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Preparation of enantiomerically pure (R)-3-hydroxybutyrate

이영, 이상엽
한국과학기술원 화학공학과 및 생물공정연구센터
전화 (042) 869-5970, FAX (042) 869-3910

Abstract

Enantiomerically pure hydroxycarboxylic acids have great potential as chiral building blocks in fine chemicals due to their easily modificable two functional groups. Microbial polyhydroxyalkanoates (PHAs) can have more than one hundred of hydroxycarboxylic acids as monomeric constituents by growing cells under various conditions. All of the monomeric constituents are enantiomerically pure in (R)-conformation if there is a chiral center. Therefore, efficient production of enantiomerically pure hydroxycarboxylic acids by degrading PHAs is possible. In this presentation, we report on the development of a novel method for the preparation of (R)-hydroxybutyric acid by *in vivo* depolymerization of Polyhydroxybutyrae.

Introduction

Hydroxycarboxylic acids contain two functional groups (hydroxyl and carboxyl group) which are convenient to modify. Generally, they have optical isomerism with the chiral center on the carbon position containing hydroxyl group, and the chiral center can be easily inherited by simple chemical synthesis. Therefore, enantiomerically pure hydroxycarboxylic acids have great potential as chiral building blocks for the synthesis of various chiral fine chemicals.[1-3] Unfortunately, preparation of enantiomerically pure (R)-hydroxycarboxylic acids by chemical synthesis is difficult and uneconomical.

Polyhydroxyalkanoates (PHAs), a carbon and/or energy storage material synthesized and accumulated in numerous microorganisms,[4,5] have been drawing much attention since they can be used as truly biodegradable plastics and elastomers.[5] More than 120 kinds of hydroxycarboxylic acids was known that can be incorporated into the polymer.[5,6] All of the monomeric constituents are enantiomerically pure in (R)-conformation if they have a chiral center. Therefore, it was reasoned that various enantiomerically pure (R)-hydroxycarboxylic acids might be conveniently prepared by depolymerizing biosynthesized PHAs.

Materials and Methods

Alcaligenes latus DSM 1123 was used in this experiment. Cells containing PHB were prepared by batch culture or fed-batch culture as previously reported,[7] and directly used for *in vivo* depolymerization.

The activity of PHB depolymerase was determined by measuring the (R)-3-hydroxybutyric acid (R3HB) production rate. The specific activity (unit/mg protein) was defined as the moles of R3HB released in one min by 1 mg of protein.

The activity of R3HB dehydrogenase was determined as follows. Cultured *A. latus* cells were harvested by centrifugation, and were resuspended in 0.5ml of 0.2 M potassium phosphate buffer (pH 8.0) supplemented with 10% glycerol (v/v) and 10 mM 2-mercaptoethanol at the concentration of 113.6 g cell dry weight/L, and then were disrupted by sonication. After removal of cell debris by centrifugation, the crude enzyme extract (100 L) was added to the pre-incubated assay mixture (2ml containing 7.5 mol of R3HB, 1 mol of NAD, 20 mol of 2-mercaptoethanol and 75 mol of potassium phosphate) at 37℃. The (R)-3-hydroxybutyric acid dehydrogenase activity was measured by UV spectrometric method by monitoring the production rate of NADH. The specific activity (unit/mg protein) was defined as the moles of NADH produced in one min by 1 mg of protein.

The concentration of R3HB was measured by high performance liquid chromatography (HPLC). The HPLC was equipped with Hitachi L-6000 pump, L-3300 RI detector (Tokyo, Japan), and AMINEX® HPX-87H column (Bio-Rad Co., Hercules, CA). 0.01N H_2SO_4 solution was used as the mobile phase at the flow rate of 0.5 mL/min. The total protein concentration was measured by Bradford method using bovine serum albumin as a standard.

Results and Discussion

Batch culture of *A. latus* was first carried out in a chemically defined medium to accumulate PHB. Cells were then transferred to various solutions and incubated under several different conditions for the depolymerization of PHB. Cells of *A. latus* were suspended at 13.1 g cell dry weight/L (equivalent to 10.1 g PHB/L) in the culture medium (initial pH of 7.0) containing either 20 g/L or no sucrose, and were incubated for 8 h without shaking. The concentrations of R3HB (and dimer) obtained in the media with and without sucrose were 7.0 (0.37) and 11.3 (0.69) g/L, resulting in the monomer (plus dimer) yields of 61% and 99%, respectively. These results suggest that *A. latus* has active PHA depolymerase system operational either in the presence or in the absence of carbon source. Higher yield in the medium containing no sucrose was somewhat expected since cells would degrade accumulated PHB when a carbon/energy source is limited. It was, however, unexpected that almost all PHB was depolymerized to yield R3HB monomer without further metabolic conversion. Since cells were incubated without shaking (without oxygen supply), *A. latus*,

an aerobic bacterium, seemed to be unable to operate normal metabolic pathways including that converts R3HB to acetoacetate. *In vivo* depolymerization experiments were also carried out in water. PHB was efficiently depolymerized to R3HB with the yield of 99% in water, which allowed much easier recovery of pure R3HB. Therefore, further experiments were carried out in water.

In order to understand the physiological conditions leading to polymer degradation and possibly to find the optimal condition for *in vivo* depolymerization, the time profiles of depolymerization as well as the effects of varying pH and temperature were examined. Cells of *A. latus* were dispersed in water (pH 7.0) so that the initial PHB concentration was 10.9 g/L, and were incuba6ted at 30℃, 37℃, or 45℃. When cells were incubated at 30℃ and 37℃, rapid degradation started at 7 h and 5 h, respectively. When cells were incubated at 45℃, the reaction was slightly faster but stopped after 3 h. Since the pH of the reaction mixture decreased with time due to acid production and depolymerization rate increased suddenly after 5 h, it was reasoned that low pH might enhance depolymerization. The effect of the initial pH on *in vivo* depolymerization was therefore examined (Table 1).

Table 1. Concentrations of the (R)-(-)-3-hydroxybutyric acid (R3HB) and the dimer obtained by varying the pH of degradation solution (initial PHB concentration : 1.07g/L, reaction temp. : 37℃, reaction time : 30 minutes).

Initial pH	2	3	4	5	6	6.5	7	8	9	10	11
R3HB (g/L)	0.6	0.96	0.99	0.35	0.03	0.04	0.04	0.05	0.11	0.15	0.19
Dimer (g/L)	0.14	0.24	0.23	0.04	0.03	0.00	0.00	0.00	0.00	0.00	0.00
Final yield (mol %)	58	95	96	31	5	3	3	4	9	12	15

It can be seen that *in vivo* depolymerization of PHB was best at the initial pH of 3 and 4. The yield of R3HB was as high as 95-96% after only 30 min. To examine the possible reasons for this, the activities of intracellular PHA depolymerase and R3HB dehydrogenase were measured. The activity of intracellular PHA depolymerase was highly pH dependent. The activity was the highest at pH 3 and 4, at which the monomer production rate was also the highest. On the other hand, R3HB dehydrogenase activities at pH 4, 5, 6 and 7 were 0, 1.2, 5.0, and 15.1 unit/mg protein, respectively. Since no activity of R3HB dehydrogenase was detected at pH 4 at which the intracellular PHA depolymerase activity was highest, there was no loss of produced R3HB and the yield was as high as 96%. Therefore, it was concluded that *in vivo* depolymerization of PHB could be best carried out at pH 4 and 37℃.

As demonstrated above, PHB of low concentration (10-15 g/L) could be

depolymerized *in vivo* with high efficiency. However, the process will be more economical if the *in vivo* depolymerization reaction can be carried out at higher polymer concentration. *A. latus* cells containing 85% of PHB was prepared by fed-batch culture using sucrose as a carbon source as previously described.[7] The final cell dry weight and PHB concentration obtained in 23 h were 135.6 g/L and 115.3 g/L, respectively. Cells were immediately collected by centrifugation, and were resuspended in the same volume of water. In an actual large scale process, diafiltration can be used for this step. *In vivo* depolymerization reaction was carried out by incubating the cells at pH 4 and 37℃. After 1 h, 117.8 g/L of R3HB was obtained, resulting in the monomer yield of 84%. Therefore, it is possible to produce (R)-3-hydroxybutyric acid monomers with high efficiency by *in vivo* depolymerization of PHB at a high concentration.

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