C-1. 環境にやさしい有機材料設計のための劣化の制御に関する研究

Preparation of the artificial poly(3-hydroxybutyrate) granules which is a suitable substrate for intracellular poly(3-hydroxybutyrate) depolymerase

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1. Introduction

Poly(3-hydroxybutyrate) (PHB) is a major bacterial intracellular deposit found in a wide variety of bacteria [1, 2]. Recently industrial application of PHB and similar polyesters, polyhydroxyalkanoates (PHA) has been tried [3-5]. One of the chracteristics properties of PHB including PHA is its biodegradability. The extracellular PHB degradation by microorganisms has been studied extensively [6-12]. Intracellular metabolism of PHB may be important in relation to bacterial physiology and mass production of PHB in bacteria. However, only a few studies on intracellular PHB degradation in bacteria have been reported. Merrick and Doudoroff studied a hybrid system consisted of the native PHB granules from *Bacillus megaterium* and the soluble enzyme fraction from polymer-depleted cells of *Rhodospirillum rubrum* [13]. They found that the native PHB granules easily lost their degradability by repeated centrifugation and freezing and thawing. Intracellular degradation of PHB in *Alcaligenes eutrophus* was described by Hippe and Schlegel [14]. They observed only low hydrolysis activity against [^{14}C]PHB granules independent of the harvest time of the cells.

Recently we reported the intracellular PHB depolymerase activity in *Zoogloea ramigera* I-16-M and *A. eutrophus* using protease-treated native PHB granules [15, 16]. In the study of PHB depolymerase, it is important to prepare stable substrate that is degradable with the enzyme. The native PHB granules isolated from *Z. ramigera* and *A. eutrophus* showed autodigestion activity (i.e. release of D(-)-3-hydroxybutyrate, 3HB), but addition of cell extract did not increase 3HB from the native PHB granules. The idea to use protease-treated PHB granules as a substrate for intracellular PHB depolymerase was that proteins on the surface of native PHB granules may interfere PHB degradation by depolymerase outside. The protease-treated native PHB granules showed activity against cell extracts, but still has some autodigestion activity [15]. Recently Horowitz and Sanders reported the artificial PHB granules prepared from purified PHB and detergents [17]. These granules retained amorphous condition same as the PHB granules in cells. Since intracellular PHB depolymerase can not degrade the purified (crystalline) PHB, and repeated centrifugation or freezing and thawing abolish degradability of the native PHB granules reported by Merrick and Doudoroff as described above, it is highly possible that the intracellular PHB depolymerase only degrades PHB in amorphous state. Preliminary experiment showed that the artificial PHB granules made by procedure of Horowitz and Sanders using cetyltrimetylammonium bromide (CTAB)

had poor degradation activity with the bacterial cell extracts. In this work, we examined preparation conditions of the artificial PHB granules focusing on types of detergents to make a suitable substrate for intracellular PHB depolymerase.

2. Materials and Methods

2.1. Preparation of cell extract for enzyme assay

Ralstonia eutropha H16 (ATCC 17699) (formerly *A. eutrophus*, Yabuuchi et al. 1995 [18]) was grown at 30°C on a shaker in a nutrient-rich medium containing 1% yeast extract, 1% polypeptone, 0.5% meat extract, and 0.5% $(NH_4)_2SO_4$. The cells grown in the nutrient-rich medium for 24 h were harvested and washed with 0.9% NaCl. The washed cells were then suspended in a nitrogen-free medium containing 2% fructose and cultivated at 30°C for 72 h. The PHB-rich cells suspended in 5 volumes of Tris-HCl, pH 7.5 were disrupted with sonication. The ammonium sulfate fraction (0-45%) of the supernatant was used for the PHB depolymerase in this study.

2.2. Enzyme assay

PHB depolymerase activity was measured from the released 3HB [15]. The standard assay mixture (0.5 ml) containing 0.1 M Tris-HCl (pH 8.0), PHB granules (1.0 mg as PHB) and ammonium sulfate fraction from *R. eutropha* (~0.5 mg protein) was incubated at 30°C for 20 min. The reaction was stopped by heating at 100°C for 3 min, and the amount of D(-)-3-hydroxybutyric acid released was determined enzymatically.

2.3. Preparation of PHB granules for enzyme assay

PHB used in this study was extracted from *R. eutropha* with chloroform using a Soxhlet extractor. PHB was purified and recovered by precipitation with ethanol.

The artificial PHB granules were prepared according to Horowitz and Sanders [17]. Briefly, the purified PHB was dissolved in 6 ml of chloroform, then 120 ml of water containing a detergent was added. The mixture was sonicated (200 W, 20 kHz) for 10 min, and the emulsion was heated at 75°C for 90 min with stirring to remove chloroform and dialyzed for 24 h against 0.01% detergent at room temperature.

2.4. Wide-angle X-ray scattering

Artificial PHB granules (3 mg ml^{-1}, 100 ml) were collected by centrifugation (7 000 x g, 10 min) and the precipitate was added by supernatant and was adjusted to 1 ml. This suspension was analyzed immediately by wide-angle X-ray scattering (WAXS) using a MAC Science MXP-18 diffractometer, using Cu Kα radiation (λ=1.5405 Å).

3. Results and Discussion

Since detergents generally inhibit extracellular PHB depolymerase, inhibitory effect of detergent in the artificial PHB granules on intracellular PHB depolymerase was suspected. Therefore, the inhibition activity of various detergents on the intracellular PHB depolymerase was examined using the protease-treated PHB granules [15] as a substrate (Fig. 1). Among detergents examined, CTAB and sodium dodecyl sulfate strongly inhibited the enzyme activity. Sodium oleate moderately inhibited the enzyme activity at 0.01%. Sodium cholate and sodium laurate showed weak inhibition. From these results, for the artificial PHB granules made by using sodium laurate, sodium cholate or sodium oleate, their suitability as a substrate for intracellular PHB depolymerase was examined (Fig. 2). Among three artificial granules, PHB granules made with laurate and oleate showed relatively high degradation activity. However the PHB granules made with laurate tended to aggregate in preservation. Therefore, sodium oleate was chosen for a detergent to prepare the artificial PHB granules that work as a substrate for intracellular PHB depolymerase. Fig. 3 shows effects of PHB/oleate ratios on PHB degradation. PHB granules made from 0.15 g of PHB and 0.1% oleate showed relatively less degradation. In the case of PHB granules from 0.3 g of PHB and 0.1% oleate, some inhibition of degradation was observed at higher amounts of substrate. The PHB granules from 0.3 g of PHB and 0.05% oleate seem best among three. When this preparation was analyzed by WAXS, only an amorphous halo was observed. The other hands, crystalline PHB powder showed a series of sharp peaks in its diffraction pattern . This data agreed with that of Horowitz and Sanders [17].

This preparation showed a low autodigestion (less than 0.015 unit) at pH 6-11. No enzymatic degradation was observed against the artificial PHB granules that were freezed and dried, and resuspended in 0.01% sodium oleate (data not shown). Compared with the protease-treated PHB granules as a substrate for intracellular PHB depolymerase, the artificial PHB granules have several good points as follows: (i) Since the artificial PHB granules can be made from identified materials, purified PHB and sodium oleate, they show rather low autodigestion activity at wide range of pH. On the other hand, the protease-treated PHB granules contain still several unknown proteins in their granules and retain some autodigestion activity. (ii) It is easier to control quality of the artificial PHB granules than the protease-treated PHB granules. (iii) It probably be possible to make artificial polyester granules having various PHA composition that are susceptible to intracellular degradation enzymes.

Using the artificial PHB granules described here, we can expect further progress in biochemistry of intracellular PHB depolymerase in near future.

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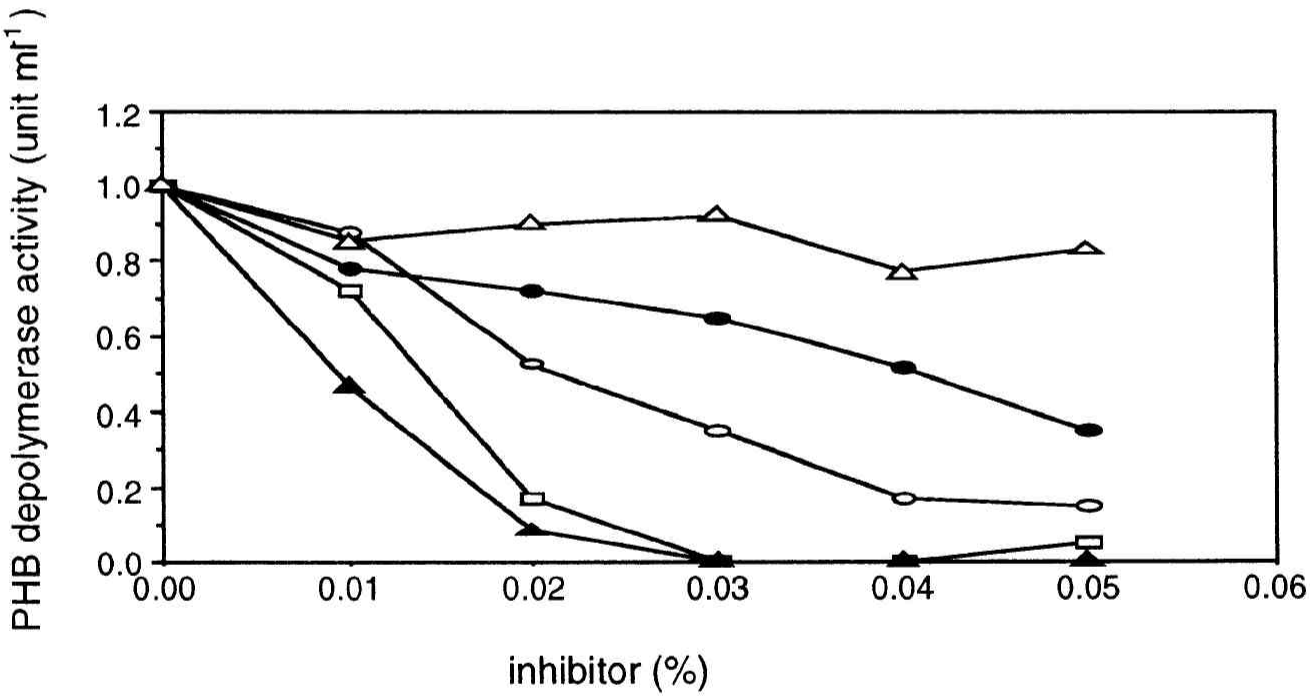

Fig. 1. Inhibition of the intracellular PHB depolymerase activity in the crude extract from *R. eutropha* by detergents. Reaction was performed as described in text using the crude extract from *R. eutropha* (0.5 mg protein). The protease-treated PHB granules were used as a substrate. Sodium oleate (○); sodium dodecylsulfate (□); cetyltrimethylammonium bromide (▲); sodium laurate (●); sodium cholate (△). One unit represents 1.0 μmol D(-)-3-hydroxybutyrate released per min under assay conditions.

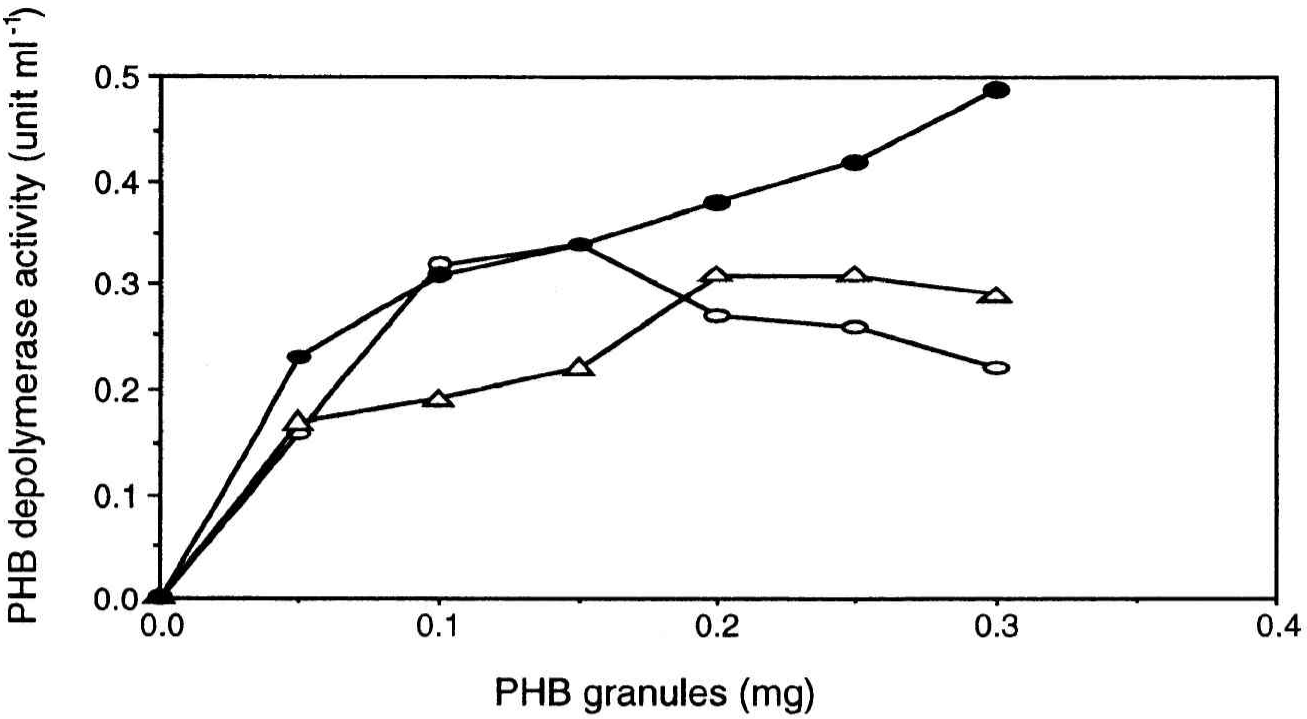

Fig. 2. Biodegradation of the artificial PHB granules with the crude extract from *R. eutropha*. The artificial PHB granules were prepared from 0.3 g PHB in 6 ml of chloroform with 120 ml of sodium oleate (0.1%) (○), sodium laurate (0.1%) (●) and sodium cholate (50 mM) (△), respectively.

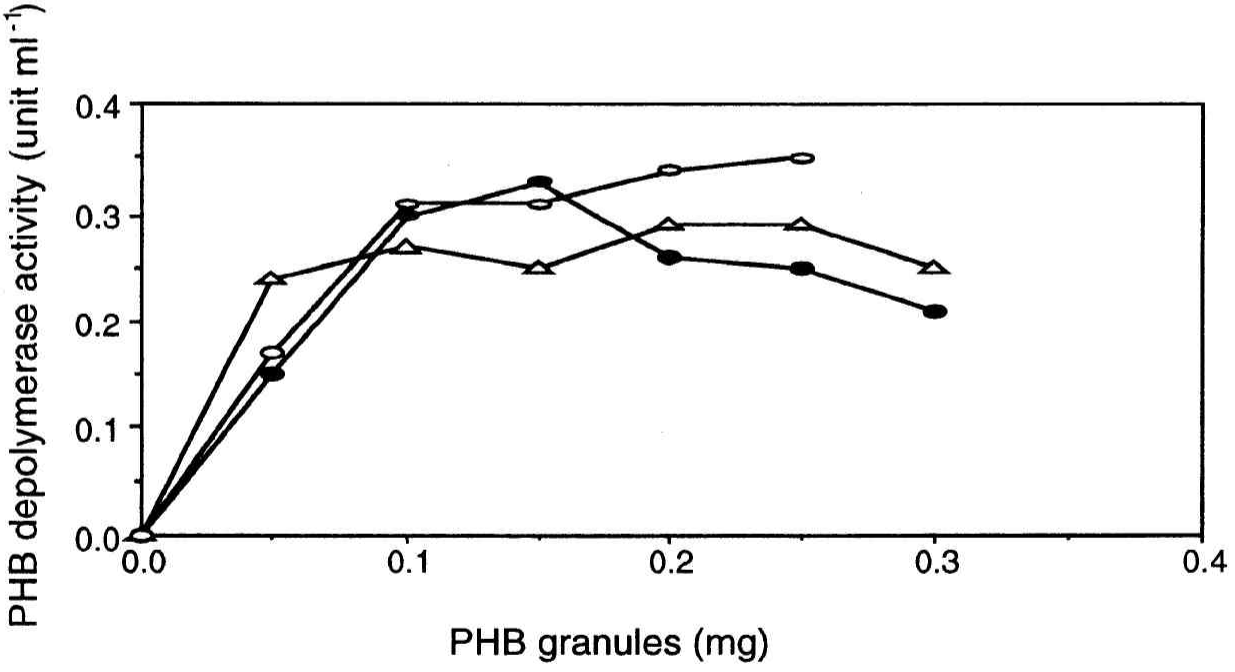

Fig. 3. Effects of PHB/oleate ratios on degradation of the artificial PHB granules with the crude extract from *R. eutropha*. The degradation of the artificial PHB granules prepared from PHB (0.15 or 0.3 g) in 6 ml of chloroform and 120 ml of sodium oleate (0.05 or 0.1%) was assayed using the crude extract from *R. eutropha* (0.5 mg protein). 0.3 g PHB and 0.1% oleate (●); 0.15 g PHB and 0.1% oleate (△); 0.3 g PHB and 0.05% oleate (○).