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An Intracellular PHB Depolymerase from the Supernatant Fraction of *Ralstonia eutropha* H16 Cells

Tomoko Abe[1], Teruyuki Kobayashi[1] and Terumi Saito[1,2,3]

[1] Laboratory of Molecular Microbiology, Department of Biological Sciences, Faculty of Science, Kanagawa University, 2946 Tsuchiya, Hiratsuka, Kanagawa 259-1293, Japan

[2] Research Institute for Integrated Science, Kanagawa University, 2946 Tsuchiya, Hiratsuka, Kanagawa 259-1293, Japan

[3] To whom correspondence should be addressed. E. mail: 43saito-bio@kanagawa-u.ac.jp

Abstract: A poly (3-hydroxybutyrate) (PHB) depolymerase was purified 4,400-fold from the supernatant fraction of *Ralstonia eutropha* cells through fractionation with ammonium sulfate and chromatography with Toyopearl DEAE, Q Sepharose, and Toyopearl Ether. The partially purified preparation showed a high level of specific activity to hydrolyze amorphous PHB, comparable to PhaZd from *R. eutropha*. The degradation was inhibited by diisopropylphosphate (10 mM) and dithiothreitol (100 mM). Most of the degradation products were 3-hydroxybutyrate oligomers. The properties of the enzyme closely resembled those of PhaZd, but some differences were observed. This distinction from PhaZd was discussed.

Keyword: poly(3-hydroxybutyrate), PHB, enzyme purification, intracellular PHB depolymerase, *Ralstonia eutropha* H16

Introduction

Poly (3-hydroxybutyrate) (PHB), a homopolymer of R(-)-3-hydroxybutyrate (3HB), is a storage material produced by a variety of bacteria under certain conditions[1]. Intracellular PHB, which is accumulated in an amorphous state, is degraded by several hydrolases for use as a source of carbon/energy[2]. When PHB-accumulating bacteria die, PHB released into the environment is hydrolyzed by microorganisms which secrete extracellular PHB depolymerases[3]. In the past few decades, the potential use of PHB in biodegradable polymers/plastics has been studied extensively[4]. As a result, the extracellular metabolism of PHB has been fully clarified in many bacteria such as *Ralstonia pickettii* T1 and *Paucimonas lemoignei,* and some fungi[5].

The intracellular PHB mobilization system is not as well understood as the extracellular PHB degradation system. Some intracellular PHB depolymerases have been reported[6-11]. The intracellular PHB depolymerase system was first identified in *Rhodospirillum rubrum* in 1964[8]. The system consists of a thermostable activator and a thermolabile esterase and it was recently reinvestigated[6, 7]. The amino acid sequence of the intracellular soluble PHB depolymrase in *Rhodospirillum rubrum* ($PhaZ1_{Rru}$) shows similarity with extracellular PHB depolymerases in, for example, *Acidovorax* sp. TP4[7,12].

In *Ralstonia eutropha* H16, four types of intracellular PHB depolymerase or 3HB-oligomer hydrolase genes have been cloned and some aspects of the gene products have been reported[9, 13-17]. PhaZa1 (formerly PhaZ1) exists in PHB inclusion bodies and degrades artificial amorphous PHB[9,15]. The existence of some PhaZa1 homologs has also been reported in *R. eutropha*[18]. Additionally, some enzymes having similar properties to PhaZa1 have been studied in other bacteria[19, 20]. PhaZb (formerly PhaZ2)[14, 16] and PhaZc mainly degrade 3HB-oligomers and the degradation product is a 3HB-monomer. PhaZd has been characterized recently[17]. This enzyme showed a high level of specific activity for the hydrolysis of amorphous PHB.

The existence of an intracellular PHB depoly-

merase in the soluble fraction of disrupted cells has been suggested in bacteria such as *Rhodospirillum rubrum* [8] and *Zoogloea ramigera* I-16-M[10]. In *R. eutropha* H16, the enzyme has been purified partially[11, 21]. Although the enzyme has different properties from PhaZa1, PhaZb, PhaZc, and PhaZd, its participation in the mobilization of PHB remains little understood. To clarify how the mobilization system works, it is important to understand the function of this enzyme.

In this report, a novel intracellular PHB depolymerase was partially purified from the soluble fraction of *R. eutropha* H16 cells.

Materials and Methods

Bacterial culture

R. eutropha H16 was cultured in a mineral medium (0.27% [wt/vol] KH_2PO_4, 0.99% [wt/vol] $Na_2HPO_4 \cdot 12\ H_2O$, 0.02% [wt/vol] $MgSO_4 \cdot 7\ H_2O$, 0.1% [vol/vol] mineral solution [0.1 M HCl, 119 mg/l $CoCl_2$, 9.7 g/l $FeCl_3 \cdot 6H_2O$, 7.8 g/l $CaCl_2$, 118 mg/l $NiCl_2 \cdot 6H_2O$, 62 mg/l $CrCl_3 \cdot 6H_2O$, 156 mg/l $CuSO_4 \cdot 5H_2O$]) containing 2% (wt/vol) fructose and 0.1% (wt/vol) ammonium sulfate at 30ºC with shaking[22]. To preserve *R. eutropha* H16, cells were grown in a nutrient (N)-rich medium (1% [wt/vol] yeast extract, 1% [wt/vol] polypeptone, 0.5% [wt/vol] beef extract, and 0.5% [wt/vol] ammonium sulfate) at 30ºC and then stocked at -80ºC, mixing in glycerol (final concentration, 25% [vol/vol]).

Enzyme assays

PHB depolymerase activity and 3HB-oligomer hydrolase activity were assayed by measuring the amount of 3HB released during the reaction. The reaction mixture (50 µl) was composed of 100 mM Tris-HCl (pH 8.0), artificial amorphous PHB granules (0.5 mg/ml), and enzyme. The reaction was started by adding substrate at 30ºC, and stopped (30 min) by adding 6 M HCl to pH 2. The reaction mixture was then centrifuged. The supernatant was used treated with for 3HB -oligomer hydrolase (1.0 U [3HB-dimer-hydrolyzing activity] of $PhaZb_{Reu}$ or $PhaZc_{Reu}$) for 10 min before the quantification of 3HB. The amount of 3HB was measured from the increase in $A_{340\ nm}$ by an enzymatic method using R(-)-3HB dehydrogenase and hydrazine hydrate [23].

Preparation of substrates

Artificial amorphous PHB granules were prepared using the method described by Horowiz and Sanders[24] as follows. The PHB isolated from *R. eutropha* H16 cells was dissolved in chloroform, and 0.05% (wt/vol) sodium deoxycholate was added. The mixture was sonicated (20 kHz, 100 W) for 2 min. The emulsion was heated to remove the chloroform and dialyzed for 24 h against 0.01% (wt/vol) sodium deoxycholate at room temperature. The linear 3HB-dimer and trimer were synthesized and then purified by HPLC. The linear 3HB-tetramer and pentamer were purified from the digest of artificial amorphous PHB granules with PhaZ7 of *Paucimonas lemoignei* [25] as described by Sugiyama et al.[26]. The chemically synthesized cyclic 3HB- oligomers were donated by D. Seebach of ETH, Switzerland[27].

Partial purification of intracellular PHB depolymerase from the soluble fraction in *R. eutropha* H16

Fifty five grams of *R. eutrtopha* H16 cells cultured in a mineral medium containing 2% (wt/vol) fructose and 0.1% (wt/vol) ammonium sulfate for 72 h was suspended in 50 mM Tris-HCl buffer (pH 7.5). The cells were sonicated twice on ice for 4 min (20 kHz, 50 W) and centrifuged at 10,000 x g for 30 min. The supernatant ("soluble fraction"), crude extract (250 ml), was fractionated with ammonium sulfate (0 to 35% saturation). The pellet was resuspended in 50 mM Tris-HCl (pH 7.5) buffer and dialyzed overnight against 20 mM Tris-HCl (pH 8.5) containing 20% (vol/vol) glycerol (buffer A). The dialyzed fraction was applied to a TOYOPEARL DEAE-650M (Tosoh, Japan) column (2.5 x 24 cm) and the column was washed with buffer A. PHB depolymerase activity was eluted with a linear gradient of NaCl (total volume, 1000 ml; 0 to 0.4 M) in buffer A. The eluted fraction was dialyzed against 20 mM Tris-HCl (pH 7.5) containing 20% (wt/vol) glycerol (buffer B) overnight and applied to a Q Sepharose FF (Amersham) column (2.5 x 5 cm). The column was washed with buffer B, and then PHB depolymerase activity was eluted with a linear gradient of NaCl (total volume, 180 ml; 0 to 0.34 M) in buffer B. To the eluted fraction was added

$(NH_4)_2SO_4$ (final concentration, 1.7 M) and the mixture was applied to a TOYOPEARL Ether- 650M column (1.5 x 6 cm) equilibrated with buffer A containing 1.7 M $(NH_4)_2SO_4$. The column was washed with buffer A and PHB depolymerase activity was eluted with a linear gradient of $(NH_4)_2SO_4$ (total volume, 60 ml; 1.7 to 0 M) in buffer A. The eluted enzyme was dialyzed overnight against 20 mM Tris-HCl (pH 8.0) containing 50% glycerol.

Immunoblot analysis

Samples were subjected to immunoblotting according to standard procedures using a rabbit antiserum against PhaZd as primary antiserum and alkaline phosphatase-conjugated goat anti-rabbit IgG (SIGMA-ALDRICH, MO, U.S.A.) as secondary antibody. The immunocomplex was visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine.

Other analytical methods

The amount of PHB was quantified as crotonic acid by HPLC as described by Karr et al.[28] Protein concentrations were measured by the method of Lowry et al.[29] with bovine serum albumin as the standard. The purity and size of proteins were estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli[30]. Proteins on the gel were stained with a Silver Stain II kit (Wako, Osaka, Japan).

Results

Partial purification of soluble PHB depolymerase from *R. eutropha* H16

R. eutropha H16 cells were cultivated in minimum salt medium containing 2% (wt/vol) fructose (carbon source) and 0.1% (wt/vol) ammonium sulfate (nitrogen source) (PHB-accumulating conditions). Both the PHB content of cells and PHB depolymerase activity in the soluble fraction reached a maximum after 72 h (Fig. 1). The soluble fraction obtained from cells cultivated for 72 h was used as the starting material for purification.

The crude extract was fractionated with $(NH_4)_2SO_4$ (0 to 35% saturation) and dissolved in 50 mM Tris-HCl (pH 7.5) buffer. Compared to the crude extract, the total activity in the ammonium sulfate fraction was increased 1.6-fold (Table 1). The ammonium

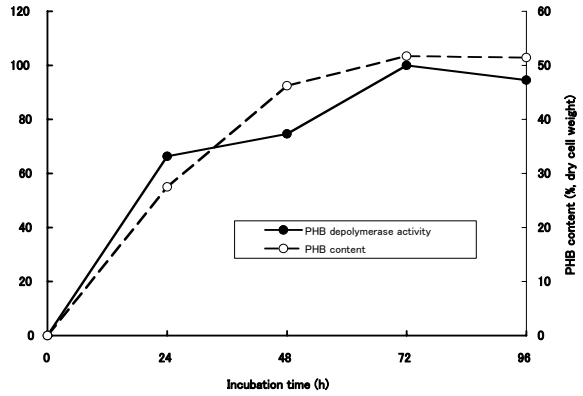

Fig. 1. PHB depolymerase activity (●)in the soluble fraction (crude extract) and PHB content (○, wt/wt) of the cells of *R. eutropha* H16 grown in PHB-accumulating conditions. The 100% value for PHB depolymerase activity was 0.019 μmole/min/mg.

sulfate fraction was purified using a TOYOPEARL DEAE-650M (anion exchanger) column, a Q Sepharose FF (anion exchanger) column, and a TOYOPEARL Ether-650M (hydrophobic resin) column in sequence. In the TOYOPEARL DEAE- 650M column and Q Sepharose FF column (Fig. 2), PHB depolymerase activity was eluted in about 0.15 M NaCl. In a TOYOPEARL Ether-650M column (Fig. 3A), the activity was eluted near 0.25 M NaCl. In step using TOYOPEARL Ether-650M, the activity of PHB depolymerase incresed more than 10-fold compared to that in the Q Sepharose FF step. However, no fractions from TOYOPEARL Ether-650M showed a single homogeneous band on SDS-PAGE when visualized with silver stain (Fig. 3B). The enzyme was partially purified 4,400-fold with 59% yield (Table 1). An increase of the total activity

Table 1. Partial purification of a intracellular PHB depolymerase from the soluble fraction of *R. eutropha*

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Crude extract	810	15	0.019	100
Ammonium sulfate	680	24	0.035	160
DEAE-650M	97	5.7	0.058	38
Q-Sepharose FF	6.4	0.76	0.12	5
Ether-650M	0.11	8.8	83	59

The enzyme was purified from a 4.4-liter culture (55 g of wet cells). Artificial amorphous PHB was used as a substrate.
U = μmol/min

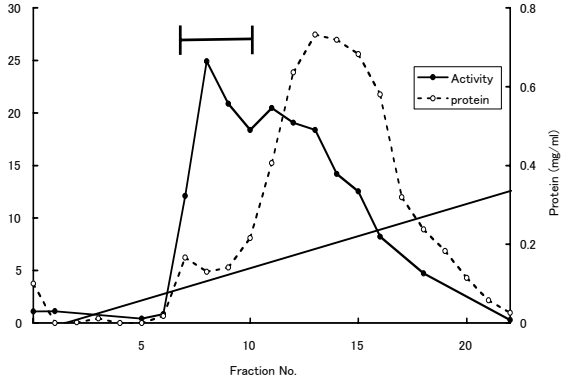

Fig. 2. Q Sepharose FF column chromatography. Artificial amorphous PHB granules were used as a substrate. Fractions 7-10 (total 40 ml) were collected. Solid circle, activity; open circle, protein.

after ammonium sulfate fractionation or the use of TOYOPEARL Ether-650M was observed. When the partially purified enzyme was mixed with crude extract, the PHB-hydrolyzing activity was partially inhibited (data not shown).

Properties of the partially purified PHB depolymerase

Table 2. shows the substrate specificity of the final preparation in the TOYOPEARL Ether-650M step. No 3HB-oligomer hydrolase activity was detected in the final preparation. The specific activity toward artificial amorphous PHB of the partially purified enzyme was much stronger than that of any other intracellular PHB depolymerase or 3HB-oligomer hydrolase in *R. eutropha* H16 reported except for PhaZd whose specific activity is 110[13-15, 17]. This enzyme, like other intracellular PHB depolymerases, did not degrade semicrystalline PHB. The release of 3HB from cyclic 3HB-oligomers or linear 3HB- oligomers by the enzyme was not detected.

Figure 4. shows the time course of the degradation of amorphous PHB by the partially purified enzyme. Most of the products were 3HB-oligomers. Even when the reaction time was lengthened, little 3HB was detected.

The effect of various reagents on the PHB depolymerase activity of the partially purified enzyme was examined (Table 3). The activity to degrade PHB was almost completely inhibited by diisopropylfluorophosphate (DFP) at 10 mM, but not by 10 mM of phenylmethane sulfonylfluoride (PMSF). 1,4-Dithiothreitol (DTT) (100 mM), by EDTA, but the purified soluble PHB EDTA (10 mM),

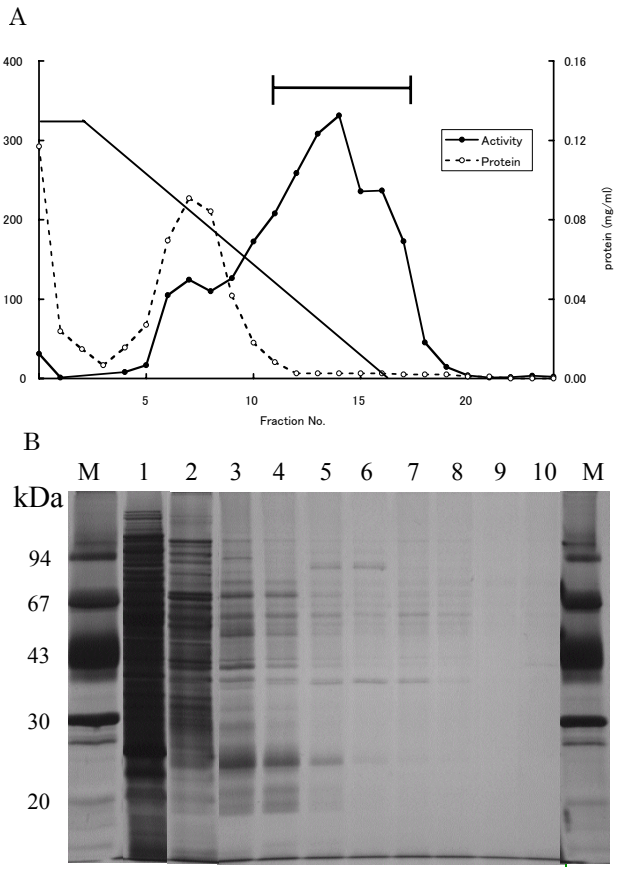

Fig. 3. Toyopearl Ether-650M column chromatography (A) and SDS-polyacrylamide gel electrophoresis of fractions at various steps in the purification process (B). A. Artificial amorphous PHB granules were used as a substrate. Fractions 11-17 (35 ml) under a bar were collected as a final preparation. B. Proteins were visualized by silver staining. Lanes 1, Toyopearl DEAE-650 fraction (5.3 mg); 2, Q Sepharose FF fraction (1.6 mg); 3, Toyopearl Ether-650M fraction 10 (0.2 mg); 4, fraction 11 (0.08 mg); 5, fraction 12 (0.02 mg); 6, fraction 13 (0.02 mg); 7, fraction 14 (0.02 mg); 8, fraction 15; 9, fraction 16 (0.02 mg); 10, fraction 17 (0.02 mg); M, molecular makers.

Table 2. Substrate specificity of the partially purified PHB depolymerase

Substrate	3HB released (μmol/min/mg)
artificial amorphous PHB granules	83
semicrystalline PHB granules	0
linear 3HB-oligomers[a]	0
cyclic 3HB-oligomers[b]	0

a; linear 3HB-dimer, -trimer, -tetramer, -pentamer
b; cyclic 3HB-trimer, -pentamer

or Triton X-100 (1%) did not completely inhibit the activity, while 0.005% Triton X-100 seemed to activate the enzyme.

The pH optimum of this enzyme was 8.5 in Tris-HCl. During storage at 4 ºC, the enzyme lost

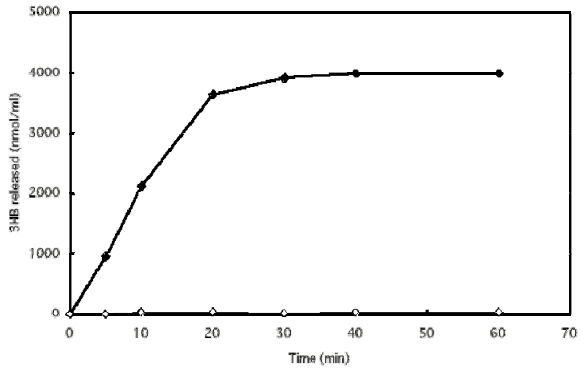

Fig. 4. Degradation products of artificial amorphous PHB granules. 0.15 mg of the partially purified PHB depolymerase was used. Reaction conditions were as described in Materials and Methods except for the reaction time. Open circle, 3HB-monomer; solid circle, 3HB-oligomer.

60% activity within 24 h. For long-term storage, freezing at -20°C was required.

Immunological analysis of the partially purified enzyme

To examine whether the partially purified soluble PHB depolymerase contains PhaZd which is a novel intracellular PHB depolymerase present in both cytosol and PHB inclusion bodies[17], the final preparation obtained in the TOYOPEARL Ether-650M step was analyzed by SDS-PAGE and stained immunologically using antibody against PhaZd. Even when the sample was concentrated 50-fold (containing about 2.5 μg of protein) or 100-fold (containing about 5 μg of protein), no significant band was detected (data not shown).

Table 3. Effect of various reagents on the partially purified PHB depolymerase

Reagents	Concentration	Activity (%)
DFP	1	63
(mM)	10	8
PMSF	1	99
(mM)	10	100
DTT	1	100
(mM)	10	86
	100	11
EDTA	1	59
(mM)	10	77
TritonX-100	0.005	136
(%,wt/vol)	0.05	62
	1	56

DFP, diisopropylfluorophosphate; PMSF, phenylmethane sulfonylfluoride; DTT, 1,4-dithiothreitol. 0.02 μg of the partially purified enzyme was used. Artificial amorphous PHB was used as a substrate.

Discussion

The intracellular PHB depolymerase partially purified from the soluble fraction of *R. eutropha* H16 in this study has several properties different from those of known intracellular PHB depolymerases or 3HB-oligomer hydrolases, PhaZa1, PhaZb, PhaZc, and PhaZd. The strong specific PHB-hydrolyzing activity is a particularly remarkable feature. The products of degradation were mainly 3HB-oligomers when artificial amorphous PHB granules were used as a substrate. Since only a very small amount of 3HB-monomer was detected in the products obtained from amorphous PHB granules, this enzyme hydrolyzes PHB in an endo-type fashion.

The partially purified enzyme was strongly inhibited by DFP, suggesting that the active center is serine. The enzyme probably has a "lipase box (G-X-S*-X-G)" which is the active center common to PhaZb, PhaZc and other PHB depolymerases except for PhaZa1. Activation by Triton X-100 at low concentrations has been reported for the extracellular PHB depolymerase of some bacteria such as *Acidovorax* sp. TP4[12]. The peak of activity was not singular in the column chromatography, suggesting that several depolymerases might be contained in the soluble fraction.

These properties are very similar to those of PhaZd[17]. Sucrose density gradient centrifugation revealed that PhaZd was distributed nearly equally in the cytosolic fraction and the PHB inclusion bodies[17]. Although these two enzymes had similar properties, they differed in how they were affected by reagents: PhaZd was activated depolymerase was partially inhibited. PhaZd was inhibited by DTT completely at 1 mM, but the purified soluble PHB depolymerase was not inhibited at that concentration of DTT at all. High concentrations of DTT (10 mM and more) partially inhibited the purified soluble PHB depolymerase (Table 3). It is possible that the partially purified sample was a mixture of PhaZd and some other PHB depolymerase(s) not inhibited by DTT. Immunostaining of the final preparation from the TOYOPEARL Ether-650M step using antiserum against PhaZd revealed no significant band. The partially purified PHB

depolymerase from the soluble fraction contains a different intracellular PHB depolymerase from PhaZd, although a minute amount of PhaZd seems to be included. The PHB depolymerase contained in the preparation purified from the soluble fraction may be an unique enzymes different from PhaZd. To clarify this, further purification is needed.

The increase in total activity after fractionation with ammonium sulfate or chromatography with TOYOPEARL Ether-650M suggests the existence of some inhibitor(s) in the soluble fraction (Table 1).

References

1) Daws EA and Ribbons DW (1964) Some aspects of the endogenous metabolism of bacteria. *Bacteriol. Rev.* **28**: 126-149.
2) Saito T and Kobayashi T (2002) Intacellular degradation of PHAs. In: *Biopolymers. 3b. Polyesters II.* Doi Y and Steinbüchel A, eds., Wiley-VCH Weinheim. pp. 23-39.
3) Jendrossek D (2002) Extracellular polyhydroxyalkanoate depolymerases: the key enzymes of PHA degradation. In: *Biopolymers. 3b. Polyesters II.* Doi Y and Steinbüchel A, eds., Wiley-VCH Weinheim. pp. 41-83.
4) Merrick JM (2002) Microbial water-insoluble aliphatic polyesters (PHA). In: *Biopolymers. 3b. Polyesters II.* Doi Y and Steinbüchel A, eds., Wiley-VCH Weinheim. pp. 105-122.
5) Jendrossek D and Handrick R (2002) Microbial degradation of polyalkanoates. *Annu. Rev. Microbiol.* **56**: 403-43
6) Handrick R, Reinhardt S, Schultheiss D, Reichart T, Schuler D, Jendrossek V and Jendrossek D (2004) Unraveling the function of the *Rhodospirillum rubrum* activator of polyhydroxy- butyrate (PHB) degradation: the activator is a PHB-granule-bound protein (Phasin). *J. Bacteriol.* **186**: 2466-2475.
7) Handrick R, Reinhardt S, Kimmig P and Jendrossek D (2004) The "intracellular" poly(3-hydroxybutyrate) (PHB) depolymerase of *Rhodospirillum rubrum* is a periplasm-located protein with specificity for native PHB and with structural similarity to extracellular PHB depolymerases. *J. Bacteriol.* **186**: 7243-7253.
8) Merrick JM and Doudoroff M (1964) Depolymerization of poly-ß-hydroxybutyrate by an intracellular enzyme system. *J. Bacteriol.* **88**: 60-71.
9) Saegusa H, Shiraki M, Kanai C and Saito T (2001) Cloning of an intracellular poly[D(-)-3- hydroxybutyrate] depolymerase gene from *Ralstonia eutropha* H16 and characterization of the gene product. *J. Bacteriol.* **183**: 94-100.
10) Saito T, Saegusa H, Miyata Y and Fukui T (1992) Intracellular degradation of poly(3-hydroxybutyrate) granules of Zoogloea ramigeta I-16-M. *FEMS Microbiol. Rev.* **103**: 333-338.
11) Saito T, Takizawa K and Saegusa H (1995) Intracellular poly(3-hydroxybutyrate) depolymerase in *Alcaligenes eutrophus*. *Can. J. Microbiol.* **41**(Suppl. 1): 187-191.
12) Kobayashi T, Sugiyama A, Kawase Y, Saito T, Mergaert J and Swings J (1999) Biochemical and genetic characterization of an extracellular poly (3-hydroxybutyrate) depolymerase from Acidovorax sp. strain TP4. *J. Environ. Polym. Degrad.* **7**: 9-17.
13) Kobayashi T, Uchino K, Abe T, Yamazaki Y and Saito T (2005) Novel intracellular 3-hydroxy- butyrate-oligomer hydrolase in *Wautersia eutropha* H16. J. Bacteriol. **187**: 5129-5135.
14) Kobayashi T, Shiraki M, Abe T, Sugiyama A and Saito T (2003) Purification and properties of an intracellular 3-hydroxybutyrate-oligomer hydrolase (PhaZ2) in *Ralstonia eutropha* H16 and its identification as a novel intracellular poly (3-hydroxybutyrate) depolymerase. *J. Bacteriol.* **185**: 3485-3490.
15) Kobayashi T and Saito T (2003) Catalytic triad of intracellular poly(3-hydroxybutyrate) depolymerase (PhaZ1) in *Ralstonia eutropha* H16. *J. Biosci. Bioeng.* **96**: 487-492.
16) Saegusa H, Shiraki M and Saito T (2002) Cloning of an intracellular D(-)-hydroxybutyrate-oligomer hydrolase gene from *Ralstonia eutropha* H16 and identification of the active site serine residue by site-directed mutagenesis. *J. Biosci. Bioeng.* **94**: 106-112.
17) Abe, T, Kobayashi, T and Saito T (2005) Properties of a novel intracellular poly(3-hydroxybutyrate) depolymerase with high specific activity (PhaZd) in *Wautersia eutropha* H16. *J. Bacteriol.* **187**: 6982-6990.
18) York GM, Lupberger J, Tian J, Lawrence AG, Stubbe JA and Sinskey AJ (2003) *Ralstonia eutropha* H16 encodes two and possibly three intracellular poly [D-(–)-3-hydroxybutyrate] depolymerase genes. *J. Bacteriol.* **185**: 3788-3794.
19) Gao D, Maehara A, Yamane T and Ueda S (2001) Identification of the intracellular polyhydroxyalkanoate depolymerase gene of *Paracoccus denitrificans* and some properties of the gene product. *FEMS Microbiol. Lett.* **196**: 159-164.
20) Kobayashi T, Nishikori K and Saito T (2004) Properties of an intracellular poly (3-hyroxybutyrate) depolymerase (PhaZ1) from *Rhodobacter sphaeroides. Curr Microbiol.* **49**: 199-202.
21) Ozawa K (1995) Thesis for degree of master of Kanagawa university.
22) Kawaguchi Y and Doi Y (1992) Kinetics and mechanism of synthesis and degradation of poly (3-hydroxybutyrate) in *Alcaligenes eutrophus*. *Macromolecules* **25**: 2324-2329.
23) Williamson DH, Mellanby J and Krebs HA (1962) Enzymic determination of D(-)-ß-hydroxybutyric acid and acetoacetic and in blood. *Biochem. J.* **82**: 90-97.
24) Horowitz DM and Sanders KM (1994) Amorphous, biomimetic granules of polyhydroxybutyrate: preparation, characterization, and biological implications. *J. Am. Chem. Soc.* **116**: 2695-2702.
25) Handrick R, Simone R, Focarete ML, Scandola M, Adamus G, Kowalczuk M and Jendrossek D (2001)

A new type of thermoalkalophilic hydrolase of *Pseudomonas lemoignei* with high specificity for amorphous polyesters of short chain-length hydroxyalkanoic acids. *J. Biol. Chem.* **276**: 36215-36224.

26) Sugiyama A, Kobayashi T, Shiraki M and Saito T (2004) Roles of poly(3-hydroxybutyrate) depolymerase and 3HB-oligomer hydrolase in bacterial motabolism. *Curr. Microbiol.* **48**: 424-427.

27) Bachmann BM, and Seebach D (1999) Investigation of the enzymatic cleavage of diastereomeric oligo (3-hydroxybutanoates) con- taining two to eight HB unit. A model for the stero selectivity of PHB depolymerase from *Alkaligenes faecalis* T1. *Macromolecules* **32**: 1777-1784

28) Karr DB, Waters JK and Emerich DW (1983) Analysis of poly-ß-hydroxybutyrate in *Rhizobium japonicum* bacteroids by ion-exclusion high-pressure liquid chromatography UV detection. *Appl. Environ. Microbiol.* **46**: 1339-1344.

29) Lowry OH, Rosebrough NJ, Farr AJ and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275

30) Leammli, UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.