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In vitro synthesis of poly(3-hydroxybutyric acid) by using an enzymatic coenzyme A recycling system

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Abstract

Purified recombinant poly(hydroxyalkanoic acid), PHA, synthase from *Chromatium vinosum* was used to examine in vitro poly(3-hydroxybutyric acid) (P(3HB)) formation. In combination with purified propionyl-coenzyme A transferase of *Clostridium propionicum* a two-enzyme in vitro P(3HB) biosynthesis system was established which allowed the synthesis of P(3HB) from free D-(−)-3-hydroxybutyric acid as substrate. The coenzyme A residue for the activation of this hydroxyacid was provided by acetyl-coenzyme A. By adding acetyl-coenzyme A synthetase to this system, a three-enzyme in vitro P(3HB) biosynthesis system was established. Coenzyme A that was released during the polymerization reaction was coupled to acetate which again served as the coenzyme A donor for the activation of 3-hydroxybutyric acid. The energy for the in vitro P(3HB) synthesis was provided by ATP hydrolyses resulting in acetyl-coenzyme A synthesis catalyzed by the acetyl coenzyme A synthetase. In this way the in vitro synthesis of P(3HB) became independent of the consumption of the expensive coenzyme A. By this procedure a handy system is available to produce in vitro PHA on a semipreparative scale.

Keywords: Poly(hydroxyalkanoic acid); Poly(3-hydroxybutyric acid); Poly(hydroxyalkanoic acid) synthase; In vitro synthesis; *Chromatium vinosum*; Propionyl-coenzyme A transferase

1. Introduction

A wide range of bacteria accumulate poly(hydroxyalkanoic acids) (PHA) as a carbon and energy storage material and deposit them as insoluble granules in the cells [1,2]. Because of the properties of these polymers as biodegradable thermoplastics they have attracted much interest. Due to the low substrate specificity of the PHA synthases, which catalyze the polymerizing step during PHA biosynthesis, more than 100 different hydroxyalkanoic acids were found to be incorporated in these polyesters in vivo [3]. The in vivo synthesis of PHA with new constituents and of defined combinations of various constituents is limited by the availability of appropriate precursor substrates for the PHA synthase and insufficient fermentation control, respectively. These limitations can be overcome by in vitro biosynthesis of PHA using isolated PHA synthases. The PHA synthase of *Chromatium vinosum* ($PhaEC_{Cv}$) consists of two different types of subunits (39 kDa and 41 kDa) and prefers coenzyme A thioesters of short carbon-chain length hydroxyalkanoic acids as substrates [4].

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After overproduction of the PHA synthases of *C. vinosum* and *Alcaligenes eutrophus* ($PhaC_{Ae}$) in *Escherichia coli*, the soluble forms of the enzymes were purified from the respective recombinant strains [5,6]. Recently, it was shown that polymer formation occurs when purified recombinant PHA synthase was incubated with 3-hydroxybutyryl coenzyme A. As in vivo, in vitro synthesized P(3HB) accumulated in the form of insoluble granules in an aqueous solution [7,8]. Because of the availability of the hydroxyacid-coenzyme A thioesters, the in vitro synthesis of PHA was restricted to an analytical scale only. We established a three-enzyme P(3HB) biosynthesis system consisting of the purified PHA synthase of *C. vinosum*, the purified propionyl-coenzyme A transferase of *Clostridium propionicum* [9] and a commercially available acetyl-coenzyme A synthetase (Fig. 1). In this way the coenzyme A, which was released by the polymerization reaction catalyzed by the PHA synthase, was recycled via acetyl-coenzyme A, synthesized by the acetyl-coenzyme A synthetase, and transferred on 3-hydroxybutyric acid, catalyzed by the propionyl-coenzyme A transferase. Therefore, in vitro P(3HB) synthesis became independent on coenzyme A consumption, thus allowing in vitro P(3HB) formation also on a semipreparative scale.

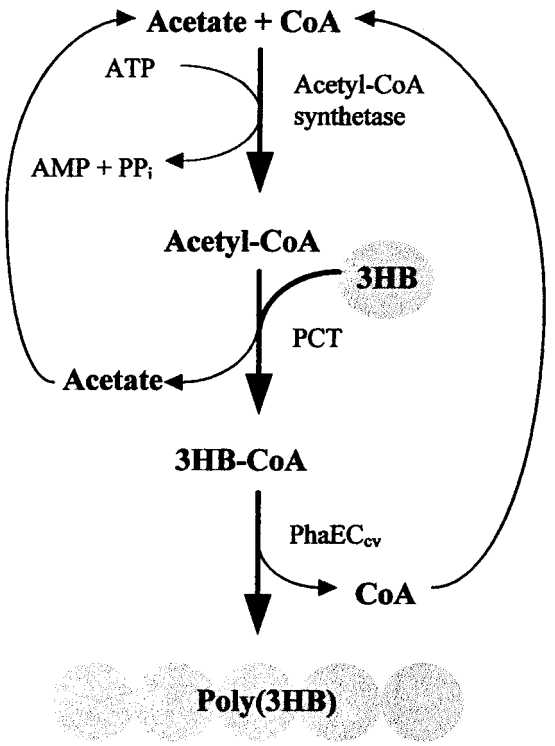

Fig. 1. Scheme of CoA recycling in the in vitro P(3HB) biosynthesis system.

2. Materials and methods

2.1. Bacterial strains and growth of bacteria

Escherichia coli XL1-Blue harboring plasmid pds37, which expressed $phaEC_{Cv}$, was grown at 37°C in Luria-Bertani medium containing 1 mM isopropyl β-D-thiogalactopyranoside and 75 μg ml^{-1} ampicillin [10]. *Clostridium propionicum* (DSMZ 1682) was grown in 20 l carboys in yeast extract/alanine medium according to Schweiger and Buckel [9]. Cultures were inoculated with 1 l precultures and were grown anaerobically at 37°C.

2.2. Purification of PHA synthase from C. vinosum ($PhaEC_{Cv}$)

The PHA synthase was purified from a recombinant strain of *E. coli* (pds37), which overexpressed *phaC* and *phaE* from *C. vinosum*. The purification of $PhaEC_{Cv}$ was performed by a two-step procedure including chromatography on DEAE-Sephacel and Procion Blue H-ERD as described by Liebergesell et al. [5]. The enzyme preparation was stable for several months at −20°C.

2.3. Purification of propionyl-CoA transferase (PCT, EC 2.8.3.1)

PCT was purified from cells of *C. propionicum* according to a procedure derived by a method of Schweiger and Buckel [9]. All preparation steps were performed under aerobic conditions at 4°C. PIPES (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)) buffer, pH 7.0, containing 1 mM $MgCl_2$, 1 mM EDTA and 1 mM dithiothreitol (DTT) was used throughout the purification. Cells were resuspended in buffer and were broken by a threefold passage through a French pressure cell at 96 MPa. The crude cell extract was centrifuged for 60 min at 160 000 × *g*,

and the supernatant was applied to a Superdex 200 prep-grade FPLC-column (Pharmacia, Uppsala, Sweden). Fractions with high PCT activity were combined and applied to a Q-Sepharose HP FPLC-column (Pharmacia, Uppsala, Sweden) from which PCT was eluted at approximately 120 mM NaCl. Fractions with high PCT activity were combined again, and the molarity of the combined fractions was decreased to 50–100 mM by dilution with buffer. The fractions were applied to a Procion-Blue column and PCT eluted at approximately 370 mM NaCl. The enzyme was concentrated by precipitation with ammonium sulfate at 100% saturation. Aliquots of the protein were stored at −20°C.

2.4. Synthesis of acetyl-CoA and 3-hydroxybutyryl-CoA

Acetyl-CoA was synthesized according to Simon and Shemin [11] from acetanhydride and coenzyme A. (*R*)-3-Hydroxybutyryl-CoA (3HBCoA) was obtained by transesterification of acetyl-CoA and 3HB employing propionyl-CoA transferase [12].

2.5. PHA synthase activity

The specific activity of the PHA synthase was determined spectrophotometrically at 232 nm as described previously [13].

2.6. Poly(3HB) formation and analysis

2.6.1. Two-enzyme system

The polyester formation was started by adding PCT (60 U ml^{-1}) to $PhaEC_{Cv}$ (1 U ml^{-1}) in 100 mM PIPES, pH 7.0, containing 20 mM $MgCl_2$, 150 mM D-(−)-3-hydroxybutyric acid and 7 mM acetyl-CoA. The progress of the reaction was monitored by assaying the release of free coenzyme A and the decrease of the acetyl-CoA concentration with DTNB. For this an aliquot of the reaction mixture was incubated in 100 mM Tris-HCl, pH 8.0, containing 1 mM DTNB. The absorbance at 412 nm was monitored, and from this the CoA concentration was calculated ($\varepsilon^{-}_{CoA-TNB} = 13.61$ cm^2 μmol^{-1}). The acetyl-CoA concentration was determined in the same way by adding citrate synthase and 0.4 mM oxalacetate to the assay.

2.6.2. Three-enzyme system

The P(3HB) synthesis was started by adding acetyl-CoA synthetase (EC 6.2.1.1) from yeast (4.6 U ml^{-1}) and as purchased from Boehringer Mannheim (Mannheim, Germany) to PCT (56 U ml^{-1}) and $PhaEC_{Cv}$ (1.2 U ml^{-1}) in 100 mM PIPES, pH 7.5, containing 20 mM $MgCl_2$, 5.0 mM Na-acetate, 20–30 mM D-(−)-3-hydroxybutyric acid, 10–20 mM ATP and 0.5 mM CoA. The progress of the reaction was monitored by assaying the ATP consumption. For this an aliquot of the reaction mixture was incubated in 100 mM TEA buffer, pH 7.6, containing 1 mM $NADP^+$, 8 mM $MgCl_2$, 10 mM glucose and hexokinase/glucose-6-phosphate dehydrogenase. The absorbance at 340 nm was monitored, and from this the ATP concentration was calculated. The P(3HB) granule formation was observed by phase-contrast microscopy. The in vitro synthesized P(3HB) was extracted with chloroform and analyzed by gel-permeation chromatography and gas chromatography as described previously [8,14].

3. Results and discussion

Recently it was demonstrated that in vitro P(3HB) was synthesized by incubation of $PhaEC_{Cv}$ in the presence of sufficient amounts of 3-hydroxybutyryl-CoA (3HBCoA) and $MgCl_2$ [8]. A scale up of the in vitro synthesis of PHA was limited by the availability and costs of 3HBCoA. The aim of this work was to establish an in vitro P(3HB) biosynthesis system in which coenzyme A, which is released after incorporation of the monomeric unit into the polymer chain, will be recycled by activating another 3HB unit for polyester synthesis.

It was confirmed that propionyl-coenzyme A transferase, isolated from *C. propionicum*, activated 3HB by esterification with CoA in the presence of the PHA synthase. In this two-enzyme system acetyl-CoA served as the CoA donor since only in the presence of both enzymes, $PhaEC_{Cv}$ and PCT, acetyl-CoA was consumed and CoA was released and vice versa (Fig. 2). By adding acetyl-coenzyme A synthetase from yeast to this system a three-enzyme P(3HB) biosynthesis system was obtained in which coenzyme A released during the polymerization reaction was subsequently coupled to acetate, which

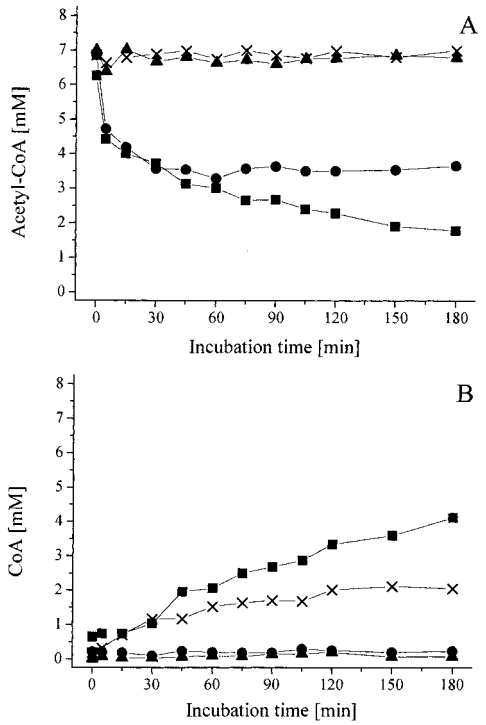

Fig. 2. Two-enzyme system for in vitro P(3HB) biosynthesis. PHA synthase ($PhaEC_{Cv}$) was incubated in the presence of propionyl-CoA transferase (PCT) in 100 mM PIPES, pH 7.0, containing 20 mM $MgCl_2$, 150 mM D-(−)-3-hydroxybutyric acid and 7 mM acetyl-CoA for 3 h at 30°C (■). The progress of the reaction was monitored by determination of the concentration of acetyl-CoA (A) and CoA (B). As controls, assays containing $PhaEC_{Cv}$ but no PCT (▲), $PhaEC_{Cv}$ and 5 mM 3HBCoA but no PCT (×) or PCT without $PhaEC_{Cv}$ (●) were also analyzed.

again served as CoA donor for the activation of 3HB. Only in the presence of all three enzymes a progress of the reaction was observed (Fig. 3).

By successive addition of 3HB and ATP to the three-enzyme system more than 30 μmol ATP was consumed in a 1-ml reaction mixture containing only 0.5 μmol CoA. As in vivo [15] or in the in vitro system, in which 3HBCoA was directly used as substrate for polyester formation [8], the P(3HB) synthesized in vitro in the three-enzyme system accumulated in the form of insoluble granules. The granules aggregated to large flocks which sedimented at the bottom of the reaction vessel. After chloroform extraction of the reaction assay and evaporation of the chloroform a polymeric film was obtained. If one of the three enzymes was missing, the solution did not become turbid and no polyester could be extracted. A 2-ml reaction assay in which 60 μmol ATP was consumed within 12 h incubation time is shown in Fig. 4A. From this, 5.5 mg polymeric material was extracted (Fig. 4B); this is up to 73% of the polymerized 3HB (44 μmol) which is calculated with reference to the ATP consumption. Compared with 5.5 mg in vitro P(3HB), formed by using a system without CoA recycling, more than 88 μmol DL-3HBCoA must have been used, which is commercially available for a price of more than DM 2000 (Sigma-Aldrich, Deisenhofen, Germany).

GPC- and GC-analysis proved that the polymeric material consisted of P(3HB), exhibiting a molar mass in the range of 10^6 g mol^{-1} (M_w/M_n 1.2). In comparison with P(3HB) synthesized in vivo by *C. vinosum* (M_w 1.6×10^6 g mol^{-1}, M_w/M_n 2.9, [16]) and in vitro by incubation of $PhaEC_{Cv}$ in the presence of sufficient amounts of 3HBCoA (M_w $1–2\times10^6$ g mol^{-1}, M_w/M_n 1.8, [8]), P(3HB) synthesized in vitro by using the three-enzyme system exhibited approximately the same molecular mass whereas the polydispersities of the polyester synthesized by using the CoA recycling system were significantly lower, indicating a much narrower molecular mass distribution of the polyester molecules.

The results demonstrate that a CoA recycling sys-

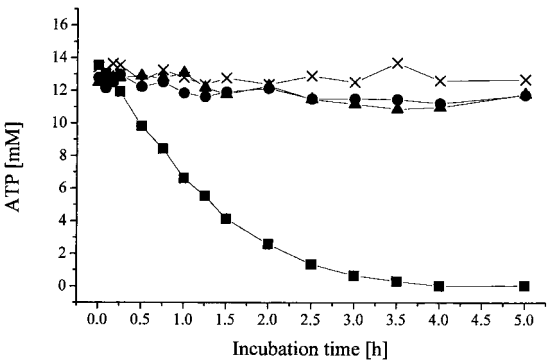

Fig. 3. Three-enzyme system for in vitro P(3HB) biosynthesis. PHA synthase ($PhaEC_{Cv}$) was incubated in the presence of propionyl-CoA transferase (PCT) and acetyl-CoA synthetase in 100 mM PIPES, pH 7.5, containing 20 mM $MgCl_2$, 5 mM Na-acetate, 20 mM D-(−)-3-hydroxybutyric acid, 13 mM ATP and 0.5 mM CoA for 5 h at 30°C. The progress of the reaction was monitored by determination of the ATP concentration (■). As controls, assays containing the same components as described above but without acetyl-CoA synthetase (×), without PCT (▲) or without $PhaEC_{Cv}$ (●) were also analyzed.

tem was obtained by using purified $PhaEC_{Cv}$ in combination with PCT and acetyl-CoA transferase. The energy for the in vitro P(3HB) synthesis was provided by ATP hydrolyses catalyzed by the acetyl-CoA synthetase. This system required only catalytic amounts of CoA for in vitro PHA formation. In addition to this cost factor, the low level of CoA in this in vitro assay prevents an inhibitory effect of free CoA on the PHA synthase activity (Fig. 5).

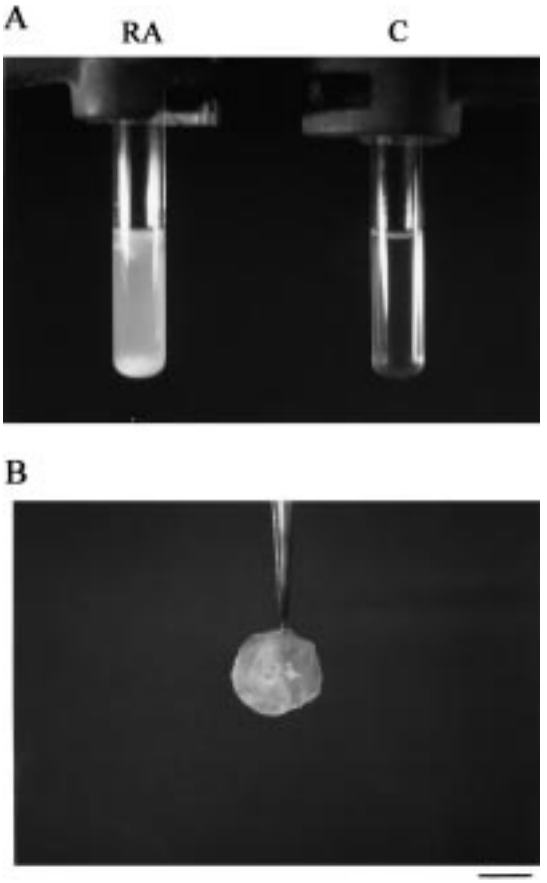

Fig. 4. In vitro P(3HB), synthesized by using the CoA recycling system. PHA synthase ($PhaEC_{Cv}$) was incubated in the presence of propionyl-CoA transferase (PCT) and acetyl-CoA synthetase in 100 mM PIPES, pH 7.5, containing 20 mM $MgCl_2$, 5 mM Na-acetate and 0.5 mM CoA at 30°C. The reaction was started by adding 30 mM D-(−)-3-hydroxybutyric acid and 20 mM ATP. After 3.5 and 8.5 h incubation, two times each 30 mM D-(−)-3-hydroxybutyric acid and 20 mM ATP were added to the reaction mixture. A: Three-enzyme in vitro P(3HB) biosynthesis system (RA) and a corresponding control assay containing no PCT (C) after incubation for 12 h at 30°C. B: Extracted in vitro P(3HB) (bar = 1 cm).

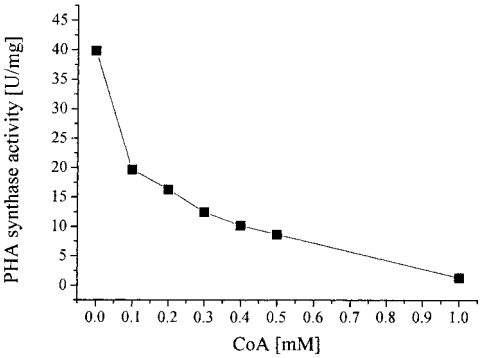

Fig. 5. Effect of CoA on the specific activity of the PHA synthase. In 500 μl 25 mM Tris-HCl, pH 7.5, containing 120 μM 3HBCoA and various concentrations of CoA, 0.4 μg purified PHA synthase ($PhaEC_{Cv}$) was incubated. The changes in absorbance at 232 nm were monitored, and from this the specific PHA synthase activity was calculated.

Furthermore, due to P(3HB) granule formation and CoA recycling the products of the polymerization step were removed from the equilibrium, thus shifting the reaction towards polyester formation. The established CoA recycling P(3HB) biosynthesis system may provide a useful strategy for the synthesis of diverse PHAs on a semipreparative scale, which can be investigated as model polymers in the area of biodegradable thermoplastics and elastomers.

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