Polymer Journal, Vol. 35, No. 4, pp 407—410 (2003)

NOTES

Synthesis of Poly(3-hydroxybutyrate) by Immobilized Poly(3-hydroxybutyrate) Synthase

Kenji TAJIMA,† Hatsumi TANNAI, Yasuharu SATOH, and Masanobu MUNEKATA

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

(Received December 9, 2002; Accepted February 13, 2003)

KEY WORDS Poly(3-hydroxybutyrate) / PHB / Immobilized Enzyme / PHB Synthase /

At present, biodegradable and biocompatible polymers are being widely sought by materials industries, and poly(3-hydroxyalkanoate) (PHA) appears to be one of the most promising material sources of such polymers. Poly(3-hydroxybutyrate) (PHB) is a typical PHA and has been investigated in detail. Although there have been several reports on *in vitro* PHB synthesis by soluble enzymes,[1–8] PHB synthesis using immobilized enzymes has not, to our knowledge, yet been reported.

In this paper, we describe PHB synthesis on the surface of a carrier resin by immobilized PHB synthase (PhbC).

EXPERIMENTAL

Immobilization and Assay of PhbC

The method of enzyme preparation has been described previously.[9] For immobilization, purified PhbC (2 μg) was mixed with 10 mg of wet resin (Chitopal; Fuji Spanning Co., Ltd., Tokyo) in 100 mM Tris-HCl (pH 7.2) at 4 °C for 2 h. The immobilized enzyme was put into a reaction buffer containing 100 mM Tris-HCl (pH 7.2), 1 mM DL-3HBCoA, and 0.05% HECAMEG in a total volume of 180 μL; the mixture was then maintained at 25 °C with mixing. The reaction was quenched with 5%(V/V) trichloroacetic acid. After centrifugation to remove the precipitated protein, the quenched reaction mixture was added to 0.1 mM DTNB [5,5′-dithio-bis(2-nitrobenzoic acid)] in 0.5 M potassium phosphate (pH 7.5), and A_{412} was measured.[10]

Polymerization

The monomer was synthesized by an enzyme-catalyzed system according to a method we have developed.[9] Four enzymes were used for monomer synthesis in this system, the scheme of which is shown in Figure 1. The enzymes used for monomer synthesis and PHB synthase (PhbC) were purified and immobilized on the carrier resin Chitopal BCW2603. For immobilization, the purified enzymes (2 mg each) were mixed with 500 mg of wet resin in 100 mM Tris-HCl (pH 7.5) at 4 °C for 2 h. The polymerization reaction was started by the addition of wet immobilized enzymes to the reaction buffer containing 100 mM Tris-HCl (pH 7.5), 0.25 mM NADPH, 0.50 mM CoA, 30 mM ATP, 40 mM acetate, 20 mM glucose, and 1 mM DTT in a total volume of 1 mL. The polymerization reaction was carried out at 25 °C.

Scanning Electron Microscope Observation

Surface views of the resin, which was coated with a 200 Å thick layer of vacuum-evaporated gold, were obtained using a Hitachi SEM S-430.

Measurements

The ^{1}H NMR spectrum of PHB was obtained at 400 MHz for protons using a Bruker MSL400 spec-

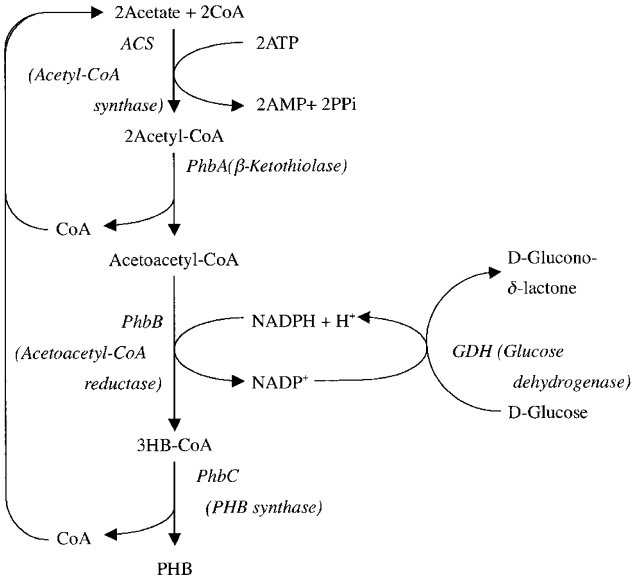

Figure 1. Schematic illustration of the enzyme-catalyzed PHB synthesis system.

†To whom correspondence should be addressed.

trometer. The ^{1}H-decoupled NMR spectrum was obtained at a 90° pulse with a 4 ms, 3,000 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, Tokyo) using chloroform as an eluent, and calibration was performed using standard polystyrene samples.

RESULTS AND DISCUSSION

There are various methods for enzyme immobilization, and many resins have been developed. In the PHB polymerization reaction, a product with high molecular weight and insolubility was produced. We therefore selected resins that immobilize enzymes *via* carrier binding. Chitopal is one of these resins, and those with various binding modes can be purchased. Based on the results of an enzyme assay using enzymes immobilized on various Chitopal resins (BCW2503, BCW2603, BCW3003, and BCW3503), Chitopal BCW2603 was chosen for the immobilization resin (Figure 2). The binding mode of Chitopal BCW2503 and BCW2603 is ionic binding, and the activity of PhbC immobilized *via* hydrophobic interactions (BCW3003 and BCW3503) was found to be very low (Figure 2). PHB formed by a polymerization reaction was hydrophobic; the low PhbC activity on BCW3003 and BCW3503 could be due to the hydrophobic interaction between PHB and the resin.

The enzymes used for monomer synthesis and PHB synthase (PhbC) were immobilized on the carrier resin Chitopal BCW2603, and PHB was synthesized from

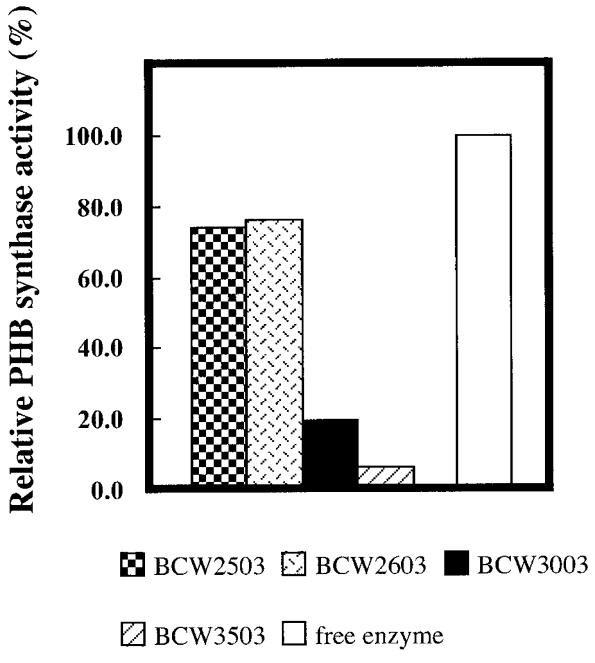

Figure 2. Assay of PhbC immobilized on Chitopal.

acetate. The progress of the polymerization reaction was confirmed by measuring glucose concentrations in the reaction mixture, based on the fact that glucose is consumed as the reaction progress. The reaction progressed rapidly up to 4 h, after which it progressed more gradually. After the polymerization reaction (72 h), the resin was air-dried *in vacuo*. Later, the conversion rate from acetate to 3HBCoA at 72 h was 28.1%, which was calculated based on the consumption of glucose. The surfaces of the resin before (Figure 3a, 3c) and after polymerization (Figure 3b, 3d) were observed with a scanning electron microscope. Many small granules were found only on the surface of the resin after polymerization. To analyze the chemical structure of these granules, the resin was treated with chloroform (1 mL) at 50 °C for 2 h. The mixture was centrifuged to remove insoluble materials, and the supernatant was transferred to another glass tube. Methanol (10 mL) was added to the tube, and the mixture was maintained at 4 °C overnight. The white precipitates that formed were filtered through a 0.2 μm poly(tetrafluoroethylene) (PTFE) membrane. The final yield of PHB was 2.2 mg, and the extraction efficiency of PHB by chloroform in the case of assuming that all 3HB-CoA formed was converted to PHB was approximately 90%. After chloroform extraction, no PHB synthase activity was observed. The lack of activity could have been due to the denaturing and elimination of PhbC. Figure 4 shows the ^{1}H NMR spectrum of the polymer. Peaks corresponding to methyl, methylene, and methyne protons were observed in the spectrum of the extract. Peak areas agreed with the structure of PHB, confirming that the granules were PHB. The number-average molecular weight and molecular weight distribution of the polymer were 204000 and 2.56, respectively. These values were smaller than those of the polymer as synthesized by soluble PhbC,[9] suggesting that polymerization is hindered by immobilization.

During the polymerization reaction, CoA was released into the reaction solution, which is known to be an inhibitor of PhbC.[11] In the case of using free PhbC, the reaction was prevented with an increase in free CoA concentrations. In contrast, in the case of using immobilized PhbC, exchange of the reaction mixture became easier by filling an empty column with immobilized PhbC, thus avoiding inhibition of PhbC by CoA. In addition, PHB can be collected by washing the column with an organic solvent such as chloroform. The above are advantages to the immobilization of PHB synthase.

The proposed mechanism for the formation of PHB granules *in vivo* is as follows.[12] Soluble enzyme con-

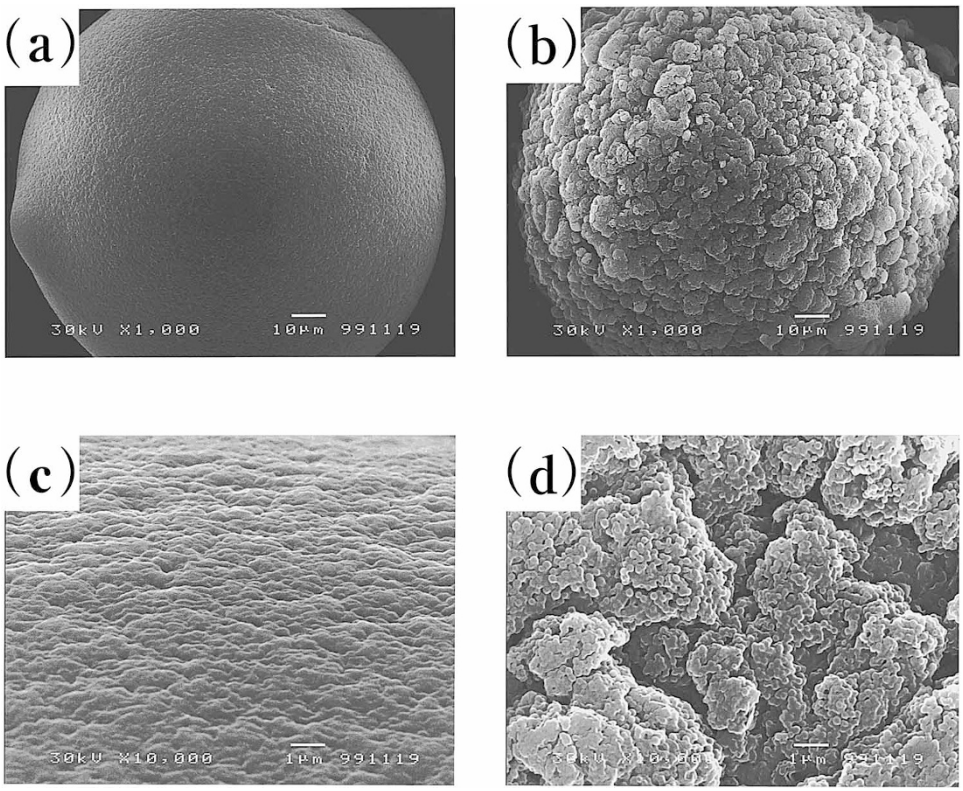

Figure 3. SEM photographs of the surface of carrier resins, a, c: before polymerization; b, d: after polymerization. Magnifications were 1000 for a, b, and 10000 for c, d.

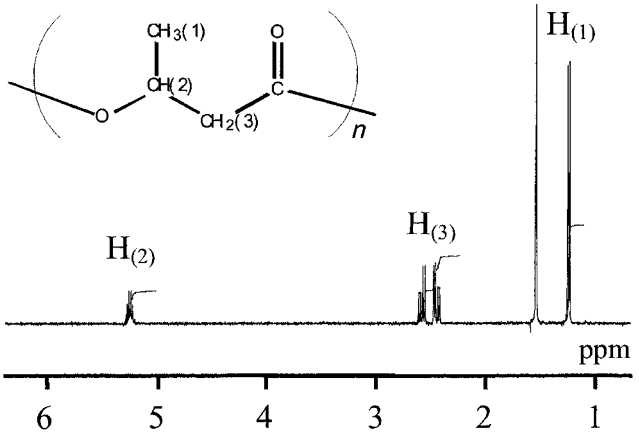

Figure 4. ^{1}H NMR spectrum of granules formed on the surface of carrier resin.

verts 3HB-CoA to oligomers (step 1). At a critical oligomer length and enzyme-oligomer concentration, the enzyme-oligomer complexes form micelles with the enzyme located at the interface, separating the PHB from the solution (step 2). Because of this compartmentalization, PHB polymerization is facilitated. Because the hydrophobic polymer can now be extruded into a hydrophobic environment instead of the aqueous phase, the reaction proceeds more rapidly. The micelles are then expanded (step 3). As the number of granules increase, they may fuse and coalesce, giving rise to large aggregates of PHB (step 4). After the polymerization reaction, small granules were observed on the surface of the resin immobilizing the enzymes (Figure 3); this result was similar to that when free PhbC was used for the reaction (data not shown). In the case that the polymerization reaction and granule formation occurred on the surface of resin, it was thought that PhbC left the resin surface with the growth of granules. For the immobilization of PhbC, covalent binding using glutaraldehyde resulted in a remarkable decrease in PhbC activity (data not shown); this decrease could be caused by the covalent binding between PhbC and resin preventing the movement of PhbC. In addition, abrasion of PHB granules was observed at the latter half, these suggested that granules grew on the resin surface.

These results suggest that PHB can be polymerized on the surface of carrier resin by immobilized PHB synthase. As such, PHB could possibly be continuously produced by using this method.

Acknowledgments. The authors are most grateful to Dr. Kouki Itoyama of Fuji Spanning Co., Ltd., for providing Chitopal. The authors would like to thank Dr. Tomoki Erata for his valuable discussions and Mr. Eiji Yamada for help with the NMR spectroscopy.

This work was supported by a grant from the Hokkaido Foundation for the Promotion of Scientific and Industrial Technology.

REFERENCES

1. T. Fukui, A. Yoshimoto, M. Matsumoto, S. Hosokawa, T. Saito, H. Nishikawa, and K. Tomita, *Arch. Microbiol.*, **110**, 149 (1976).
2. H. T. U. Gerngross and D. P. Martin, *Proc. Natl. Acad. Sci. USA*, **92**, 6279 (1995).
3. R. Jossek, R. Reichelt, and A. Steinbüchel, *Appl. Microbiol. Biotechnol.*, **49**, 258 (1998).
4. R. Jossek and A. Steinbüchel, *FEMS Microbiol. Lett.*, **168**, 319 (1998).
5. S.-J. Liu and A. Steinbüchel, *Appl. Microbiol. Biotechnol.*, **53**, 545 (2000).
6. L. Su, R. W. Lenz, Y. Takagi, S. Zhang, S. Goodwin, L. Zhong, and D. P. Martin, *Macromolecules*, **33**, 229 (2000).
7. J. J. Song, S. Zhang, R. W. Lenz, and S. Goodwin, *Biomacromolecules*, **1**, 433 (2000).
8. Q. Qi, A. Steinbüchel, and B. H. Rehm, *Appl. Microbiol. Biotechnol.*, **54**, 37 (2000).
9. Y. Satoh, K. Tajima, H. Tannai, and M. Munekata, *J. Biosci. Bioeng.*, in press.
10. Y. Satoh, N. Minamoto, K. Tajima, H. Tannai, and M. Munekata, *J. Biosci. Bioeng.*, **94**, 343 (2002).
11. G. W. haywood, A. J. Anderson, and E. A. Dawes, *FEMS Microbiol. Lett.*, **57**, 1 (1989).
12. L. L. Madison and G. W. Hisman, *Microbiol. Mol. Biol. Rev.*, **63**, 21 (1999).