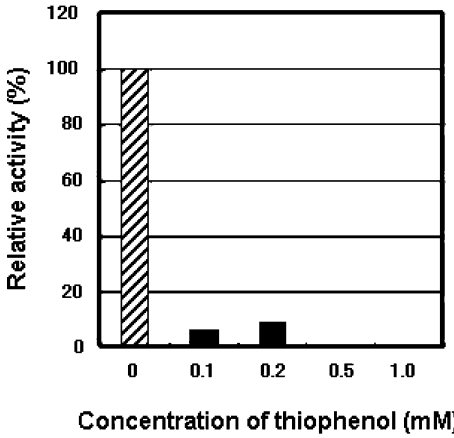


Figure 1. PHA synthase assay in the presence of thiophenol.

Table 1. Effects of Organic Solvents on Yields and Molecular Weights of P(3HB)

organic solvent	log P_{ow}	yield (%)	$M_n \times 10^{-6}$ [a]	M_w/M_n
n-dodecane	7.0	47	1.4	3.0
n-decane	6.0	40	1.5	2.6
n-octane	4.9	37	1.7	3.0
n-hexane	3.9	42	1.6	2.9

[a] Determined by GPC.

advantages of enzymatic synthesis; hence, the above could be a valuable method for preparing PhaC suitable for in vitro PHA synthesis.

The polymerization reaction was started by the addition of PhaC/PEG complex or PhaC without PEG (as a control) to the water phase in the reaction mixture. The both reaction mixtures started to become turbid after 2 h, and a white precipitate formed after 3 h. On the other hand, no precipitate was observed in the reaction mixture without CoA. Peaks corresponding to methyl, methylene, and methyne protons were observed in the NMR spectrum of the products (data not shown). Peak areas agreed with the structure of P(3HB), confirming that the product was P(3HB). The number-average molecular weights of the polymers were approximately 1.6×10^6 (Table 1), which was comparable to those of PHAs reported by other investigators. The yield of P(3HB) using PhaC/PEG complex was 1.8 mg (42%). The yield of P(3HB) using PhaC without PEG was lower than that using the complex; this could be due to the differences in the specific activities and stabilities of PhaC/PEG complex and PhaC.

To dissect the progress of the polymerization reaction, CoA derivatives in the water phase were analyzed by HPLC.[16] Free CoA was observed after 8 h, suggesting that the reaction was progressing, as CoA was released by the polymerization reaction. Free CoA, however, was not observed after 24 h, suggesting that the polymerization reaction had terminated in 24 h. Free CoA is known to inhibit PhaC; it is therefore desirable for CoA concentrations to be maintained as low as possible. The free CoA concentrations in the reaction mixture were maintained at low levels by the ester exchange, and it appeared that the inhibition of PhaC by CoA was avoided. After the reaction was complete, all CoA existed as (*R*)-3-HBCoA in the water phase, suggesting that the polymerization reaction was terminated by the inhibition and/or denaturation of PhaC. A small amount of thiophenol can dissolve in the water phase, and termination of the polymerization reaction would be due to the inhibition of PhaC by the thiophenol.

To analyze the effects of organic solvent on P(3HB) yield, hexane, octane, decane, and undecane were used for the organic solvent. The value of P_{ow}, the distribution coefficient, is the ratio of the concentrations of a substance in 1-octanol and water, respectively, and means its hydrophobicity. The logP_{ow} values of these solvents are 3.9, 4.9, 6.0, and 7.0, respectively. Thiophenyl (*R*)-3-hydroxybutyrate was dissolved in each organic solvent, and the polymerization reaction was performed under the same conditions as those described above. The apparent effects of organic solvent on the yields and molecular weights of P(3HB)s were not observed (Table 1), despite the hydrophobicities of the organic solvents.

Conclusion

We succeeded in developing a new method for in vitro P(3HB) synthesis with coenzyme A (CoA) recycling. In this method, P(3HB) was synthesized from thiophenyl (*R*)-3-hydroxybutyrate by using the water−organic solvent two-phase reaction system. This novel system makes it possible for monomers with various structures to be continuously supplied to PHA synthase. At present, we are attempting to prepare new PHA synthases with high stability, high activity, and low (wide ranging) substrate specificity by means of error-prone PCR mutagenesis, and it should be possible to synthesize novel PHAs with new structures and functions with the combination of the water−organic solvent two-phase reaction system and the new PHA synthases.

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