Chemo-enzymatic synthesis of polyhydroxyalkanoate by an improved two-phase reaction system (TPRS)

(Running title: Chemo-enzymatic synthesis of PHA by an improved TPRS)

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[**Key words:** chemo-enzymatic synthesis, polyhydroxyalkanoate (PHA), two-phase reaction system (TPRS), propionylCoA transferase (PCT), PHA synthase]

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ABSTRACT

In our previous paper, we synthesized poly-3-hydroxybutyrate [P(3HB)] by using the water-organic solvent two-phase reaction system (TPRS), in which thiophenyl (*R*)-3-hydroxybutyrate [(*R*)-3HBTP] was used as a precursor of 3HBCoA.We have developed an improved TPRS for the chemo-enzymatic synthesis of polyhydroxyalkanoate (PHA). In this method, acetyl thioester of ethyl thioglycolate (AcETG) was used as a precursor of acetylCoA (AcCoA), which was a donor of CoA. The AcCoA was formed by the ester exchange reaction between CoA in the water phase and AcETG in the organic solvent phase. The AcCoA and free 3-hydroxybutyrate (3HB) in the water phase were converted into 3HBCoA and acetate by a CoA-transfer reaction of propionylCoA transferase (PCT). The synthesized 3HBCoA was polymerized sequentially by PHA synthase, and P(3HB) was successfully formed. The maximal yield of P(3HB) was 1.2 g/l under the optimal reaction condition; this is comparable to that of in vivo PHA production. Furthermore, the number of enzymes was reduced and enzyme preparation was simplified by the construction of a fusion protein, PCT-PhaC. The chemo-enzymatic synthesis of P(3HB-*co*-3-hydroxypropionate) and

P(3HB-*co*-3-mercaptopropionate) was also achieved by the improved TPRS using the fusion protein.

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are a class of aliphatic polyesters that are accumulated as carbon and energy storage materials in numerous bacteria under nutrient-limited conditions (1, 2). PHAs have attracted much attention because they have properties similar to those of biodegradable thermoplastics and can be produced from renewable resources (3, 4). On the basis of their monomer composition, PHAs are classified into short-chain-length (scl-; C3~C5), medium-chain-length (mcl-; C6 and longer), and scl-*co*-mcl PHAs (5). Poly(3-hydroxybutyrate) [P(3HB)] is the most abundant PHA and has been widely studied by many researchers.

PHAs are produced by either in vivo or in vitro methods. For in vivo PHA production, it is possible to produce PHA by using renewable resources such as sugars or plant oils as raw materials. However, although this method is suitable for large-scale production, it has disadvantages in that the monomer structures are limited and it is relatively difficult to control the monomer composition. On the other hand, substrates with various structures can be supplied to PHA synthases and monomer compositions can be easily controlled for in vitro PHA production. These are advantages of in vitro PHA

production and the key technologies in the development of new types of PHA. In fact, we have succeeded in finding a lactate-polymerizing enzyme and producing a lactate-incorporated PHA, P(3HB-*co*-lactate), by using an in vitro production system (6).

To date, several in vitro PHA production systems have been reported (7-18). The first in vitro PHA synthesis was reported by T. U. Gerngross and D. P. Martin (7), who demonstrated that PHA synthase generated macroscopic PHB granules in the presence of sufficient (*R*)-3-hydroxybutyryl CoenzymeA [(*R*)-3HBCoA]. However, CoA, an inhibitor of PHA synthase (7) released from (*R*)-3HBCoA in the polymerization reaction, was not recycled in their system, so it was impossible to produce a large amount of P(3HB). Thus, it is necessary to introduce a CoA recycling pathway to convert released CoA into monomer molecules for efficient in vitro PHA production. R. Jossek and A. Steinbuchel reported a P(3HB) synthesis system using three enzymes: AcetylCoA synthetase (ACS), propionylCoA transferase (PCT), and PHA synthase (PhaC) (8). In their system, CoA released during polymerization was connected with acetic acid by ACS to form acetylCoA (AcCoA), which again served as the CoA donor for the activation of 3HB. The system could recycle CoA, but needed three enzymes and ATP to supply

energy for the synthesis of AcCoA. On the other hand, we reported in vitro PHA synthesis using a water-organic solvent two-phase reaction system (TPRS) (10). This system required only PHA synthase, and the polymerization reaction proceeded without ATP under a CoA recycling condition. In the TPRS, chemically synthesized thiophenyl (*R*)-3HB [(*R*)-3HBTP] was used as a substrate precursor and (*R*)-3HBCoA was formed by the ester exchange reaction between CoA in the water phase and (*R*)-3HBTP in the organic solvent phase, and the (*R*)-3HBCoA formed was polymerized sequentially by PHA synthase. The thiophenol (TP) released during the ester exchange reaction was trapped in the organic solvent phase. However, in that system, it is necessary to convert hydroxyalkanoate (HA) into HATP, a free HA cannot be used directly, and PHA synthase is inhibited by a part of TP that is not trapped in the organic solvent phase and that is dissolved into the water phase. In this way, in vitro PHA synthesis systems have several problems, which have limited their use to an analytical scale.

To overcome these problems, in this paper we described a new chemo-enzymatic in vitro PHA synthesis system, an improved TPRS (Fig. 1). To improve the TPRS, we synthesized various PHAs in three steps: 1) AcCoA formation by ester

exchange reaction; 2) conversion of a free HA into hydroxyalkanoylCoA (HACoA) by PCT; 3) polymerization of HACoA by PhaC. In this improved TPRS, acetyl thioester of ethyl thioglycolate (AcETG) was used instead of thiophenyl acetate (AcTP) as a precursor of AcCoA to prevent TP from inhibiting the enzyme. An ester exchange reaction between AcETG in the organic solvent phase and CoA in the water phase was performed on the interface of their phases to synthesize AcCoA, and 3HACoA was formed by the transfer reaction between the AcCoA and a free 3HA by PCT. Then, the synthesized 3HACoA was polymerized sequentially by PhaC, and PHA formed, successfully.

In this improved TPRS, two enzymes, PCT and PhaC, were used for PHA synthesis. To reduce the number of enzymes and to simplify the process of enzyme preparation, a fusion protein of PCT and PhaC (PCT-PhaC) was prepared. This fusion protein had both PCT and PHA synthase activities and could be used for not only the synthesis of P(3HB) but also for that of P(3HB-*co*-3-hydroxypropionate) [P(3HB-*co*-3HP)] and P(3HB-*co*-3-mercaptopropionate) [P(3HB-*co*-3MP)].

MATERIALS AND METHODS

Chemicals Hexane, chloroform, ethyl acetate, dichloromethane, acetonitrile, thiophenol (TP), ethyl thioglycolate (ETG), dicyclohexyl carbodiimide (DCC), acetic acid, methanol, and coenzymeA (CoA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). (*R*)-3-hydroxybutyric acid [(*R*)-3HB] sodium salt and 3-hydroxypropionic acid (3HP) were products of MP Biomedicals, Inc. (Solon, OH, USA) and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), respectively. 3-mercaptopropionic acid (3MP) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals were of reagent grade or better.

Measurements The ^{1}H-NMR spectra of the polymers were obtained using a Bruker MSL400 spectrometer (400 MHz) at a 90° pulse with a 4 ms, 3,000 Hz spectral width and a 4 s repetition rate. The molecular weights of the obtained polymers were determined by gel-permeation chromatography (GPC) using tandem TSK gel Super HZM-H columns (6.0 mm I.D. × 150 mm; TOSOH, Tokyo, Japan) with chloroform as an eluate. The molecular weights were calibrated using polystyrene samples as standards. CoA derivatives by HPLC were analyzed by the method of Satoh et al. (18).

Synthesis of AcTP and AcETG esters The synthesis of acetylTP (AcTP) and acetylETG (AcETG) is shown in Fig. 2. Dicyclohexylcarbodiimide (DCC) (3.54 g, 17.1 mmol) was added to a mixture of dichloromethane (10 ml) containing acetic acid (1.00 g, 16.6 mmol) and thiophenol (1.89 g, 17.1 mmol) at 0 ^{o}C under a nitrogen atmosphere. The reaction was stirred at room temperature for 16 h under a nitrogen atmosphere. Then diethyl ether (25 ml) was added to the reaction mixture, and the formed white precipitate (N, N-dicyclohexylurea (DCU)) was removed by filtration. The solvent was evaporated, and then dried in vacuo. To purify the products of AcTP, flash column chromatography was carried out on a silica gel 60 (230-400 mesh, Merck, Darmstadt, Germany) using hexane containing 10 % ethyl acetate as an eluate. Thin-layer chromatography was also performed on silica gel plates with a fluorescent indicator (0.25 mm, Merck), and compounds were detected by ultraviolet light. ETG was used instead of the TP for the synthesis of AcETG..

Preparation of PHA synthase (PhaC) from *Ralstonia eutropha* Recombinant *Escherichia coli* (*E. coli*) BL21 (DE3) harboring both pREP-4 and pQEREC was used to produce PhaC. PhaC was purified and assayed by the protocol described by Satoh et al.

(18).

Preparation of PCT from *Clostridium propionicum* To construct plasmid pQECPP, which expressed His-tagged PCT, propionyl CoA transferase gene (*pct*) was amplified by PCR using genome DNA from *Clostridium propionicum* (*C. propionicum*) JCM1430 as a template and the two specific primers--sense primer (CGGGATCCATGAGAAAGGTTCCCATTATTACCGCAGAT) and anti-sense primer (AACTGCAGGAAATCAGGACTTCATTTCCTTCAGAC)--as specific primers. The amplified DNA fragment was digested with *Bam*HI and *Pst*I restriction enzymes, then inserted into pQE80 (Qiagen K.K., Tokyo, Japan) digested with the same restriction enzymes. The constructed plasmid pQECPP was introduced into *E. coli* BL21 (DE3), and the prepared cells were used to produce PCT. PCT was purified by the protocol described by Satoh et al. (18). PCT was assayed by detecting CoA released by a combination of CoA transferase and PHA synthase reaction. The CoA concentration was determined with 5, 5'-dithio-bis (2-nitrobenzoic acid) (DTNB) as described before (18). The PCT activity was defined as the formation of 1 μmol CoA per minute. The assay mixture (a total volume 0.5 ml) contained 100 mM sodium phosphate buffer (pH 7.5), 2 mM AcCoA, 200

mM 3HB, PhaC, and PCT.

Preparation of PCT-PhaC fusion protein PCT from *C. propionicum* and PhaC from *R. eutropha* were used in the improved TPRS (Fig. 1). To simplify the preparation of the enzymes, a plasmid to express $(His)_6$-PCT-PhaC fusion protein was constructed. The PCT gene was amplified by PCR using genomic DNA from *C. propionicum* as a template and two specific primers--sense primer (CGGGATCCATGAGAAAGGTTCCCATTATTACCGCAGAT) and anti-sense primer including linker region (CGGGATCCACCACCACCACCACCACCACCGGACTTCATTTCCTTCAGACCCA TTAA)--as specific primers. The amplified DNA fragment was digested with *Bam*HI restriction enzyme, then inserted into pQEREC digested with the same restriction enzyme. The constructed plasmid (pQEHPC) was introduced into *E. coli* BL21 (DE3). The $(His)_6$-PCT-PhaC fusion protein was purified and assayed by the same procedures described in preparation of PCT.

Chemo-enzymatic synthesis of P(3HB) by an improved TPRS An improved TPRS was used for PHA synthesis (Fig. 1). The water phase (5 ml) contained 100 mM

sodium phosphate (pH 7.5), 2.0 mM CoA, 100 mM 3HB, and enzymes PCT (20 U) and PhaC (5 U). AcTP or AcETG was dissolved in hexane (0.5–5 ml) to give 100–10 mM solutions. The water phase without PhaC 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 to the water phase. The polymerization reaction was carried out at 30 °C in static for 72 h.

RESULTS AND DISCUSSION

Synthesis of thioesters and confirmation of ester exchange reaction and transfer reaction We have developed our TPRS as an in vitro PHA synthesis method (10). In this TPRS, HATP was used as a substrate precursor and the HATP was synthesized in three steps (19): 1) protection of a hydroxyl group, 2) thioesterification, and 3) deprotection. For the PHA synthesis using the TPRS, it is necessary to convert a HA into HATP, and a free HA cannot be used directly. In addition, TP was released as the reaction progressed, and PhaC was inhibited by a small amount of TP that was not trapped in the organic solvent phase and was dissolved into the water phase. These are obstacles to efficient in

vitro PHA production using the TPRS.

Therefore, we attempted to improve TPRS to overcome these problems (Fig. 1). First, PCT was introduced into the TPRS to make the direct use of a free HA possible. PCT can convert a free HA into HACoA in the presence of AcCoA (8). Although AcCoA can be enzymatically synthesized from acetic acid and CoA by ACS, ATP is needed to supply energy for the synthesis. Hence, we attempted to produce AcCoA from AcTP by the ester exchange reaction, because it is expected that AcTP could be converted into AcCoA without ATP and ACS. In addition, AcTP could be easily synthesized in one-step (Fig. 2), because acetic acid has no hydroxyl group in the molecule. Second, ETG was also used as a leaving group in addition to TP (20). ETG has a thiol group and can make a thioester linkage with acetic acid, the same as with TP, and it was expected that ETG's inhibition of enzymes was lower than that of TP, based on ETG's structure.

AcTP or AcETG was synthesized by the condensation reaction of acetic acid and TP or ETG using DCC (Fig. 2). The result of NMR analysis revealed that acetic acid was successfully and efficiently converted into AcTP or AcETG in the one-step reaction (data not shown). Since the synthesis of AcTP and AcETG was confirmed, we tested the

formation of AcCoA by the ester exchange reaction. AcTP or AcETG in acetonitrile was added to 100 mM sodium phosphate buffer (pH 7.5) containing CoA. The mixture was incubated at 30 ^{o}C with stirring. After the incubation, 1 M H_3PO_4 was added to the solution to stop the reaction, and the mixture was then analyzed by HPLC. The peak corresponding to CoA decreased as the reaction progressed, and the peak corresponding to AcCoA appeared in each sample using AcTP or AcETG as a precursor (data not shown). This result confirmed that AcCoA was produced from AcTP or AcETG by the ester exchange reaction.

Then, a TPRS including PCT in the water phase was constructed to confirm the formation of HACoA by the conjugated reaction of the ester exchange and CoA transfer reactions. In this TPRS, AcTP or AcETG was dissolved in the organic solvent phase, and PCT and 3HB were added into the water phase. After incubation at 30 ^{o}C, an aliquot of the water phase was analyzed by HPLC. HPLC analysis revealed the formation of 3HBCoA in each sample using AcTP or AcETG as a precursor of AcCoA (data not shown), suggesting that it was possible to produce 3HBCoA from AcTP or AcETG by combining the formation of AcCoA by the ester exchange reaction and CoA transfer

reaction by PCT.

Chemo-enzymatic synthesis of P(3HB) by an improved TPRS To produce P(3HB), PHA synthase from *R. eutropha* was added to the reaction mixture in which 3HBCoA was formed. The progress of the reaction was monitored by measuring the turbidity of the reaction mixture. We showed the time course change in turbidity at 600 nm of the reaction mixture including AcTP and AcETG (Fig. 3). The turbidity of the reaction containing AcETG was rapidly increased until 300 min, then decreased with the formation and sedimentation of precipitates. On the other hand, the turbidity of the reaction mixture including AcTP was gradually increased, and the maximal turbidity value was significantly lower than that for AcETG. The difference in the PHA formation rates for AcTP and AcETG could be due to the difference in the rates in AcCoA formation, because the AcCoA formation rate was faster for AcETG than for AcTP. This result suggested that sufficient amounts of the enzymes existed in the reaction mixture and that the step producing AcCoA was the rate-determining step in the improved TPRS. In addition, it has been reported that TP inhibited PHA synthase even at low concentrations (10). Since TP's structure is similar to that of phenol, which has a high ability to denature

proteins, it is supposed that TP has the same features as phenol. In addition, it was confirmed that a small amount of TP could be dissolved into the water phase (unpublished data); therefore, the slow reaction rate for AcTP could be based on the inhibition of the enzymes by TP in addition to the rate of the ester exchange reaction.

White precipitate was obtained by the improved TPRS using AcTP or AcETG as a precursor of AcCoA. To confirm that this precipitate was P(3HB), it was collected by filtration, dissolved in chloroform, and then reprecipitated by adding methanol into the solution. The precipitate was removed by filtration and analyzed by ^{1}H-NMR spectroscopy. The peaks corresponding to methyl, methylene, and methyne protons were observed in the ^{1}H-NMR spectra of the products (data not shown). The observed peaks corresponded to the structure of P(3HB), suggesting that the product was P(3HB). The yields of P(3HB) for AcTP and AcETG were 0.04 g/l and 0.58 g/l, respectively. After this experiment, we decided to use AcETG as a precursor of AcCoA.

Optimization of reaction conditions for chemo-enzymatic synthesis of P(3HB) by the improved TPRS To increase the production rate and yield of P(3HB) by using this TPRS, we optimized the reaction conditions. The amount of AcETG in the

organic solvent phase and that of 3HB in the water phase were maintained (0.5 mmol) and the volume of organic solvent phase was changed (volume of organic solvent phase: volume of water phase = 1:1, 0.5:1, and 0.1:1); the reaction rates were analyzed by measuring the turbidities (at 600 nm) of the reaction mixtures (Fig. 4). The production rate and the maximal values of the turbidities increased as the volume of the organic phase decreased. When the ratio of the organic solvent phase to the water phase was 0.1:1, the reaction mixture started to become turbid after 10 min, and the turbidity of the reaction mixture peaked with growing polymer molecule deposition after 1 h. The maximal yield of P(3HB) was 1.2 g/l, comparable to that of in vivo flask scale PHA production (21). The number-average molecular weight and the molecular weight distribution of the polymer were 8.5×10^4 and 1.7, respectively, and these were smaller than those of P(3HB) produced in vivo. From these results, the volume ratio of hexane to the water phase was determined to be 0.1:1.

Chemo-enzymatic synthesis of P(3HB) by the improved TPRS using a fusion protein PCT-PhaC In this improved TPRS, PCT and PhaC were used for PHA synthesis. Hence, we attempted to prepare PCT and PhaC as a fusion protein, PCT-PhaC,

to reduce the number of the enzymes and to simplify the process of enzyme preparation. The order of PCT and PhaC was determined based on the report that the insertion of green fluorescent protein (GFP) into the N-terminus of PhaC did not influence the PHA granule formation (22). Seven glycine residues were inserted between the C-terminus of PCT and the N-terminus of PhaC as a linker to reduce the effects of steric hindrance. The PCT-PhaC fusion protein was purified by using the His-tag system according to the same method used for the purification of PCT and PhaC.

Although the specific activities of PCT and PhaC were decreased by their fusion, it was confirmed that PCT-PhaC had both activities. To determine the optimal amount of PCT-PhaC, 0.5, 1.0, and 2.0 mg of PCT-PhaC were added to the reaction mixture. From the results of yields and GPC analyses, 2.0 mg was selected as the optimal amount (data not shown). The same units of PCT and PhaC as those of PCT-PhaC fusion protein were added to a reaction mixture, and the yield of P(3HB) produced by PCT and PhaC or PCT-PhaC was compared to confirm the fusion's effect on PHA synthesis. The yield of P(3HB) produced by the fusion protein was higher than that produced by the two enzymes. In addition, fusion protein had a faster reaction rate than the two enzymes. This

could be due to the effective reaction of PhaC and 3HBCoA by the fusion of PhaC and PCT supplying 3HBCoA. Based on this result, we decided to use the fusion protein, PCT-PhaC in the following experiments.

Chemo-enzymatic synthesis of P(3HB-*co*-3HP) by the improved TPRS using a fusion protein PCT-PhaC In general, the physical properties of PHA vary with the monomer compositions in the polymers. It is easy to control the monomer composition in PHA in vitro; this is one advantage of in vitro synthesis. In the improved TPRS, a free HA in the water phase is converted into a CoA derivative by CoA transfer activity of PCT. That is, the activity and substrate specificity of PCT are significantly important for in vitro PHA synthesis using PCT. The kinds of monomers and the monomer compositions in polymers are also regulated by PhaC' s substrate specificity as well as by that of PCT. The substrate specificity of PCT was analyzed to estimate the possibility of copolymer production using the improved TPRS. We chose 3HP as a substrate of PCT because it was known that PCT has substrate specificity to the short-chain fatty acid (23) and that *R. eutropha* can produce PHA incorporating 3HP (13, 15). From the result of HPLC analysis of the reaction mixture, it was confirmed that 3HPCoA was produced from 3HP and

AcCoA by the CoA transferase activity of PCT (data not shown).

(*R*)-3HB and 3HP were added to the reaction mixture at various molar ratios (3HB/3HP: 100/0, 95/5, 85/15, 50/50, 0/100), and the reaction was started by the addition of PhaC to the water phase. The polymerization reaction was carried out at 30 °C in static for 72 h. The syntheses of P(3HB), P(3HB-*co*-38 mol% 3HP), and P(3HP) were confirmed by ^{1}H-NMR analysis (Fig. 5). The monomer compositions of PHAs synthesized at various 3HB/3HP ratios are summarized in Table 1. The molar ratios of 3HP in the product increased with the increase in the 3HP concentration in the reaction mixture, indicating that it is possible to control the monomer ratios in the polymers. The molar ratios of 3HP units in the polymers were significantly higher than those in the corresponding reaction mixture compositions. To analyze the reason for this, we measured the production rates of 3HPCoA and 3HBCoA in the reaction mixture including (*R*)-3HB, 3HP, or both (*R*)-3HB and 3HP with the same concentration. The production rates of 3HBCoA and 3HPCoA in the reaction mixture containing 3HB or 3HP were 0.08 and 0.12 μmol/s, respectively. On the other hand, the production rates of 3HBCoA and 3HPCoA in the reaction mixture containing both (*R*)-3HB and 3HP were 0.02 μmol/s and

0.12 μmol/s, respectively (Fig. 6). These results suggested that PCT had higher affinity toward 3HP than 3HB, and it was thought that Michaelis-Menten's constant (Km) of PCT for 3HP was smaller than that for (*R*)-3HB. Thus, the high ratios of 3HP unit in P(3HB-*co*-3HP) could be due to the high affinity of PCT toward 3HP.

The processability of P(3HB) is narrower (24) than that of the other homopolymer. However, it was reported that P(3HP) has good mechanical properties to process (25). The melting point (T_m), heat of fusion (ΔH), and glass transition temperature (T_g) of P(3HB-*co*-3HP) strongly depend on the 3HP composition, and these values are monotonously decreased with increase in the 3HP ratio (26). As shown in Table 1, the 3HP ratios could be controlled; therefore, it is possible to synthesize PHA with desirable properties by using this TPRS.

Chemo-enzymatic synthesis of P(3HB-*co*-3MP) by the improved TPRS using a fusinon protein PCT-PhaC Because of the structural similarity between 3MP and 3HP, it was expected that PHA containing 3MP as a monomer unit could be synthesized by using the improved TPRS. To confirm this, 3MP was used as a substrate instead of 3HP. A certain amount of polymer was obtained when the concentrations of 3HB and 3MP were

80 mM and 20 mM, respectively. The result of ^{1}H-NMR analysis of the product revealed that P(3HB-*co*-18 mol% 3MP) was successfully synthesized from 3HB and 3MP by using the improved TPRS (Fig. 7). Unlike the case with 3HP, the monomer ratio of 3MP in the polymer agreed with the molar ratio of 3MP fed into the reaction mixture.

Poly(3-mercaptopropionate) has greater thermal stability and a higher melting point (T_m) than the equivalent oxygen analogue P(3HP) (27, 28). The thermal properties of P(3HB-*co*-3MP) depend on the monomer composition, and the melting point of P(3HB-*co*-3MP) was increased with the increment of 3MP fraction. By using the improved TPRS, it will be possible to make PHAs with various thermal properties.

We succeeded in developing a new method for in vitro PHA synthesis derived from the TPRS. In the improved TPRS, the organic solvent phase included AcETG and the water phase included CoA, HA, PCT, and PHA synthase. An ester exchange reaction to form AcCoA between ETG and CoA was performed on the interface of the water and the organic solvent phases, and a free 3HA was converted into 3HACoA by the transfer reaction between AcCoA and 3HA by PCT. Then, the synthesized 3HACoA was polymerized sequentially by PhaC, and PHA was formed, successfully. The advantages

of this system are as follows. (i) A free HA can be directly used as a substrate. (ii) PHA can be synthesized by using the improved TPRS without ATP. (iii) PHA can be produced at a high production rate and yield.

Though PCT from *C. propionicum* was used in this experiment, CoA transferase genes have been found in several bacteria. The kinds of monomers supplied to PHA synthase depend on the substrate specificity of the CoA transferase used; therefore, it is important to find CoA transferase with wide substrate specificity for the production of various PHAs. Since a free HA can be directly used as a substrate in the improved TPRS, it would be possible to synthesize novel PHAs with new structures and functions by applying various kinds of CoA transferases and PHA synthases to this system.

We recently succeeded in the synthesis of P(lactate-*co*-3HB) using an engineered PHA synthase in vitro (6) and in vivo (29). In those papers, the TPRS was used as a screening system to find a lactate-polymerizing enzyme from PHA synthases. The improved TPRS described in the present is superior to the former TPRS; the improved TPRS will be useful as an effective screening method. Moreover, the new TPRS will be a key technology for producing various novel PHAs.

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Figure legends

FIG. 1. An improved water-organic solvent two-phase reaction system (TPRS).

FIG. 2. Synthetic pathways of AcTP and AcETG.

FIG. 3. Time course changes of the turbidity of the reaction mixtures containing AcETG (circle) or AcTP (triangle) as a precursor of AcCoA.

FIG. 4. Time course changes of the turbidities of the reaction mixtures with various volumes of the organic solvent phase containing the same amount of AcETG (the volume ratios of organic solvent phase to that of water phase: circle: 0.1:1; square: 0.5:1; triangle: 1:1).

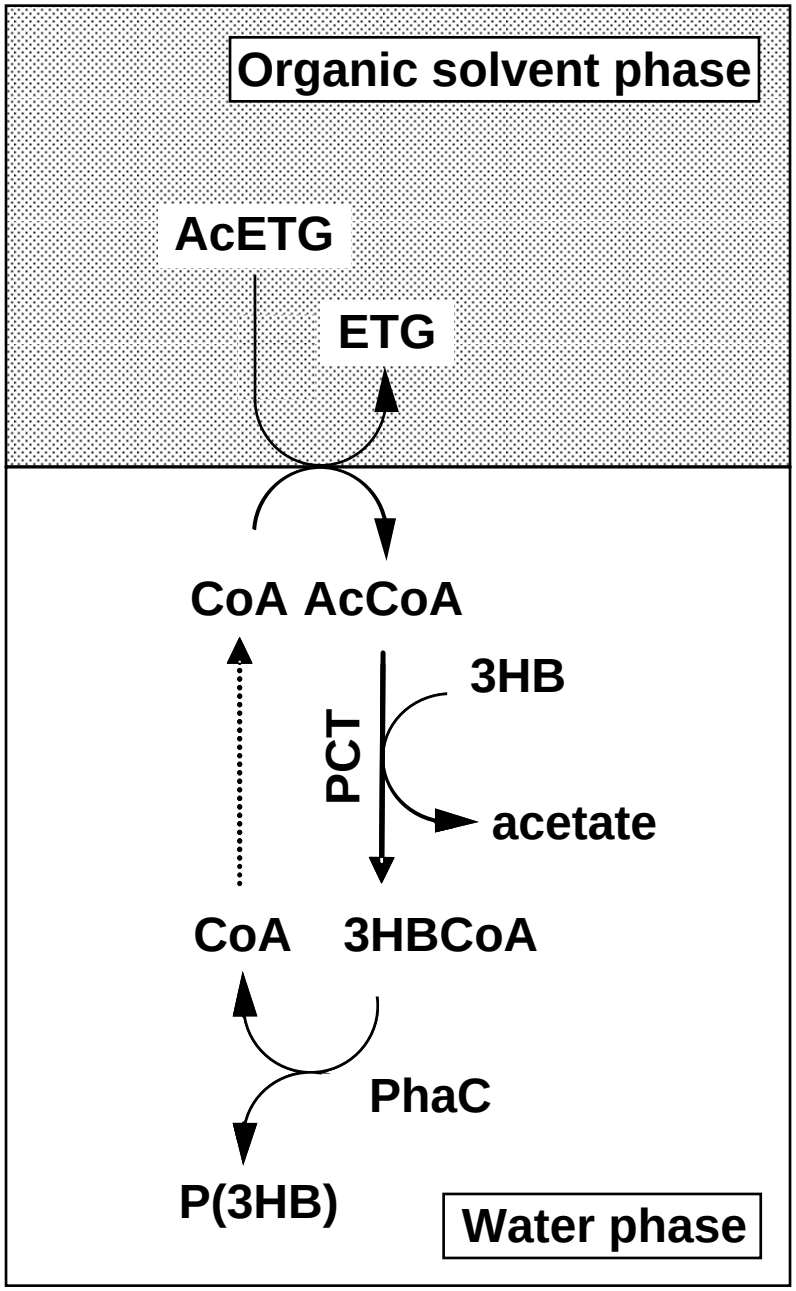
FIG. 5. ^{1}H-NMR spectra of (a) P(3HB), (b) P(3HB-*co*-38 mol% 3HP), and (c) P(3HP) produced by the improved TPRS using a PCT-PhaC fusion protein.

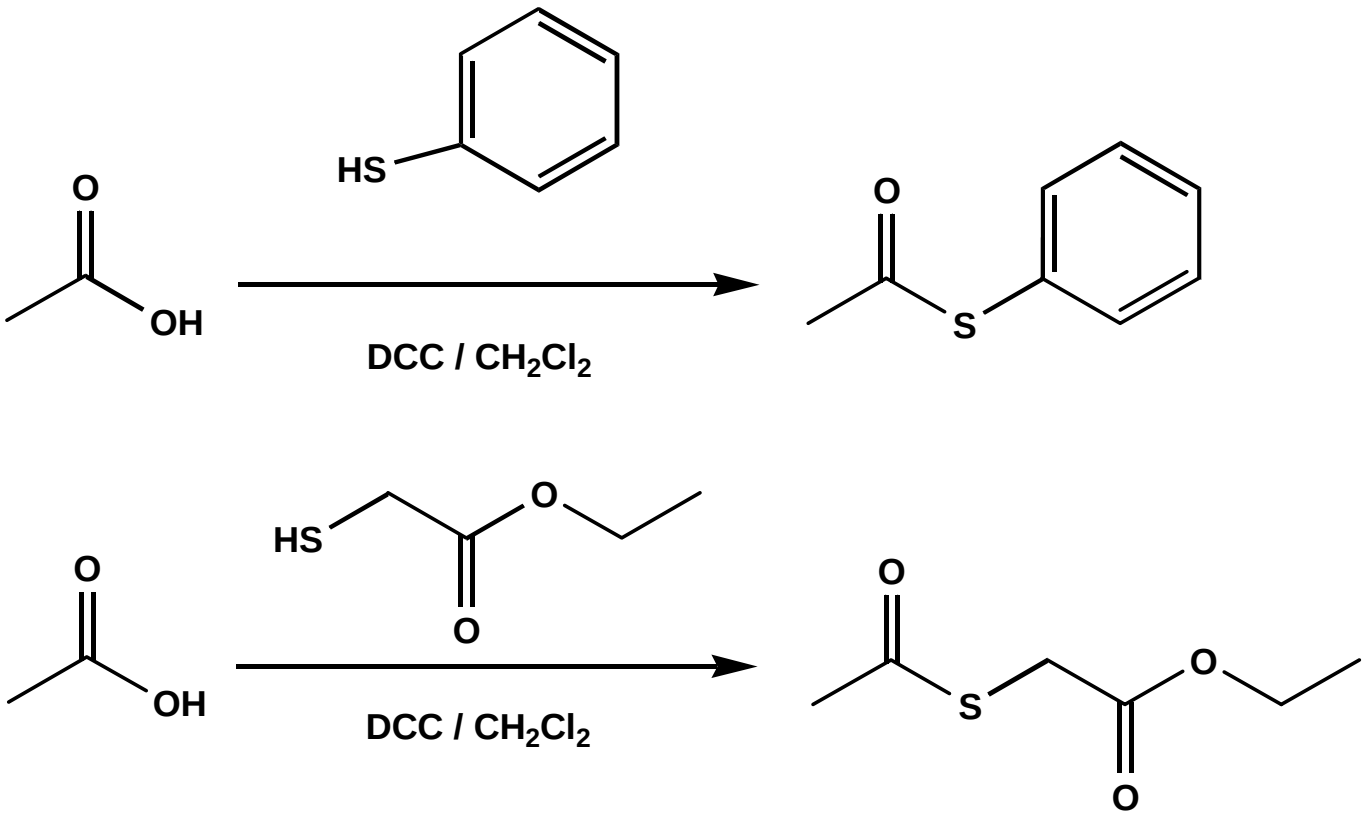
FIG. 6. Production rates of monomers in the reaction mixture containing both (*R*)-3HB and 3HP without PhaC (circle: 3HPCoA; triangle: 3HBCoA).

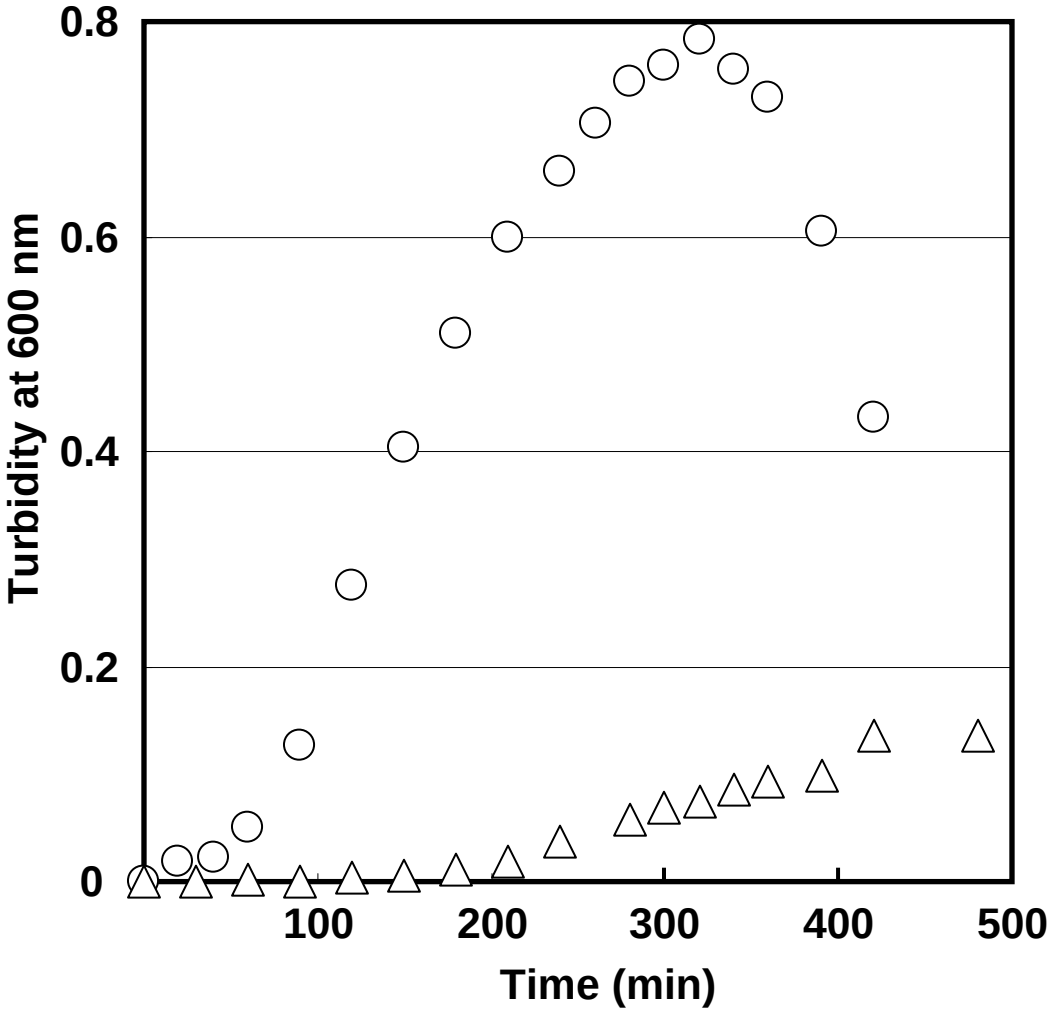
FIG. 7. ^{1}H-NMR spectrum of P(3HB-*co*-18 mol% 3MP) produced by the improved TPRS using a PCT-PhaC fusion protein.

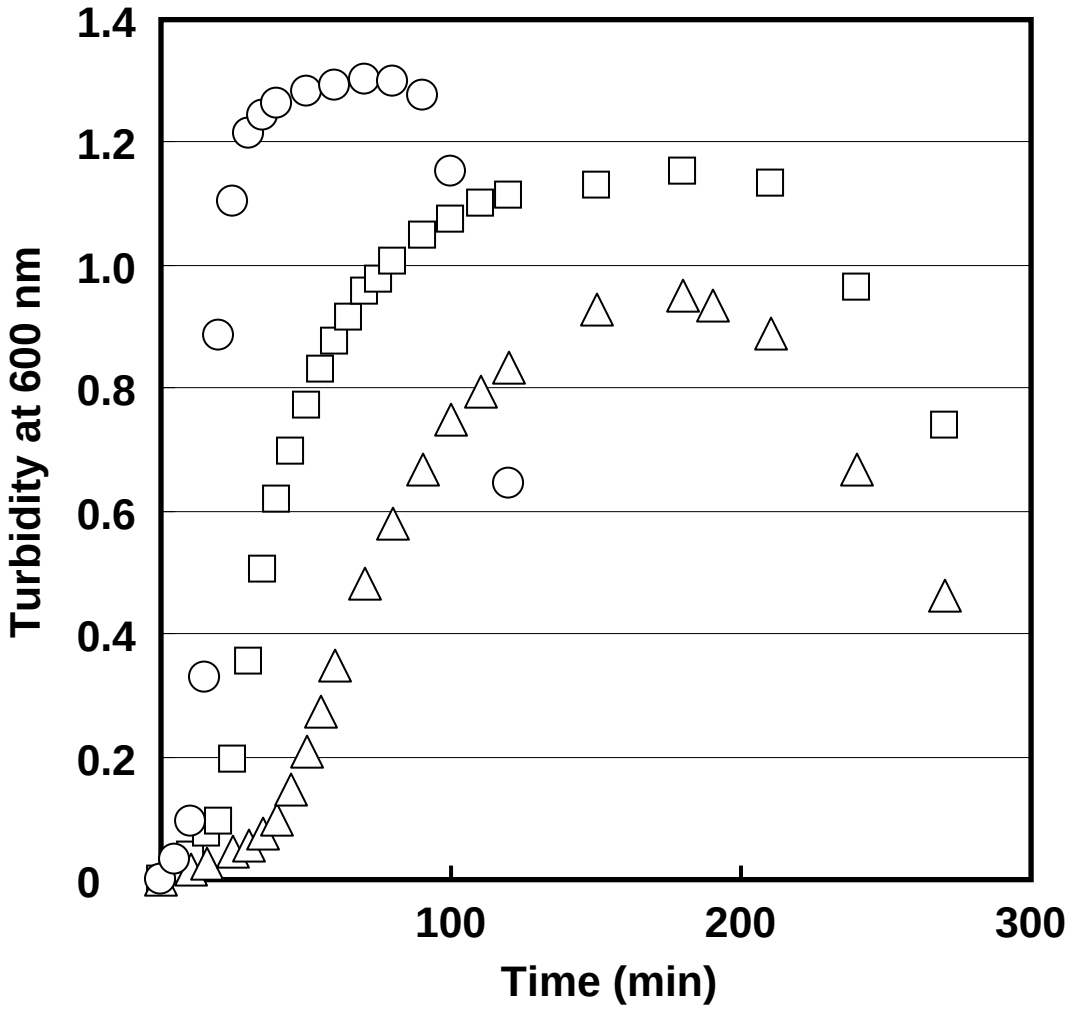
TABLE 1. Control of molar ratios of 3HB and 3HP in polymers

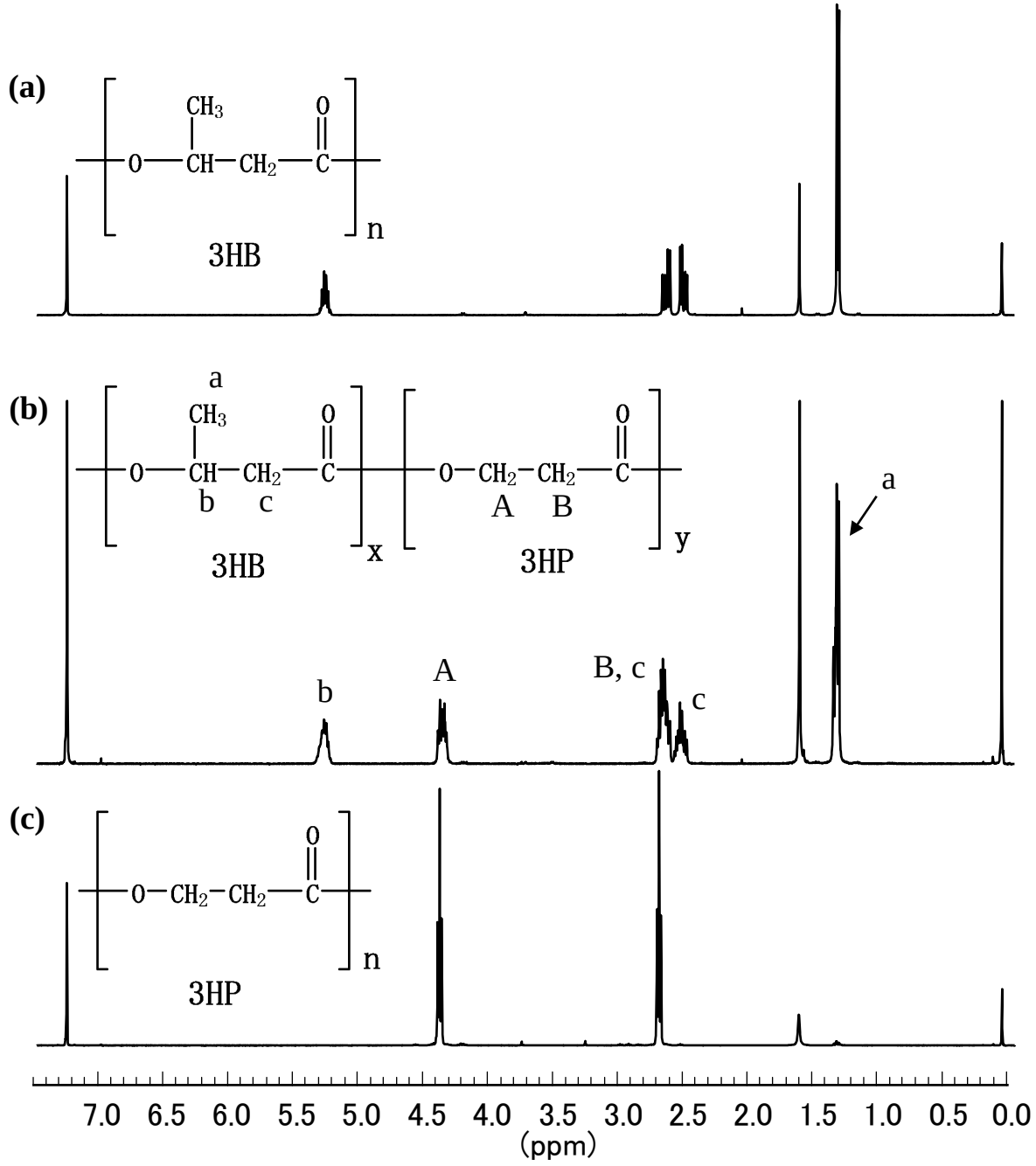
Molar ratio in a reaction mixture	Molar ratio in a polymer
(*R*)-3HB/3HP	(*R*)-3HB/3HP
100 / 0	100 / 0
95 / 5	62 / 38
85 / 15	39 / 61
50 / 50	17 / 83
0 /100	0 /100

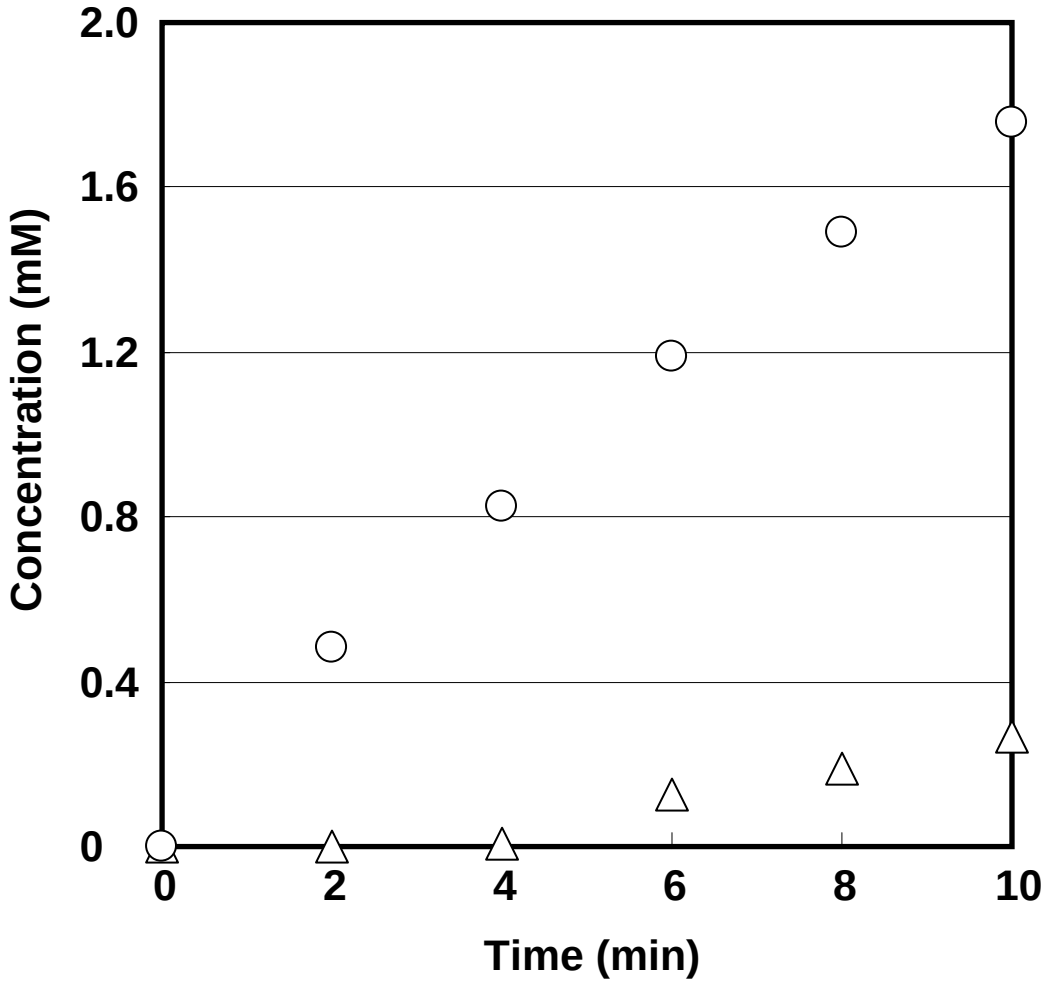

Organic solvent phase
AcETG
ETG
CoA AcCoA
3HB
PCT
acetate
CoA 3HBCoA
PhaC
P(3HB)
Water phase

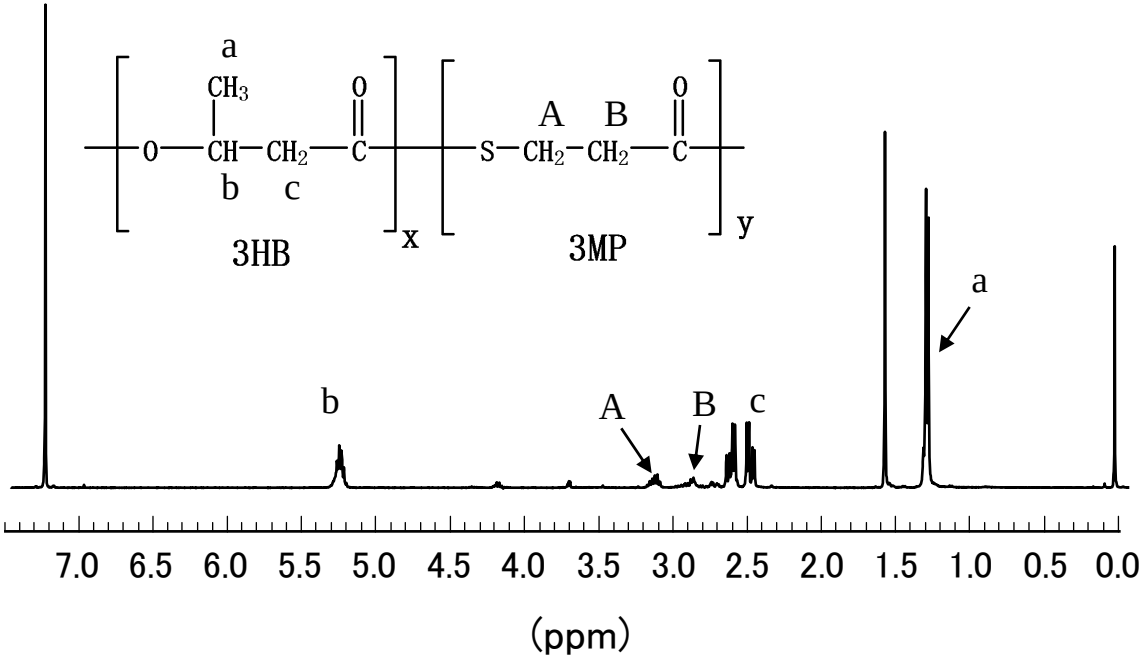

HS
O
OH
DCC / CH2Cl2
O
S
HS
O
O
O
OH
DCC / CH2Cl2
O
S
O
O


0.8
0.6
0.4
0.2
0
Turbidity at 600 nm
100
200
300
400
500
Time (min)


1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Turbidity at 600 nm
100
200
300
Time (min)


(a)
CH3
O
O
CH
CH2
C
n
3HB
(b)
a
CH3
O
O
CH
CH2
C
b
c
x
3HB
O
O
CH2
CH2
C
A
B
y
3HP
a
b
A
B, c
c
(c)
O
O
CH2
CH2
C
n
3HP
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
(ppm)


2.0
1.6
1.2
0.8
0.4
0
Concentration (mM)
0
2
4
6
8
10
Time (min)


a
CH3
O
A B
O
O CH CH2 C S CH2 CH2 C
b c
x
y
3HB
3MP
a
b
A
B
c
7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
(ppm)