# Kinetics and Mechanism of Synthesis and Degradation of Poly(3-hydroxybutyrate) in *Alcaligenes eutrophus*

**Yasushi Kawaguchi and Yoshiharu Doi***

*Research Laboratory of Resources Utilization, Tokyo Institute of Technology, Nagatsuta 4259, Midori-ku, Yokohama 227, Japan*

*Received October 2, 1991; Revised Manuscript Received January 2, 1992*

ABSTRACT: The kinetics and mechanism of the synthesis and degradation of poly(3-hydroxybutyrate) (P(3HB)) in *Alcaligenes eutrophus* have been studied. P(3HB) polymers were produced from fructose or butyric acid at 30 °C and pH 7.0 in a nitrogen-free mineral media. P(3HB) was accumulated in the cells while the carbon source was present in the media, and the accumulated P(3HB) was subsequently degraded after the carbon source was exhausted. The rates of P(3HB) accumulation were about 10 times higher than those of P(3HB) degradation. The number-average molecular weight ($\bar{M}_n$) of P(3HB) polymers increased rapidly with time during P(3HB) accumulation to reach a maximum value after around 10 h of incubation, followed by a gradual decrease in $\bar{M}_n$ with time. The maximum values of $\bar{M}_n$ were in the range of 580 000–950 000, dependent upon the type and concentration of carbon source. The polydispersities ($\bar{M}_w/\bar{M}_n$) of P(3HB) polymers were almost constant at 2.0 ± 0.3 during both P(3HB) accumulation and P(3HB) degradation. The number of P(3HB) polymer chains increased with time during P(3HB) accumulation, suggesting that a chain-transfer reaction of propagating chain takes place in this biological polymerization system. The number of P(3HB) polymerase molecules per cell was determined to be 18 000 from the time dependence of the number of P(3HB) polymer chains. A model of P(3HB) chain propagation and chain transfer on the active site of polymerase has been proposed together with a model of P(3HB) chain degradation on the active site of depolymerase.

## Introduction

A wide variety of bacteria synthesize an optically active polymer of D-(−)-3-hydroxybutyric acid as an intracellular storage material of carbon and energy, and poly(3-hydroxybutyrate) (P(3HB)) is accumulated as granules within the cytoplasm.[1-3] The isolated P(3HB) is a partially crystalline thermoplastic with biodegradable and biocompatible properties.[3] The industrial-scale production of P(3HB) has begun by using *Alcaligenes eutrophus*[4] or *A. latus*.[5]

The pathway and regulation of P(3HB) synthesis have been studied extensively in *A. eutrophus*.[6-9] P(3HB) is synthesized from acetyl-coenzyme A (acetyl-CoA) by a sequence of three enzymatic reactions. 3-Ketothiolase catalyzes the reversible condensation reaction of two acetyl-CoA molecules into acetoacetyl-CoA. The intermediate is reduced to D-(−)-3-hydroxybutyryl-CoA by NADPH-dependent acetoacetyl-CoA reductase. P(3HB) polymers are then produced by the polymerization of D-(−)-3-hydroxybutyryl-CoA by the action of P(3HB) polymerase (synthase). Recently, the DNA sequences of the genes encoding these three enzymes have been analyzed.[10-12]

The degradation of P(3HB) is initiated by P(3HB) depolymerase to form D-(−)-3-hydroxybutyric acid.[13] NAD-specific dehydrogenase oxidizes the acid to acetoacetate,[14] which is then converted to acetoacetyl-CoA.[15] Thus, acetoacetyl-CoA is an intermediate common to the synthesis and degradation of P(3HB), which occur via a cyclic metabolic process, as shown in Figure 1. Recently, we demonstrated the simultaneous operation of P(3HB) polymerase and depolymerase in *A. eutrophus* under non-growth (nitrogen-free) conditions.[16] However, the mechanisms involved in P(3HB) synthesis and degradation are little known.

In this paper we investigate the time-dependent changes in the yield and molecular weight of P(3HB) polymers produced in *A. eutrophus* cells from fructose or butyric acid during the batch fermentation. In addition, the mechanisms of P(3HB) synthesis and degradation are discussed.

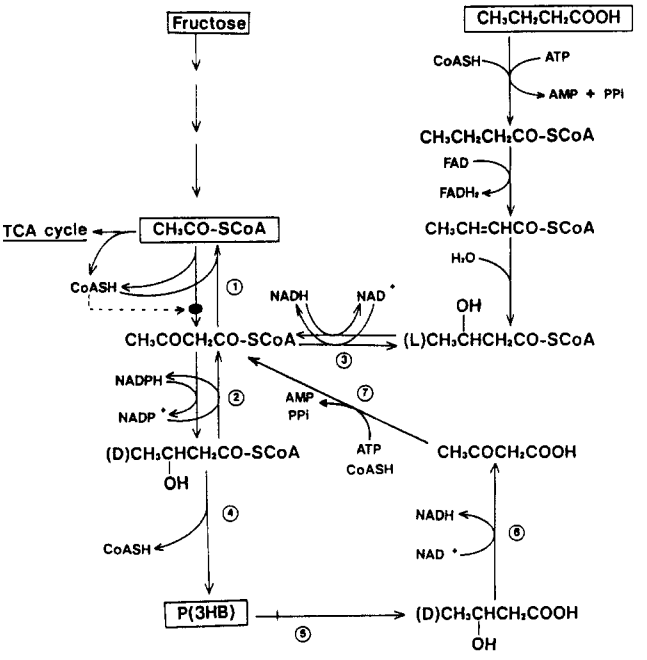


**Figure 1.** Metabolic pathway of biosynthesis and degradation of P(3HB): (1) 3-ketothiolase; (2) NADPH-dependent acetoacetyl-CoA reductase; (3) NADH-dependent acetoacetyl-CoA reductase; (4) P(3HB) polymerase; (5) P(3HB) depolymerase; (6) D-(−)-3-hydroxybutyrate dehydrogenase; (7) acetoacetyl-CoA synthetase.

## Experimental Section

P(3HB) production was carried out by two fermentation methods based on one-step and two-step batch cultivations of *A. eutrophus*. In the one-step batch cultivation, both cell growth and P(3HB) accumulation occur simultaneously, whereas cell growth does not occur during the stage of P(3HB) accumulation under the nitrogen-free conditions of the two-step incubation

* To whom correspondence should be addressed.

0024-9297/92/2225-2324$03.00/0 

system. In the one-step batch fermentation, *A. eutrophus* H16 (ATCC 17699) was inoculated into a mineral medium (1.0 L) containing 3.8 g of $Na_2HPO_4 \cdot 12H_2O$, 2.65 g of $KH_2PO_4$, 0.2 g of $MgSO_4$, 1.0 g of $(NH_4)_2SO_4$, and 1 mL of microelement solution.[17] Fructose (20 g/L) was added to the mineral medium as the sole carbon source. The cells were aerobically cultivated at 30 °C and pH 7.0 in a 2.6-L jar fermenter equipped with six conventional turbine impellers and three baffles.

In the two-step batch fermentation, *A. eutrophus* cells were first grown at 30 °C for 24 h in a nutrient-rich medium (1.0 L) containing 10 g of polypeptone, 10 g of yeast extract, 5 g of meat extract, and 5 g of $(NH_4)_2SO_4$. The cells were harvested by centrifugation at 5000*g* for 15 min. Under these growth conditions, P(3HB) was not accumulated in the cells. To promote P(3HB) synthesis, about 4 g (dry weight) of cells without P(3HB) was transferred into a nitrogen-free mineral medium (1.0 L) containing 3.8 g of $Na_2HPO_4 \cdot 12H_2O$, 2.65 g of $KH_2PO_4$, 0.2 g of $MgSO_4$, and 1 mL of microlelement solution.[17] Fructose or butyric acid was added to the nitrogen-free medium as the carbon source, and the cells were aerobically incubated at 30 °C and pH 7.0 in a 2.6-L jar fermenter.

Each 5 mL of culture was periodically collected during the one-step and two-step fermentations. The supernatant was removed by centrifugation at 5000*g* for 15 min, and the cells were lyophilized. P(3HB) was extracted from the lyophilized cells into chloroform by stirring for 40 h at room temperature, and the cell material was removed by filtration. P(3HB) samples were purified by reprecipitation with hexane and dried in vacuo.

The concentration of fructose in culture solutions was determined from optical rotation data obtained on a DIP-360 polarimeter (Japan Spectronic Co.) at 25 °C with a Na lamp (589 nm). The concentration of butyric acid was determined by gas chromatography analysis as described in a previous paper.[17] Ammonium concentration was determined by the Berthelot method.[18]

Molecular weight data of P(3HB) samples were obtianed by gel permeation chromatography (GPC) using a Shimadzu LC-9A system equipped with an RID-64 refractive index detector and a Shodex K-80M column at 40 °C. Chloroform was used as the eluent at a flow rate of 0.5 mL/min, and a sample concentration of 1.0 mg/mL was used. The number-average and weight-average molecular weights ($\bar{M}_n$ and $\bar{M}_w$) were calculated by using a Shimadzu Chromatopac C-R4A equipped with a GPC program. A molecular weight calibration curve of P(3HB) was obtained on the basis of the universal calibration method[19] with polystyrene standards of low polydispersities.

## Results

**P(3HB) Production by One-Step Fermentation.** *A. eutrophus* was cultivated at 30 °C and pH 7.0 in 1.0 L of a mineral medium containing 20 g of fructose and 1 g of $(NH_4)_2SO_4$. The initial C/N molar ratio of carbon to nitrogen sources was 37.7 mol/mol. Figure 2 shows the time course of cell growth and P(3HB) production during the one-step batch cultivation of *A. eutrophus*. The optical density (OD) of the cells increased rapidly with time during the initial stage of fermentation until the nitrogen source ($NH_4^+$) in the medium was exhausted at 19 h, indicating that the cells grow and multiply in the presence of a nitrogen source. The concentration of fructose decreased with time for 144 h, and P(3HB) polymers were accumulated up to 75% of total dry cell weight in the presence of the carbon source. At 144 h of incubation, the total dry cell weight reached 9.2 g/L, and 6.9 g/L of P(3HB) was produced from 20 g/L of fructose. The conversion of fructose into P(3HB) was as high as 35 wt %.

The number-average molecular weight ($\bar{M}_n$) of P(3HB) decreased from 580 000 to 330 000 during the course of fermentation. However, the polydispersities ($\bar{M}_w/\bar{M}_n$) of P(3HB) polymers remained almost constant at 2.0 ± 0.2 during the fermentation. Figure 3 shows the GPC elution curves of P(3HB) polymers produced for 15 h ($\bar{M}_n$ = 580 000 and $\bar{M}_w$ = 1 070 000) and for 144 h ($\bar{M}_n$ = 330 000 and $\bar{M}_w$ = 630 000). The GPC curve of P(3HB) produced for 144 h is clearly shifted toward longer elution times (lower molecular weights) without broadening of the line shape.

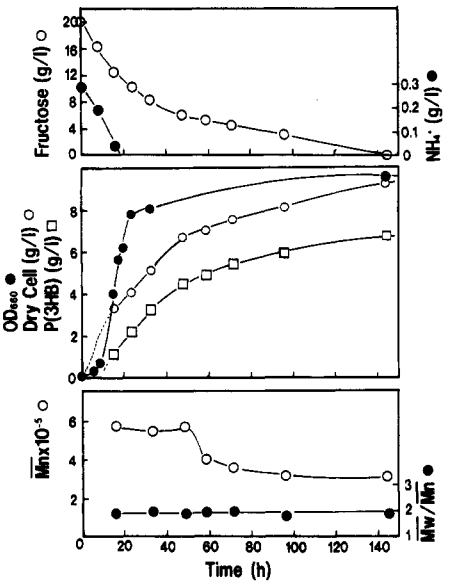


**Figure 2.** Production of P(3HB) by the one-step batch cultivation of *A. eutrophus* in the mineral medium (1.0 L) containing 20 g/L of fructose and 1 g/L of $(NH_4)_2SO_4$ at 30 °C and pH 7.0.

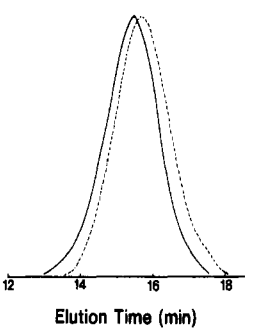


**Figure 3.** GPC elution curves of P(3HB) samples produced for 15 h (solid line) and 144 h (broken line) during the cultivation in Figure 2.

Ballard et al.[21] studied the time-dependent change in the molecular weights of P(3HB) polymers produced from glucose during the fed-batch culture of *A. eutrophus* under phosphate-limited conditions and found that the $\bar{M}_w$ of P(3HB) decreased from $2 \times 10^6$ to $6 \times 10^5$ during the course of P(3HB) accumulation. Thus, the decrease in molecular weights of P(3HB) during the course of P(3HB) accumulation under growth-limited conditions is a general phenomenon.

These results suggest that P(3HB) polymers of high molecular weights accumulated in the initial stage of fermentation are degraded in the later stage where P(3HB) polymers are still produced. The simultaneous operation of P(3HB) polymerase and depolymerase enzymes in *A. eutrophus* under nongrowth (nitrogen-free) conditions has been demonstrated by us.[16]

In the one-step batch cultivation of *A. eutrophus*, the cells accumulated 30 wt % of P(3HB) at the end (15 h) of the growth stage in the presence of the nitrogen source, indicating that both cell growth and P(3HB) accumulation occurred simultaneously. This P(3HB) accumulation during the cell-growth stage is not suited for the study on the kinetics and mechanism of the synthesis and degradation of P(3HB). Therefore, a two-step batch fermentation of *A. eutrophus* was carried out for the study.

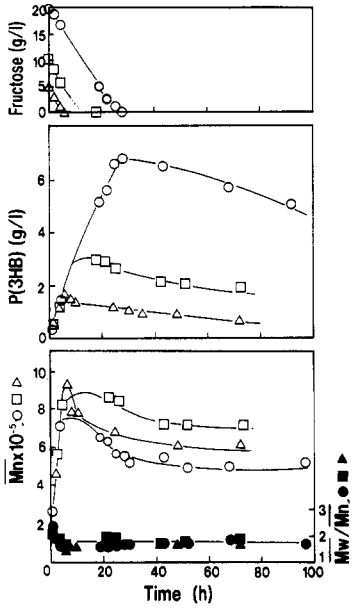


**Figure 4.** Time course of P(3HB) accumulation and degradation during the batch incubation of *A. eutrophus* in a nitrogen-free mineral medium (1.0 L) containing fructose ((○) 20 g/L; (□) 10 g/L; (△) 5 g/L)) as the sole carbon source at 30 °C and pH 7.0.

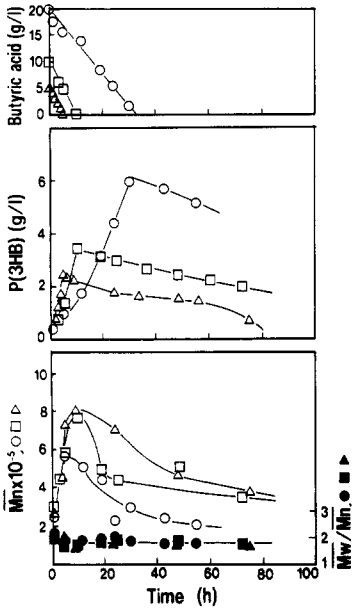


**Figure 5.** Time course of P(3HB) accumulation and degradation during the batch incubation of *A. eutrophus* in a nitrogen-free mineral medium (1.0 L) containing butyric acid ((○) 20 g/L; (□) 10 g/L; (△) 5 g/L)) as the sole carbon source at 30 °C and pH 7.0.

**P(3HB) Production by Two-Step Fermentation.** *A. eutrophus* was first grown for 24 h in a nutrient-rich medium in which P(3HB) was not accumulated within cells. About 4 g (dry weight) of the collected cells without P(3HB) was transferred into a nitrogen-free mineral medium (1.0 L) containing different amounts of fructose or butyric acid, and the cells were incubated at 30 °C and pH 7.0 to accumulate P(3HB).

Figures 4 and 5 show the time courses of P(3HB) accumulation and degradation in *A. eutrophus* cells during the batch incubation in the nitrogen-free media containing fructose and butyric acid, respectively. The initial concentrations of the carbon sources in the media were 5, 10, and 20 g/L. The concentrations of fructose or butyric acid decreased with time and reached zero within 30 h of incubation. P(3HB) was accumulated in the cells while the carbon sources were present in the media. After the carbon sources were exhausted, P(3HB) was gradually degraded with time, indicating that P(3HB) was utilized for the energy generation under conditions of carbon starvation.

**Table I**
**Maximum Weight of P(3HB), Rate of P(3HB) Accumulation, and Rate of P(3HB) Consumption in Nitrogen-Free Media Containing Fructose or Butyric Acid as the Carbon Source for *A. eutrophus***

| concn, $g \cdot L^{-1}$ | max wt, $g \cdot L^{-1}$ | rate of accum, $g \cdot h^{-1} \cdot L^{-1}$ | rate of consump, $g \cdot h^{-1} \cdot L^{-1}$ |
|---|---|---|---|
| fructose | | | |
| 5 | 1.5 | 0.25 | 0.013 |
| 10 | 2.9 | 0.29 | 0.027 |
| 20 | 6.7 | 0.26 | 0.025 |
| butyric acid | | | |
| 5 | 2.4 | 0.48 | 0.023 |
| 10 | 3.4 | 0.34 | 0.023 |
| 20 | 6.0 | 0.19 | 0.034 |

Table I lists the maximum weight of P(3HB), the rate of P(3HB) accumulation, and the rate of P(3HB) consumption in the nitrogen-free media containing fructose or butyric acid. The rates of P(3HB) accumulation from fructose were in the range 250–290 $mg \cdot h^{-1} \cdot L^{-1}$, independent of the concentration of fructose. In contrast, the rate of P(3HB) accumulation from butyric acid decreased from 480 to 190 $mg \cdot h^{-1} \cdot L^{-1}$ with increasing initial concentration of butyric acid. The rates of P(3HB) consumption after the exhaustion of carbon sources were almost constant at 25 ± 2 $mg \cdot h^{-1} \cdot L^{-1}$ except for the two cases of P(3HB) consumption from 5 g/L of fructose and 20 g/L of butyric acid.

As can be seen in Figures 4 and 5, the $\bar{M}_n$ values of P(3HB) polymers increased rapidly with time to reach a maximum value at around 10 h of incubation period, followed by a gradual decrease in $\bar{M}_n$ with time. The maximum values of $\bar{M}_n$ were in the range 580 000–950 000, dependent upon the type and concentration of carbon source. The polydispersities ($\bar{M}_w/\bar{M}_n$) of P(3HB) polymers were almost constant at 2.0 ± 0.3 during the course of incubation.

Figure 6 shows the time-dependent changes in the weight and $\bar{M}_n$ of P(3HB) polymers in *A. eutrophus* caused by the addition of fructose to the nitrogen-free medium. After 5 g/L of fructose was added at 24 h of incubation, the cells accumulated P(3HB) again while fructose was present in the medium. The P(3HB) accumulated in the cells was degraded in the absence of the carbon source. The $\bar{M}_n$ value of P(3HB) again increased with time after the addition of fructose, followed by a gradual decrease in $\bar{M}_n$ during the incubation. Thus, the increase in $\bar{M}_n$ during the initial stage of P(3HB) accumulation is a reproducible phenomenon.

## Discussion

**Kinetics and Mechanism of P(3HB) Synthesis.** P(3HB) is well-known to be synthesized by the polymerization of D-(−)-3-hydroxybutyryl-CoA with P(3HB) polymerase.[9] The D-(−)-3-hydroxybutyryl-CoA was produced via several enzymatic reactions from fructose or butyric acid, as shown in Figure 1. A key enzyme in P(3HB) synthesis is P(3HB) polymerase with thiol groups as the active site.[20] Peoples and Sinskey[11] have demonstrated that the *A. eutrophus* P(3HB) polymerase encoded by the *phbC* gene is a polypeptide with the molecular mass of 63 940 and has five cysteine residues, $Cys^{246}$, $Cys^{316}$, $Cys^{382}$, $Cys^{438}$, and $Cys^{459}$. The P(3HB) polymerase activity

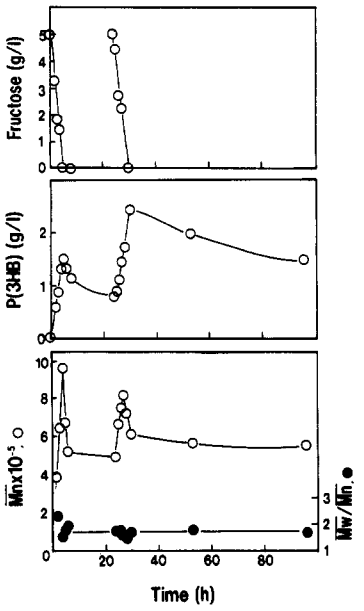


**Figure 6.** Effect of addition of fructose (5 g/L, at 24 h) on the production and number-average molecular weight ($\bar{M}_n$) of P(3HB) during the batch incubation of *A. eutrophus* in a nitrogen-free mineral medium (1.0 L).

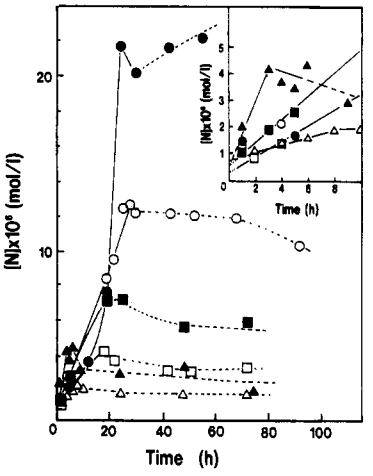


**Figure 7.** Time-dependent changes in the number of polymer chains [$N$] on the stages of accumulation (solid line) and degradation (broken line) of P(3HB) during the batch incubation of *A. eutrophus* in a nitrogen-free medium containing fructose ((○) 20 g/L; (□) 10 g/L; (△) 5 g/L) and butyric acid ((●) 20 g/L; (■) 10 g/L; (▲) 5 g/L), respectively.

of *A. eutrophus* was found to remain almost constant during the P(3HB) accumulation, independent of the content of P(3HB) within cells.[9] This result suggests that the number of polymerase molecules remains constant throughout the P(3HB) formation under nitrogen-free conditions. As described below, the number of P(3HB) polymerase molecules per cell was 18 000. Here, we determine the number of P(3HB) polymerase molecules per cell from the relation between the yield and molecular weights of P(3HB).

The number of P(3HB) polymer chains produced for time $t$, $[N]_t$ (mol/L), can be calculated by

$$[N]_t = Y_t/\bar{M}_{n,t} \quad (1)$$

where $Y_t$ and $\bar{M}_{n,t}$ are the yield and number-average molecular weight of P(3HB) polymers produced for time $t$, respectively. Figure 7 shows the time-dependent changes in the number of polymer chains [$N$] of P(3HB) produced from fructose or butyric acid under nitrogen-free conditions (see Figures 4 and 5). The [$N$] values increased with time during the stage of P(3HB) accumulation in the presence of a carbon source (as represented by the solid lines in Figure 7), indicating that a chain-transfer reaction of propagating chain takes place in this biological polymerization system. The length of growing P(3HB) chain is limited by the chain-transfer reaction, and the [$N$] value stops increasing once the carbon source is exhausted.

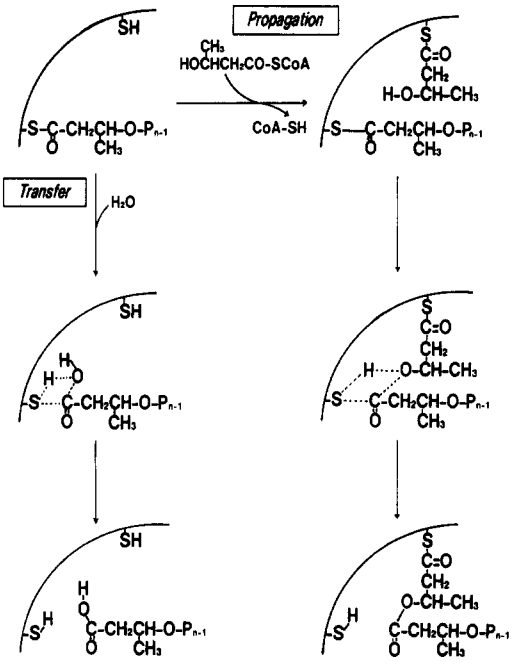


**Figure 8.** Model of chain propagation with (D)-(−)-3-hydroxybutyryl-CoA and of chain transfer with water on the active site of P(3HB) polymerase.

Here, we propose the reaction scheme of initiation, propagation, and chain transfer in the P(3HB) synthesis with polymerase as follows.

initiation

$$\text{E–SH} + \text{D-CH}_3\text{CH(OH)CH}_2\text{CO–SCoA} \xrightarrow{k_i} \text{CH}_3\text{CH(OH)CH}_2\text{CO–S–E} + \text{CoA–SH} \quad (2)$$

propagation

$$\text{P(3HB)}_{n-1}\text{OCH(CH}_3\text{)CH}_2\text{CO–S–E} + \text{D-CH}_3\text{CH(OH)CH}_2\text{CO–SCoA} \xrightarrow{k_p} \text{P(3HB)}_n\text{OCH(CH}_3\text{)CH}_2\text{CO–S–E} + \text{CoA–SH} \quad (3)$$

chain transfer with water

$$\text{P(3HB)}_{n-1}\text{OCH(CH}_3\text{)CH}_2\text{CO–S–E} + \text{H}_2\text{O} \xrightarrow{k_t} \text{P(3HB)}_{n-1}\text{OCH(CH}_3\text{)CH}_2\text{COOH} + \text{E–SH} \quad (4)$$

where CoA and P(3HB)$_n$ denote coenzyme A and a polymer chain with $n$ polymerization degree of 3HB units, respectively. The P(3HB) synthesis on the active site of P(3HB) polymerase (E–SH) is initiated by a reaction of D-(−)-3-hydroxybutyryl-CoA with P(3HB) polymerase (E–SH) (eq 2), followed by the chain propagation reaction (eq 3). The chain-transfer reaction with water may take place (eq 4).

Figure 8 shows a model of chain propagation with D-(−)-3-hydroxybutyryl-CoA and chain transfer with water on the active site of P(3HB) polymerase. Ballard et al.[21] originally proposed the active-site model of polymerase consisting of two thiol groups, one locating the incoming 3HB monomer and the other locating the propagating polymer chain. Condensation occurs through a four-

**Table II**
**Rates of Propagation ($R_p$), Transfer ($R_t$), and Degradation ($R_d$) of P(3HB) Polymer Chains in Nitrogen-Free Media Containing Fructose or Butyric Acid and Their Rate Constants ($k_p$ and $k_t$)**

| concn, g·L$^{-1}$ | $R_p$, mol·h$^{-1}$·L$^{-1}$ | $k_p$, h$^{-1}$ | $R_t$, mol·h$^{-1}$·L$^{-1}$ | $k_t$, h$^{-1}$ | $R_d$, mol·h$^{-1}$·L$^{-1}$ |
|---|---|---|---|---|---|
| fructose | | | | | |
| 5 | $2.9 \times 10^{-3}$ | $5.8 \times 10^{3}$ | $1.4 \times 10^{-7}$ | 0.28 | $1.5 \times 10^{-4}$ |
| 10 | $3.3 \times 10^{-3}$ | $6.6 \times 10^{3}$ | $1.4 \times 10^{-7}$ | 0.28 | $3.1 \times 10^{-4}$ |
| 20 | $3.0 \times 10^{-3}$ | $6.0 \times 10^{3}$ | $3.6 \times 10^{-7}$ | 0.72 | $2.9 \times 10^{-4}$ |
| butyric acid | | | | | |
| 5 | $5.6 \times 10^{-3}$ | $11.2 \times 10^{3}$ | $5.8 \times 10^{-7}$ | 1.16 | $2.7 \times 10^{-4}$ |
| 10 | $4.0 \times 10^{-3}$ | $8.0 \times 10^{3}$ | $2.9 \times 10^{-7}$ | 0.58 | $2.7 \times 10^{-4}$ |
| 20 | $2.2 \times 10^{-3}$ | $4.4 \times 10^{3}$ | $7.5 \times 10^{-7}$ | 1.50 | $4.0 \times 10^{-4}$ |

membered transition state, leaving one of the thiol groups vacant for the next 3HB monomer.

If we make the assumptions that (i) polymerization involves a chain-transfer reaction, (ii) there is a rapid initiation reaction, and (iii) the number of polymerase molecules remains constant during the P(3HB) synthesis, the number-average polymerization degree ($\bar{P}_{n,t}$) of P(3HB) polymers produced for time $t$ is given by

$$\bar{P}_{n,t} = \frac{\int_0^t R_p\,dt}{[E] + \int_0^t R_t\,dt} = \frac{k_p[E]t}{[E] + k_t[E]t} \qquad (5)$$

with $R_p = k_p[E]$ and $R_t = k_t[E]$. Here, $R_p$, $R_t$, and [E] denote the rate of propagation, the rate of chain transfer, and the concentration of polymerase molecules, respectively, and $k_p$ and $k_t$ are the pseudo-first-order rate constants for chain propagation and chain transfer. The $k_p$ and $k_t$ containing the concentrations of monomer and transfer agent are assumed to remain constant during the polymerization.

The number of P(3HB) polymer chains $[N]_t$ at time $t$ is given by eq 6 from eq 5.

$$[N]_t = k_p[E]t/\bar{P}_{n,t} = [E] + k_t[E]t \qquad (6)$$

We can estimate the values of [E] by the extrapolation of $[N]_t$ values to time zero, using the plots of $[N]_t$ vs $t$ in Figure 7. The number of polymerase molecules was (5 ± 1) × $10^{-7}$ mol/L, independent of the type and initial concentration of carbon source in nitrogen-free media. The number of *A. eutrophus* cells in the nitrogen-free medium during the stage of P(3HB) accumulation was determined to be $1.7 \times 10^{13}$ cells/L. Then the number of polymerase molecules per cell could be calculated to be 18 000.

The values of $R_t$ and $k_t$ were determined from the slopes of the $[N]_t$ vs $t$ plots in Figure 7 by using eq 6. In addition, the values of $R_p$ and $k_p$ were calculated by using the rates of P(3HB) accumulation (in Table I) and the values of [E]. The values of $R_p$, $k_p$, $R_t$, and $k_t$ are listed in Table II.

**Kinetics and Mechanism of P(3HB) Degradation.** P(3HB) polymers in cells were degraded as a carbon and energy source under conditions of carbon starvation. During the stage of P(3HB) degradation, the $\bar{M}_n$ and content of P(3HB) polymers in cells decreased with time at almost the same rate (see Figures 4 and 5). As a result, the number of P(3HB) polymer chains [$N$] was almost constant during the stage of P(3HB) degradation after the exhaustion of the carbon source (as represented by broken lines in Figure 7). This result suggests that the P(3HB) intracellular depolymerase is an exo-type hydrolase which hydrolyzes an ester bond of P(3HB) at the terminus of polymer chain and releases a monomeric 3-hydroxybutyric acid. In addition, the polydispersities ($\bar{M}_w/\bar{M}_n$) of the P(3HB) polymers remained constant at 2.0 ± 0.3 during the stage of P(3HB) degradation, suggesting that the P(3HB) depolymerase hydrolyzes the termini of all P(3HB) polymer chains.

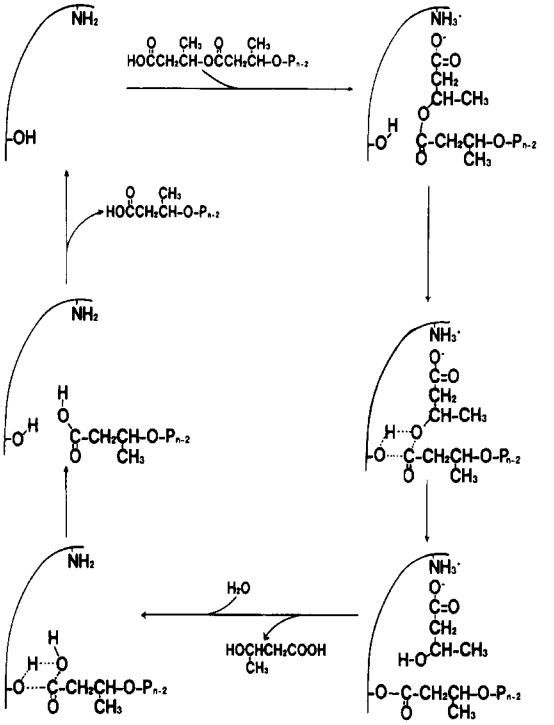


**Figure 9.** Model of P(3HB) hydrolysis on the active site of intracellular P(3HB) depolymerase.

Hippe and Schlegel[13] demonstrated that a soluble intracellular P(3HB) depolymerase isolated from *A. eutrophus* hydrolyzed native P(3HB) granules to give D-(−)-3-hydroxybutyric acid as the sole product. However, little is known about the structure and properties of intracellular P(3HB) depolymerase. Assuming that the P(3HB) depolymerase is a serine-esterase with a hydroxy group as the active site,[22,23] we propose the reaction scheme of P(3HB) degradation with depolymerase (E–OH) as follows.

$$\text{E–OH} + \text{P(3HB)}_n \rightarrow \text{P(3HB)}_{n-1}\text{O–E} + \text{D-CH}_3\text{CH(OH)CH}_2\text{COOH}$$

$$\text{P(3HB)}_{n-1}\text{O–E} + \text{H}_2\text{O} \rightarrow \text{P(3HB)}_{n-1} + \text{E–OH} \qquad (7)$$

A model of P(3HB) degradation with depolymerase is illustrated in Figure 9. The COOH terminus of the P(3HB) polymer chain may bind to a cationic residue of the depolymerase with an electrostatic interaction. The rates of P(3HB) degradation ($R_d$) were calculated from the rates of P(3HB) consumption in Table I. The result is given in Table II. The rate constants of P(3HB) degradation could not be determined since the concentration of P(3HB) depolymerase was unknown.

As can be seen from Table II, the rates ($R_p$) of P(3HB) chain propagation with polymerase were about 10 times higher than those ($R_d$) of P(3HB) chain degradation with depolymerase. As described in the Introduction, we have recently demonstrated the simultaneous operation of P(3HB) polymerase and depolymerase in *A. eutrophus* under nongrowth conditions,[16] which suggests the cyclic nature of P(3HB) metabolism (see Figure 1). In Figure 2, we showed that the $\bar{M}_n$ values of P(3HB) decreased with time while P(3HB) polymers were accumulated in

cells under nitrogen-free conditions. This result supports the simultaneous operation of P(3HB) polymerase and depolymerase in *A. eutrophus*.

## References and Notes

(1) Dawes, E. A.; Senior, P. J. *Adv. Microbiol. Physiol.* **1973**, *10*, 135.
(2) Anderson, A. J.; Dawes, E. A. *Microbiol. Rev.* **1990**, *54*, 450.
(3) Doi, Y. *Microbial Polyesters*; VCH Publishers: New York, 1990.
(4) Byrom, D. *Trends Biotechnol.* **1987**, *5*, 246.
(5) Hăggi, U. J. In *Novel Biodegradable Microbial Polymers*; Dawes, E. A., Ed.; Kluwer Aademic Publishers: Dordrecht, 1990; p 65.
(6) Oeding, V.; Schlegel, H. G. *Biochem. J.* **1973**, *134*, 239.
(7) Haywood, G. W.; Anderson, A. J.; Chu, L.; Dawes, E. A. *FEMS Microbiol. Lett.* **1988**, *52*, 91.
(8) Haywood, G. W.; Anderson, A. J.; Chu, L.; Dawes, E. A. *FEMS Microbiol. Lett.* **1988**, *52*, 259.
(9) Haywood, G. W.; Anderson, A. J.; Dawes, E. A. *FEMS Microbiol. Lett.* **1989**, *57*, 1.
(10) Peoples, O. P.; Sinskey, A. J. *J. Biol. Chem.* **1989**, *264*, 15293.
(11) Peoples, O. P.; Sinskey, A. J. *J. Biol. Chem.* **1989**, *264*, 15298.
(12) Schubert, P.; Krüger, N.; Steinbüchel, A. *J. Bacteriol.* **1991**, *173*, 168.
(13) Hippe, H.; Schlegel, H. G. *Arch. Mikrobiol.* **1967**, *56*, 278.
(14) Nakada, T.; Fukui, T.; Saito, T.; Miki, K.; Oji, C.; Matsuda, S.; Ushijima, A.; Tomita, K. *J. Biochem.* **1981**, *89*, 625.
(15) Fukui, T.; Ito, M.; Tomita, K. *Eur. J. Biochem.* **1982**, *127*, 423.
(16) Doi, Y.; Segawa, A.; Kawaguchi, Y.; Kunioka, M. *FEMS Microbiol. Lett.* **1990**, *67*, 165.
(17) Doi, Y.; Kawaguchi, Y.; Nakamura, Y.; Kunioka, M. *Appl. Environ. Microbiol.* **1989**, *55*, 2932.
(18) Sonnleiter, B.; Heinzle, E.; Braunegg, G.; Lafferty, R. M. *Eur. J. Appl. Microbiol. Biotechnol.* **1979**, *7*, 1.
(19) Grubisic, Z.; Rempp, R.; Benoit, H. *J. Polym. Sci., Part B* **1967**, *5*, 753.
(20) Griebel, R.; Smith, Z.; Merrick, J. M. *Biochemistry* **1968**, *7*, 3676.
(21) Ballard, D. G. H.; Holmes, P. A.; Senior, P. J. In *Recent Advances in Mechanistics and Synthesis Aspects of Polymerization*; Fontanille, M., Guyot, A., Eds.; Reidel Publishing Co.: Lancaster, U.K., 1987; Vol. 215, p 219.
(22) Shirakura, Y.; Fukui, T.; Saito, T.; Okamoto, Y.; Narikawa, T.; Koide, K.; Tomita, K.; Takemasa, T.; Masamune, S. *Biochim. Biophys. Acta* **1986**, *880*, 46.
(23) Huisman, G. W.; Wonink, E.; Meima, R.; Kazemier, B.; Terpstra, P.; Witholt, B. *J. Biol. Chem.* **1991**, *266*, 2191.

**Registry No.** P(3HB), 26063-00-3; fructose, 57-48-7; butyric acid, 107-92-6.