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In vitro Polymerization and Copolymerization of Poly-3-hydroxypropionyl-CoA with the PHB Synthase from *Ralstonia eutropha*

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Abstract

The poly(3-hydroxybutyrate) (PHB) synthase of *Ralstonia. eutropha*, which was produced by a recombinant strain *E. coli* and purified in one-step with a methyl-HIC column to a purity of more than 90 %, was used to polymerize 3-hydroxypropionyl-CoA (3HPCoA) and to copolymerize 3HPCoA with 3-hydroxybutyryl-CoA (3HBCoA) *in vitro*. A K_m of 189 μM and a k_{cat} of 10 sec^{-1} were determined for the activity of the enzyme in the polymerization reaction of 3HPCoA based on the assumption that the dimer form of PHB synthase was the active form. Free coenzyme A was found to be a very effective competitive inhibitor for the polymerization of 3HPCoA with a K_i of 85 μM. The maximum degree of conversion of 3HPCoA to polymer was less than 40 %. In the simultaneous copolymerization reactions of these two monomers, both the turnover number for the copolymerization reaction and the maximum degree of conversion of 3HPCoA and 3HBCoA to copolymers increased with an increase in the amount of 3HBCoA in the monomer mixture. However, the maximum conversion of 3HPCoA to a copolymer was less than 35 % regardless of the ratio of 3HPCoA to 3HBCoA. Block copolymers were obtained by the sequential copolymerization of the two monomers and these copolymers had a much narrower molecular weight distribution than those obtained by the simultaneous copolymerization of the same molar ratio of 3HPCoA and 3HBCoA.

Introduction

Many species of bacteria synthesize poly-3-hydroxyalkanoates (PHAs) as storage polymers.[1,2] The PHAs can be divided into two types according to the length of the alkyl group at the 3-position in the repeating unit. The two types are : (1) short chain length (SCL) PHAs, in which the alkyl group has from 3 to 5 carbon atoms as synthesized by *Rastonia eutropha,* and (2) medium chain length (MCL) PHAs with alkyl groups having 6 carbons or more as synthesized by many *Pseudomonas* species. The type of PHA

produced by a bacterium is most likely determined by the substrate specificity of the PHA synthase.

Haywood, *et al.* demonstrated that *R. eutropha* PHB synthase only catalyzed the polymerization of the 3HBCoA)which had the [R]-configuration at the 3-position, not the [S] monomer, for both granule bound and soluble enzymes in natural cells[3]. These results were consistent with the observation that all of the PHAs in natural cells which have been examined for configurational purity contained only repeating units with the [R]-configuration. They also found that enzyme activity for the polymerization of 3HBCoA is much greater than for that of 3-hydroxyvaleryl-CoA (3HVCoA).

Overexpression of the PHB synthase from *R. eutropha* in recombinant bacterial and insect cells has allowed the enzyme to be obtained in an active form in high purity. The PHB synthase is water-soluble and has a molecular weight of approximately 64,000. The purified PHB synthase exists in both monomeric and dimeric form.[4]

In this present study, the properties of the PHB synthase from *R. eutropha* for the *in vitro* polymerization of 3HPCoA and copolymerization of 3HPCoA and 3HBCoA are described.

Experimental Section

Production and Purification of PHB synthase

The recombinant *E. coli* strain UT5600, which hosts the chimeric plasmid pKAS4 harboring the PHB synthase from *R. eutropha* was obtained from Metabolix Inc. of Cambridge, MA and was used for producing the PHB synthase.

Ammonium sulfate in the amount of 15 %(w/v) was added to 20 ml of the crude extract with stirring and centrifuged at 15,000 g for 20 minutes. After the supernatant was collected, ammonium sulfate was added in the amount of 50 %(w/v) with stirring and the resulting suspension was centrifuged. The precipitate was dissolved in 20 ml of phosphate buffer (pH 7.0) containing 1 M of ammonium sulfate, 5% glycerol, and 0.05 % hecameg, and the PHB synthase was purified by liquid chromatography with a methyl-HIC column (Bio-Rad Co.).

The PHB synthase activity was assayed spectrophotometrically by measuring the hydrolysis of thioesters in substrates according to the method of Fukui, *et al*[5]. The reaction was performed in 1 ml of total volume with 100 mM Tris-Cl at pH 8.0.

Synthesis of 3HPCoA and 3HBCoA

The 3HPCoA and 3HBCoA were synthesized from β-propiolactone, β-butyrolactone and free Coenzyme A. One hundred mg of Coenzyme A lithium salt (Sigma Co.) was dissolved in 5 ml of 200 mM $KHCO_3$ solution, the solution container was placed in an

ice bath and the lactone solution was dropped into it with stirring. The molar ratio of CoASH to lactone is 1 to 10. The reaction was followed by measuring spectrophotometrically an increase in the absorption at 236 nm, which increases with increasing thioester bond formed. Ellman's reagent, DTNB[5,5'-dithiobis(2-nitrobenoic acid)], was also used for determining the concentration of free coenzyme A in the final reaction solution. After completion of the reaction, an excess lactone was removed by extracting with ether three times and the solution was lyophilized.

In vitro Polymerization and copolymerization of 3-Hydroxyalkanoyl-CoA monomers

The polymerization reactions were carried out in 10 ml of 100 mM Tris-Cl buffer solution at pH 8.0 containing 10 μg of BSA, 0.25% hecameg, and suitable amounts of monomers. After shaking this mixture gently, the polymerization reaction was initiated by adding aliquots of the PHB synthase solution. The relative amounts of enzyme solution and substrates were varied. During the polymerization reaction, the degree of conversion of the monomers to the polymers was determined by periodically taking a 5 μl aliquot from the reaction mixture, adding it to 20 μl of 5% trichloroacetic acid solution, then adding 1 ml of 1 mM DTNB solution to that solution. The absorbance of the resulting mixture was measured at 412 nm to determine the conversion of the substrate using a molar ε_{412} of 13,700.

Results and Discussion

Enzyme purification. After removing the extraneous proteins in the crude extract by precipitation with 15% (w/v) ammonium sulfate, the PHB synthase was separated on a methyl-HIC column using a concentration gradient of ammonium sulfate. The fractions showing the highest PHB synthase activity were collected together. The purity of the PHB synthase in these fractions was more than 90% and the specific activity of the PHB synthase increased by approximately 27-fold (Table 1). In this manner, approximately 5 mg of the PHB synthase was obtained from 1L of culture.

Kinetic Studies of the *in Vitro* polymerization of 3HPCoA. A kinetic study was carried out in the presence of fructose to obtain the basic parameters for the polymerization of 3HPCoA by the PHB synthase. Figure 1 shows the double reciprocal plot of the initial reaction rate as a function of the concentration of 3HPCoA. The effect of adding coenzyme A to the initial solution on the rate is also shown in Figure 1. The K_m value for 3HPCoA was found to be 119 μM, which is a little higher than the value previously found for 3HB-CoA of 91 μM[6]. Furthermore, the turnover number of the enzyme for 3HPCoA, k_{cat}, was only 10 sec^{-1} based on the dimer form of PHB synthase,

which was approximately 35 times smaller than that for 3HBCoA.

Table 1. Purification of the PHB synthase with methyl-HIC column.

Sample [a]	Total amount of Protein (mg)	Total Activity (unit)	Specific Activity (unit/mg)	Yield (%)
Crude extract	312	99.8	0.32	100
Supernatant [b]	6	0.3	0.05	0
Precipitate [b]	287	80.4	0.28	81
Purified sample [c]	5	42.5	8.50	43

a, From 1 L culture of *E. coli* harboring the PHB synthase b, The samples that were separated with 50%(w/v) of ammonium sulfate. c, Purified PHB synthase with methyl-HIC column.

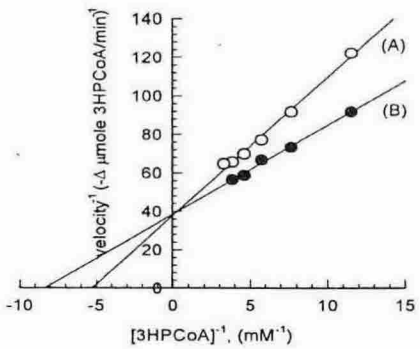

Figure 1. Double reciprocal plot of the initial rate versus 3HPCoA concentration in the reaction with PHB synthase. The reaction solution contained 5.6 μg of PHB synthase and either (A) 50 μM coenzyme A or (B) no coenzyme A.

Characteristics of the *in vitro* polymerization reactions. With a synthase solution containing 50% fructose, the polymerization of 3HPCoA had a turnover number of 4.8 mol of 3HPCoA of 3HPCoA/mol of dimer PHB synthase/sec with the molar ratio of monomer-to-enzyme ratio of 1 x 10^4.

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