The first order intracellular degradation of various polyhydroxyalkanoic acid (PHA) inclusions in *Hydrogenophaga pseudoflava* cells was investigated by analyzing the compositional and microstructural changes of the PHA using gas chromatography, $^{13}$C NMR spectroscopy, and differential scanning calorimetry. Two types of PHA, copolymers and blend-type polymers, were separately accumulated in cells for comparison. The constituent monomers were 3-hydroxybutyric acid (3HB), 4-hydroxybutyric acid (4HB), and 3-hydroxyvaleric acid (3HV). It was found that the 3HB-4HB copolymer was degraded only when the polymer contained a minimal level of 3HB units. With the cells containing a 3HB/4HB blend-type polymer, only poly(3HB) was degraded, whereas poly(4HB) was not degraded, indicating the totally inactive nature of the intracellular depolymerase against poly(4HB). On the basis of the magnitude of the first order degradation rate constants, the relative substrate specificity of the depolymerase toward the constituting monomer units was determined to decrease in the order 3HB $>$ 3HV $>$ 4HB. $^{13}$C NMR resonances of the tetrad, triad, and dyad sequences were analyzed for the samples isolated before and after degradation experiments. The results showed that the intracellular degradation depended on the local monomer sequence of the copolymers. The relative substrate specificity of the depolymerase determined from the NMR local sequence analysis agreed well with that obtained from the kinetics analysis. It is suggested that, without isolation and purification of the intracellular PHA depolymerase and “native” PHA substrates, the relative specificity of the enzyme as well as the microstructural heterogeneity of the PHA could be determined by measuring in situ the first order degradation rate constants of the PHA in cells.

Many microorganisms synthesize poly(3-hydroxybutyrate) (P(3HB)) intracellularly and accumulate it in granular inclusion bodies as a carbon and energy reserve (1–3). They also synthesize other types of polyhydroxyalkanoic acids (PHA) in the form of homopolymers, copolymers, or polyester blends. More than 100 different monomer units are known to be incorporated into the polymer chain (3). Accordingly, various kinds of copolymers are expected when a bacterium is grown on the mixtures of different precursors. However, even if any two copolymers happen to have similar overall ratios of the comonomer units, the microstructure (e.g. local sequence of monomers) of the two copolymers may be quite different, resulting in totally different physicochemical properties, e.g. thermal transition temperatures, biodegradability, and mechanical strength (2). Such microstructural heterogeneity is usually caused by the different assimilation rate of the monomeric precursors into the bacterium. The width and randomness of comonomer distribution in the copolymers may depend on the availability of the comonomers in the form of their CoA thioesters and the specificity of the PHA synthase.

Most studies of PHA degradation have been performed on extracellular depolymerases using “denatured” crystalline PHA as substrates. Many extracellular PHA depolymerases have been isolated and characterized in terms of their biochemical and molecular biological properties (6). The rate of enzymatic degradation of microbial PHA is determined by the specificity of the depolymerases and physicochemical factors such as the crystallinity and monomer composition of the PHA (2, 6). However, little is known about the bacterial intracellular PHA depolymerases, probably because of the difficulty in the isolation and purification of these enzymes without loss of activity. It is well known that the “native” PHA inclusions are completely amorphous (7–9). They are readily crystallized when isolated from cells. Stabilization of the isolated amorphous granular substrates to prevent them from crystallizing could be another difficulty in the study of the intracellular depolymerases because they exhibit little activity with crystalline PHA (6).

In a previous study, we found that the polyester synthesized by *Hydrogenophaga pseudoflava* from glucose plus γ-butyrolactone, using a one-step cultivation, was not a homogeneous random copolymer but a mixture of 3HB-rich and 4HB-rich chains (10). This microstructural heterogeneity is considered to result from the different assimilation rate of the two precursors. However, the 3HB-3HV copolymers prepared from glucose and γ-valerolactone were relatively homogeneous copolymers. PHA producing microorganisms usually have intracellular PHA depolymerases. Therefore, any difference in the microstructural heterogeneity among intracellular polymers, which even have a similar composition of monomers, is expected to be strongly reflected in the intracellular degradation reaction by the depolymerase(s) because of its probable high substrate specificity. According to our recent study (11), P(4HB) homopolymer was found to be not degraded in *H. pseudoflava* cells, whereas P(3HB) homopolymer was almost completely degraded within 24 h in the presence of ammonium sulfate. This totally different susceptibility of the two homopolymers to intracellular degradation suggested that per-
forming an in situ kinetics study of the intracellular degradation reactions might enable us to obtain information on the microstructures of the 3HB-4HB copolymer inclusions as well as the specificity of the intracellular PHA depolymerase.

High resolution NMR spectroscopy has been used as a powerful tool to determine the microstructural heterogeneity (specifically, heterogeneity in local sequences) of the microbial copolymers (2, 4, 5). It is possible to assign the 13C resonances present in local dyad, triad, and tetrads sequences. Therefore, a comparative analysis of the NMR spectra obtained before and after degradation of a copolyester in cells should enable us to determine the local sequence specific degradation of the PHA by the intracellular depolymerase.

In this study, we analyzed the microstructure of three different types of copolymers in H. pseudoflava cells, which included 3HB-3HV, 3HB-4HB, and 3HV-4HB copolymers, by applying first order reaction kinetics to the intracellular degradation. An additional set of polymers in the form of blend-type polymers (a mixture of two different homopolymers) 3HB/3HV, 3HB/4HB, and 3HV/4HB were also analyzed for comparison. Each blend-type polymer had a similar ratio of two component monomers to that in the corresponding copolymer. All data obtained from the analyses of intracellular degradation kinetics, NMR, and thermal transition correlated well with one another. These analyses showed that the H. pseudoflava intracellular depolymerase is the most active against 3HB-rich sequences and the least active against 4HB-rich sequences.

EXPERIMENTAL PROCEDURES

Organism and Culture Media—Strain ATCC 33668 of H. pseudoflava was purchased from the American Type Culture Collection. Inocula were grown in 5-ml test tubes containing nutrient-rich media (1% yeast extract, 0.5% tryptone, and 0.2% ammonium sulfate) for 48 h. The following two media were used in the cultivation of the bacterium for PHA accumulation: 1) LB medium (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl in 1 liter of distilled water) and 2) modified PHA synthesis mineral medium (11, 12). All growth experiments were performed under aerobic conditions in a temperature-controlled shaker (Korea Instrument Co., Seoul, Korea) at 35 °C and 190 rpm.

3HB Homopolymer Accumulation—The precultured (nutrient broth grown) cells were transferred to a PHA synthesis mineral medium containing 12 g/liter of glucose and cultivated for 30 h. Cells were harvested by centrifugation in a Beckman J2-HS (rotor JA-10, 6000 rpm for 10 min).

3HB-4HB Polymer Accumulation—Two types of 3HB-4HB polymers, differing in their structural heterogeneity, were accumulated in cells. The cells containing a 3HB-4HB copolyester were prepared by transferring the precultured cells to a PHA synthesis mineral medium containing 10 g/liter of glucose and 2 ml/liter of γ-butyrolactone and cultivating them at 35 °C for 72 h. The harvested cells contained 43 wt% (by dry cell weight) of the copolyester composed of 41 mol% 3HB and 59 mol% 4HB. The 3HB/4HB blend-type polymer, having a ratio of monomer unit composition similar to that of the 3HB-4HB copolyester just described, was accumulated in cells by cultivating the nutrient broth grown preculture in an LB medium containing 4 g/liter of glucose for 22 h and then transferring first step grown cells to a second step PHA synthesis mineral medium containing 1 ml/liter of γ-butyrolactone and cultivating for 18 h. The blend-type polymer in the cells was composed of 45 mol% 3HB and 56 mol% 4HB, and the cells contained 24 wt% of PHA.

The 3HB-4HB copolymer having more than 90% of 4HB was accumulated by cultivating the LB grown cells in a PHA synthesis mineral medium containing 2 ml/liter of γ-butyrolactone plus 0.6 g/liter of ammonium sulfate for 48 h. The cells containing 3HB-free P(4HB) homopolymer were prepared using a three-step cultivation method (11).

3HB-3HV Polymer Accumulation—To accumulate 3HB-3HV copolymers in cells, the precultured cells were transferred to an LB medium and cultivated for 22 h, and then the LB grown cells were incubated in a PHA synthesis mineral medium containing only ammonium sulfate (0.6 g/liter) for 12 h to remove the residual P(3HB). The cells were transferred to a PHA synthesis medium containing γ-valerolactone (2 ml/liter) and ammonium sulfate (0.6 g/liter) and cultivated for 18 h. The harvested cells contained 24.5 wt% of PHA composed of 21 mol% 3HB and 79 mol% 3HV.

The cells containing a copolymer with a higher level of 3HB (57 mol% 3HB and 43 mol% 3HV) were prepared by cultivating the precultured cells in a PHA synthesis mineral medium containing glucose (10 g/liter), γ-valerolactone (2 ml/liter), and ammonium sulfate (0.6 g/liter) for 24 h. The blend-type polymer composed of 3HB- and 3HV-rich chains, having a similar level of comonomer mole ratio (3HB/3HV = 55:45), was accumulated by cultivating the precultured cells in an LB medium containing glucose (4 g/liter) for 23 h and then cultivating the LB grown cells in a PHA synthesis mineral medium containing γ-valerolactone (3 ml/liter) and ammonium sulfate (0.6 g/liter) for 24 h. The cells contained 30.4 wt% of PHA.

3HV-4HB Polymer Accumulation—For the preparation of the cells containing a 3HV-4HB copolymer, the LB grown cells were transferred to a PHA synthesis mineral medium containing γ-butyrolactone (1 ml/liter), γ-valerolactone (1 ml/liter), and ammonium sulfate (0.6 g/liter) and cultivated for 36 h. The harvested cells contained 18 wt% of PHA composed of 42 mol% 3HV and 58 mol% 4HB. In the preparation of the cells containing a 3HV/4HB blend-type polymer having a similar level of the comonomer ratio to that in the copolymer just described, the LB grown cells were transferred to a PHA synthesis mineral medium containing γ-butyrolactone (2 ml/liter) and ammonium sulfate (0.6 g/liter) and cultivated for 30 h. The cells were transferred to a three-step PHA synthesis mineral medium containing γ-valerolactone (2 ml/liter) and ammonium sulfate (0.6 g/liter) and cultivated for 18 h. The cells contained 26 wt% of PHA blend composed of 43 mol% 3HV and 57 mol% 4HB. Only less than 1–2 mol% of 3HB was detected in the PHAs.

Monitoring the Intracellular Degradation of Various Polymers in Carbon-Free Media—Cells containing various types of polymers were recovered by centrifugation and transferred to a carbon-free mineral medium (a medium containing the same mineral as a PHA synthesis mineral medium) containing ammonium sulfate (1.0 g/liter) (or not containing ammonium sulfate for the control experiment). The cells were incubated under an aerobic shaking condition at 35 °C and 190 rpm. 5 ml of the culture was removed every 5 h to analyze the medium and the cells. The amount of NH₃ remaining in the medium was measured by the Nessler reagent method (12). The monomer unit composition of the PHA remaining in cells was determined by gas chromatography with a Hewlett Packard HP5890A gas chromatograph equipped with a Carbowax 20M column and a flame ionization detector (11, 12). Total protein content in cells was determined by the Bradford method.

Polyester Isolation and Characterization—Polymers were extracted from an appropriate amount of cells that had been dried overnight at 50 °C under a vacuum. Extraction was performed with hot chloroform in a Pyrex Soxhlet apparatus for 6 h. The concentrated solvent extract was dried in a vacuum at 50 °C under a vacuum. The precipitated polymer was dissolved in methanol and then by measuring the intrinsic viscosity of the polymer dissolved in chloroform. The viscosity was performed with standard software.

The change in the molecular weight of P(3HB) in cells during degradation was monitored by isolating the polymer remaining in cells and purifying it by reprecipitation in methanol and then by measuring the intrinsic viscosity of the polymer dissolved in chloroform. The viscosity of the polymer solutions (1–5 mg/ml) was measured at 30 °C using a capillary viscometer of the Cannon Fenske type (capillary number 50), which was immersed in a constant temperature bath. The polymer solution was filtered with a 0.45-µm Gelman membrane filter before viscosity measurement. The molecular weight of P(3HB) was calculated using the Mark-Houwink equation [η] = k[M]ⁿ, where the constant k is equal to 7.7 × 10⁻⁵ (cm²/g) and n is equal to 0.82 in chloroform (19).
Local Sequence Dependence of Intracellular PHA Degradation

RESULTS

Intracellular Degradation of P(3HB) Homopolymer in H. pseudoflava—As shown in Fig. 1, 90% of the P(3HB) accumulated in cells was degraded after the cells were transferred to and cultivated for 24 h in a carbon-free mineral medium containing ammonium sulfate, of which the initial concentration was 1.0 g/liter. The initial dry biomass was 3.69 g/liter and the content of P(3HB) in cells was 69 wt % by dry cell weight. The NH₄⁺ in the medium was consumed exponentially and almost depleted within 24 h during degradation. The optical density at 660 nm of the medium was significantly decreased from 9.63 at 0 h to 4.00 at 30 h and finally reached 3.58 at 60 h. Thus, despite the significant consumption of NH₄⁺, a substantial increase in both OD and biomass was not observed. As a control experiment, the cells containing P(3HB) were also incubated in a medium free from both carbon and nitrogen. Only 10% of degradation was observed after 24 h of cultivation, and the extent of degradation never exceeded 15% after 60 h (data not shown).

As a first order of approximation, the degradation rate of P(3HB) in cells was analyzed in terms of first order kinetics (Fig. 2). The final value of [3HB]∞ at infinite time was approximated by averaging the last three values in Fig. 1 (hereafter referred to as the steady-state method). The same approximation was applied to the other degradation process, in which a steady-state period was reached within 60 h, leading to no further degradation caused by limitations. A linear regression analysis of the data in Fig. 2 resulted in the degradation rate constant, \( k_1 \), of 0.109 h⁻¹. The correlation coefficient \( r^2 \) of the fitted data points in the figure was 0.985 at a 95% confidence interval. The half-life for the degradation of P(3HB) was 5.6 h, and 95% of the P(3HB) in the cells was degraded after 60 h. A further increase in the concentration of ammonium sulfate, up to 2.0 g/liter, resulted in the same \( k_1 \), value of 0.123 h⁻¹ (\( r^2 = 0.975 \)) as with the concentration of 1.0 g/liter, despite slightly less degradation (85% degradation) at the increased level of nitrogen (data not shown).

Intracellular Degradation of 3HB-4HB Polymer—The time course for the degradation in cells containing a blend-type 3HB/4HB polymer initially composed of 45 mol % 3HB/55 mol % 4HB is shown in Fig. 3. The concentration of the 4HB unit in the cell suspension was constant throughout the degradation period of 60 h, which indicated that the 4HB units in the blend-type polymer were not degraded. In contrast, the 3HB units in the cells disappeared according to the first order degradation process. The apparent first order degradation rate constant, \( k_1 \), for P(3HB) in the blend polymer, was determined to be 0.109 h⁻¹ (\( r^2 = 0.982 \)) for the initial 45 h degradation, in which 70% of the total 3HB units was degraded (data not shown). In this plot, the same final-value approximation as in Fig. 2 was made.

Contrary to the case of the 3HB/4HB blend-type polymer, the 4HB units in a copolymer composed of 41 mol % 3HB and 59 mol % 4HB were found to be degraded under the same incubation condition as in the blend-type polymer. A first order regressive fitting for the data points over 60 h of cultivation resulted in the apparent \( k_1 \) values 0.049 h⁻¹ and 0.015 h⁻¹ for 3HB- and 4HB units, respectively. Their correlation coefficients, \( r^2 \), were 0.976 and 0.984, respectively (Fig. 4). In this plot, the two values at infinite time, [3HB]∞, and [4HB]∞ were assumed to be zero (hereafter referred to as the initial rate method) because of continuing degradation of the copolymer even after 60 h. This means that 4HB units can be degraded if they are copolymerized with 3HB units. It is interesting to note the remarkable difference in the \( k_1 \) values of the two monomer units in the copolymer. With a real random copolymer, a similar or the same value of \( k_1 \) is expected for the adjoining two monomers because the overall reaction is controlled by the degradation of the monomer unit showing a slower rate. The dissimilarity in \( k_1 \) values for the two monomer units in a copolymer sample thus suggests the existence of two different types of polymer chains, 3HB-rich and 4HB-rich chains. Such probable microstructural heterogeneity, as in the 3HB-4HB copolymer, may be related with the different assimilation rate of the two monomer precursors, glucose and γ-butyrolactone, in PHA accumulation by the bacterium (10).
The suggested microstructural heterogeneity in the 3HB-4HB copolymer was detected in detail at a molecular level by using high resolution $^{13}$C NMR spectroscopy, in which each resonance peak is usually shifted by the presence of a different neighboring monomer unit, and thus every shifted resonance has information on the local monomer sequence of the copolyester (2, 4, 5). For the copolymers composed of 3HB and 4HB monomer units, the sequences of dyad, triad, and tetrad were assigned assuming a first order Markovian random copolymerization in cells (4). The expanded spectra of carbonyl carbon resonance at 169–173 ppm are shown in Fig. 5. The upper two spectra are for the samples recovered before and after degradation of the 3HB/4HB copolymer having an initial composition of 41 mol % 3HB and 59 mol % 4HB (the samples in Fig. 4). The lower two spectra are for the blend-type polymer having the initial overall composition of 45 mol % 3HB and 55 mol % 4HB (the samples in Fig. 3). As expected from the preparation procedure of each polyester, resonances ascribable to the sequences 43, 434, and 334 were smaller in the blend-type polymer than that in the copolymer. This clearly demonstrates that the blend-type polymer is principally a mixture of P(3HB) and P(4HB), the mixture containing a minor amount of P(3HB-co-4HB). For both the copolymer and blend-type polymer, the relative intensities of two absorption signals associated with 333 and 433 triads were significantly reduced after degradation, whereas the intensities of the signals for 4HB-rich sequences (e.g. 444 and 344) were relatively enhanced. Generally, the sequences having more 4HB units were more slowly degraded, as seen from the comparison of their signal intensities.

In a similar manner, sequence-dependent changes in the absorption for the other six $^{13}$C resonance-peaks in the region of 15–70 ppm occurred before and after degradation of the two types of 3HB/4HB polymers. In the methylene carbon CH$_2$ (2) associated with the 3HB unit (Fig. 6), the relative intensity of the 33$^*$33 sequence peak decreased, whereas the intensities for the other three tetrad sequences (43$^*$34, 43$^*$33, and 33$^*$34) and 4HB-inserted triad sequences (43$^*$4 and 33$^*$4) were strongly enhanced after 60 h degradation. As in the case of the split carbonyl absorption described, the more 4HB units in a sequence, the higher the relative intensity of the sequence after degradation. Thus, the degradation of tetrad sequences occurred in the decreasing rates of 33$^*$33 > 43$^*$33 and 33$^*$34 > 43$^*$34. In the blend-type polymer, the signals associated with the 4HB-containing tetrad sequences were only barely detectable, as expected. However, the intensity of the signal associated with the triad 43$^*$4 sequence was more strongly enhanced than that of 33$^*$4 sequence after degradation in a similar fashion to the case of the copolymer. The methyl carbon CH$_3$ (4) in 3HB was assigned to the resonances around 20 ppm. Four peaks associated with triad sequences 43$^*$4, 43$^*$3, 33$^*$4, and 33$^*$3 were identified (spectra not shown). In the blend polymer the two triad sequences 43$^*$3 and 33$^*$4 showed little absorption, which agreed with the observation of the carbonyl and methylene carbon described above. For both types of polymers, the intensity of the signal associated with the 33$^*$3 sequence decreased, whereas that with the 43$^*$4 sequence increased after degradation, as expected. The other signals associated with carbons such as CH(3), CH$_2$(6), CH$_2$(7), and CH$_3$(8) showed...
similar sequence-dependent intensity changes to that observed for the four carbons described (data not shown), except for the fact that the carbons CH\textsubscript{2}(6), CH\textsubscript{2}(7), and CH\textsubscript{2}(8) in the 4HB unit of the blend-type polymer showed little change in the ratios of the related local sequence-associated \textsuperscript{13}C NMR signals. This must be due to the preferential degradation of P(3HB) in the blend-type polymer. However, in the 3HB-4HB copolymer, a significant change in the ratios of the signal intensities of the sequences related to the three 4HB carbons was noticed after degradation, which additionally demonstrates that the initial copolymer was a mixture of 3HB-rich and 4HB-rich chains, not a homogeneous random copolymer.

The structural heterogeneity of the 3HB/4HB blend-type polymer was also confirmed by analyzing the two samples obtained before and after degradation using differential scanning calorimetry. The blend-type polymer exhibited two melt transitions at 45 and 160 °C, respectively (Fig. 7). Two different polymers are usually immiscible because of unfavorable interactions between them (14). Therefore, after melting a mixture of two crystallizable polymers, the component polymers crystallize in separate domains. Melting of the resulting immiscible polymer blend thus reveals two melting endotherms. P(3HB) homopolymer melts at 175 °C, and P(4HB) homopolymer melts at 55 °C (11). The exhibition of the two melting transitions (Fig. 7) indicates that the polymer sample was a blend, not a copolymer. The significant decrease in the relative area of the endothermic peak at 160 °C, observed after degradation, additionally supports that 3HB-rich polymer preferentially degraded in the 3HB/4HB blend-type polymer. However, the 3HB-4HB copolymer showed a single endotherm at 52 °C both before and after degradation, indicating the absence of the high degree of structural heterogeneity as in the blend-type polymer.

Intracellular Degradation of 3HB-3HV Polymers—Degradation of PHA in H. pseudoflava cells.
tion experiments were carried out for two types of cells, one containing a copolymer composed of 57 mol % 3HB/43 mol % 3HV and the other a blend-type polymer composed of 55 mol % 3HB/45 mol % 3HV. Both type of cells almost completely consumed the added NH₄Cl (1.0 g/liter) at 60 h of incubation. 24% of PHA remained in the copolymer cells, and 18% of PHA remained in the blend-type polymer cells. In addition, the amounts of the remaining P(3HB) and P(3HV) were 5.5 and 30.6%, respectively, for the blend-type polymer. Contrary to the case of the 3HB/4HB blend-type polymer, degradation of the second monomer 3HV unit in the 3HB/3HV blend-type polymer cells was observed. First order rate plots for the degradation of the two types of 3HB-3HV polymers also showed good linear correlation over the entire degradation period (data not shown). A linear regression analysis for the degradation of the copolymer resulted in the k₁ value of 0.026 h⁻¹ for the 3HB unit (r² = 0.949) and that of 0.022 h⁻¹ for the 3HV unit (r² = 0.975) at a 95% confidence interval. In this calculation, the initial rate method was employed because of continuing degradation of the copolymer after 60 h. The k₁ values for the 3HB and 3HV units in the blend-type polymer were determined to be 0.077 h⁻¹ with r² = 0.998 and 0.055 h⁻¹ with r² = 0.991, respectively, by the steady-state method. Thus, the degradation of the 3HV unit was slower than that of the 3HB unit. The similar degradation rate constants for both monomer units in the 3HB-3HV copolymer indicate the almost complete random nature of the copolymer, containing no long blocked chains as well as being less heterogeneous than in the 3HB-4HB copolymer. In addition, the higher degradation rate of the 3HB-rich chains in the blend-type polymer may imply that the H. pseudoflava intracellular depolymerase is more specific to 3HB units than to 3HV units.

First order degradation kinetics analysis was also applied to the 3HB-3HV copolymer containing a high level of 3HV, poly (21 mol % 3HB-co-79 mol % 3HV). Fifty-one % of the NH₄Cl added at the concentration of 1.0 g/liter remained unconsumed and 37 wt % of PHA remained not degraded after 60 h of incubation (data not shown). The k₁ values for 3HB and 3HV units were calculated to be 0.099 h⁻¹ (r² = 0.992) and 0.083 h⁻¹ (r² = 0.992), respectively, by the steady-state method.

Local sequence-specific degradation of 3HB-3HV polymers was also investigated by using 125 MHz ¹³C NMR spectroscopy. The sequence assignment was made according to the method of Doi and co-workers (5). The carbonyl absorption region contained the information on the distribution of dyad sequences, VV, BV, and BB (Fig. 8). For the sample before degradation, the absorption intensity of the doublet signal at 169.3 ppm associated with VB and BV was very low in the blend-type polymer (55 mol % 3HB/45 mol % 3HV). However, in the copolymer having a similar composition (57 mol % 3HB/43 mol % 3HV), it was almost comparable with the sum of the VV and BB signal intensities indicative of a rather homogeneous random copolymer, considering the similar 3HB/3HV monomer ratio in the sample before degradation. For both polymers, the relative intensity of the signal associated with BB was significantly reduced after 60 h of degradation. This again shows the higher specificity of the intracellular depolymerase to 3HB units than to 3HV units. The other dyad absorption signals associated with the carbons CH₃(9), CH(7), CH₃(2), CH₄(4), and CH₄(9) showed a similar trend (data not shown). An increase in the relative signal intensity of the BB sequence and a decrease in that of the VB sequence were generally observed for the carbons CH₃(2), CH₃(3), CH₄(2), and CH₄(4) during degradation. For the carbons CH(7) and CH₄(9) exhibiting VB and VV signals, the signal ratio of VB to VV decreased, as expected. A spectral analysis for the triad sequences associated with CH₃(6) and CH₂(8) in the 3HV unit revealed that an increase in the number of 3HV units in a sequence caused the relative intensity of the corresponding signals to increase during degradation (Fig. 9). The change is significant enough to distinguish between BBV and VBV or BVV sequences of the copolymer (Fig. 9). However, in the blend-type polymer, the change in resonance absorption of the carbons CH(7), CH₃(6), and CH₄(8) in the 3HV unit was negligible, probably because of the rather slower degradation of the 3HV-rich polymer chains giving rise to the absorption signals. Actually, 95% of the 3HB unit in the blend-type polymer was degraded, but 31% of the 3HV unit remained not degraded after 60 h of incubation.

The DSC trace of the 3HB-3HV (the initial molar ratio,
57:43) copolymer before degradation was very similar to that of the polymer remaining after degradation (data not shown). However, the DSC trace of the 3HB/3HV blend-type polymer exhibited two strong melting endothermic peaks of a similar area at 110 and 165 °C, ascribable to the melting of P(3HV) (15) and P(3HB), respectively. Similar to the case of the 3HB/4HB blend-type polymer, the presence of the two endothermic peaks indicates the existence of two separate crystalline domains associated with 3HV-rich P(3HB-co-3HV) and P(3HB). The relative area of the endothermic peak for P(3HB) was significantly reduced after degradation with the area for the 3HV-rich polymer being remarkably increased.

**Intracellular Degradation of 3HV-4HB Polymers**—For direct comparison of the relative degradation rate between 3HV units and 4HB units, we prepared two types of 3HV-4HB polymers, a blend-type polymer (P(3HV):P(4HB) = 43:57, in terms of monomer mole ratio) and a poly(42 mol % 3HV-co-58 mol % 4HB) copolymer. Both polymers contained less than 1–2 mol % of 3HB. Only 30% of the NH4+ (1.0 g/liter) was consumed for 60 h of incubation. The amount of the remaining 3HV unit was 85% for the copolymer and 47% for the blend-type polymer. This indicates that the introduction of the 4HB unit into the 3HV-containing polymer retarded the degradation of the polymer. No degradation of 4HB unit was observed for the two polymers. The k1 values for the 3HV unit were determined to be 0.076 h⁻¹ (r² = 0.987) and 0.079 h⁻¹ (r² = 0.979) for the blend-type and copolymer sample, respectively, by the steady-state method. This result clearly shows again that the intracellular depolymerase has no activity against the 4HB unit in P(4HB).

**DISCUSSION**

**NMR Parameter D as an Index of the Microstructural Heterogeneity of PHA**—Table I shows that the intracellular PHA degradation is a function of the NMR microstructure of the inclusion polyesters. A NMR structure parameter D is defined as the ratio FBB/FVV/FBV/FVB for 3HB-3HV polymers, where FBB is the fraction of the dyad sequence 3HB-3HB and the other Fij values are defined similarly (2, 4, 5). Each value for the fraction Fij was determined by measuring the intensity of the related split 13C NMR signal and normalizing it in terms of the total summed area for the carbon under consideration. Similarly, for 3HB-4HB polymers, D is defined as the ratio FBB/F43/F33F34, where F43 is the fraction of the dyad sequence 3HB-4HB. Before degradation, the initial D values for the two blend-type polymers, 3HB/4HB and 3HB/3HV, were 35.7 and 56.6, respectively. A random copolymer has the D value of 1.0 (2, 4, 5). The high D value for each blend-type polymer means that the polymer in cells was a mixture of the corresponding homopolymer and copolymers (e.g. P(3HB) and 4HB-rich P(3HB-co-4HB) or 3HV-rich P(3HB-co-3HV)). Inhomogeneity of the blend-type polymers in cells was also ascertained from the significant change in monomer composition before and after degradation as well as from the significant difference in the disappearance rate (k1) of the two comprising monomer units (Table I). Considering the low D values close to unity, the two P(3HB-co-3HV) copolymer copolymers were thought to be random copolymers. The randomness of comonomer distribution is also indicated by the little change in their monomer composition after degradation of the sample before degradation. A remarkable change in monomer composition was also observed after degradation of the copolymer. The large difference in apparent k1 value for the two comonomers, 3HB and 4HB, is another indication of the heterogeneity of the polymer. Even after degradation, the remaining polymer exhibited a high D value of 3.3, reflecting still a heterogeneous state of the polymer. A careful examination of the DSC trace for the polymer before degradation reveals at least two phase structures associated with an sharp endotherm at around 50 °C and a broad endotherm around 130 °C (Fig. 7). Thus, the parameter D can be used for the determination of the structural heterogeneity of bacterial polyesters.

**Determination of Relative PHA Depolymerase Specificity by in Situ Degradation Kinetics Analysis**—It is interesting to note the fact that the degradation of the inclusion polymers (e.g. P(3HB)) in the cells occurs by first order kinetics in the presence of additional ammonium sulfate (1.0 g/liter). The little degradation in the absence of additional nitrogen may be due to the metabolic suppression which prevents the flow of carbon derived from P(3HB) or/and a limited number of the depolymerase. The addition of nitrogen thus releases the suppres-
by synthesizing proteins, etc. Actually, the content of total protein in cells increased up to almost twice the initial amount when the added ammonium (1.0 g/liter) was nearly consumed after 20 h of incubation (Fig. 1), whereas without additional ammonium, the protein content was relatively constant through the incubation (data not shown).

Little is known about the mechanism of the intracellular PHA degradation (6). The degradation rate may presumably be governed by the concentration of a polymer substrate as well as the substrate specificity and concentration of the PHA depolymerase(s). Although it remains to be proved, the depolymerization of PHA by the depolymerase is considered to be the rate-determining step in the intracellular PHA degradation pathway. The proteins such as PHA synthases, PHA depolymerases, phasins (16–18), and other granule-associated proteins are known to be mostly located on the surface of intracellular PHA granules (16–19). The enzyme proteins constitute only a relatively small amount of the granule-associated proteins, whereas the phosph protein, contributing approximately 3–5% (w/w) of the total cellular protein, is the predominant protein present on the surface of the granules. Thus, PHA granules are covered with the granule-associated proteins and phospholipids. The internal part of the granules is almost free from proteins (19). The polymer chains within granules are in an amorphous state but slightly ordered (9). Thus, the native amorphous granules are stabilized by the presence of the surface layer that protects the granules from aggregating. So, during degradation, the granules kept the original spherical shape unaltered, which was confirmed in our electron microscopic investigation (data not shown), and polymer chains are not expected to be solubilized into the cytoplasm because of their high hydrophobicity. Furthermore, the measurement of molecular weight during degradation of P(3HB) in cells showed that the molecular weight of P(3HB) was 983,000, 512,000, and 577,000 at 0, 10, and 20 h of incubation, respectively. As seen in Fig. 1, 10 and 20 h of incubation correspond to 64 and 82% P(3HB) degradation, respectively. Polydispersity of P(3HB) in *Ralstonia eutropha* is known to be constant during degradation (20). The modest (less than 2-fold) decrease in the molecular weight of P(3HB) and the constant polydispersity of P(3HB) during degradation may thus imply that the depolymerization is an exo-type reaction (20). Therefore, the degradation started from the surface and continued without significant perturbation of the overall granular structure.

It was reported that P(3HB) depolymerase activity in *R. eutropha* increased during P(3HB) synthesis (21). It was suggested that phasins might control the amount of the catalytically active proteins bound to the granules (16). The first order dependence of the degradation reaction, with respect to the concentration of polymer substrate, may suggest a relatively constant number density of the depolymerase on the granular surface throughout the degradation, thus implying apparently zero order with respect to the concentration of the depolymerase. Only some of the depolymerase, additionally expressed in the presence of additional ammonium, is expected to be initially bound to the surface of granules, and the “excess” soluble depolymerase in the cytosol, if any, might have a minor contribution to the degradation of granular PHAs.

Depending on the type of polymer (a copolymer or blend), the first order degradation rate constant for each monomer unit has a characteristic value (Table I). The calculation was carried out by two procedures, the steady-state method for the case in which a steady-state was reached and the initial rate method for the case where the degradation was still going on even after 60 h. Generally, the steady-state method resulted in higher $k_1$ values than the initial rate method. Intracellular PHA degradation can be inhibited by various limiting factors in a cell such as a shortage of some limiting nutrients, encapsulation of degradable PHA in outer nondegradable PHA (e.g. P(3HB) core surrounded with outer P(4HB) layer) in blend-type polymers (22). Thus, the limiting factor inhibiting PHA degradation could be different from one type of polymer in cells to another type of polymer. In addition, the number density of the depolymerase on the surface of PHA inclusions may depend on the specific type of polymer. Therefore, the comparison of apparent $k_1$ values between different types of polymers is meaningless, especially for the case in which they were determined by the steady-state method. This ambiguity is further clarified when we consider the percentage of PHA remaining after 60 h of degradation (Table I). But, the comparison between the $k_1$ values in a cellular polymer system is meaningful because they were determined under the same degradation and limiting conditions. Furthermore, for all types of blend-type polymers or copolymers, the relative magnitude of $k_1$ values in a cellular polymer system agree well with the degradability of each local sequence determined from the NMR analysis. Therefore, the relative $k_1$ value determined for a cellular polymer system could be used as a measure for the determination of relative substrate specificity of the depolymerase. In addition, the dissimilarity in $k_1$ values of the two different monomer units in a cellular copolymer discloses its microstructural heterogeneity (see above). Therefore, without isolation and purification of the depolymerase, it has been possible to measure the relative specificity of the enzyme as well as the microstructural heterogeneity of polyesters in cells by applying a first order degradation kinetics analysis to intracellular PHA degradation.
Local Sequence-dependent Intracellular PHA Degradation—It is well known that the sequence distribution of comonomers in bacterial PHA is close to a statistically random distribution (4, 5). Analysis of all $^{13}$C NMR signals for the polymers in this study showed that the decrease and increase in the intensities of the signals assigned to every local sequence that occurred after degradation were internally consistent with one another as well as among different carbons (data not shown) (23). This strongly supports the view that the sequence distribution is random. All these may imply that the supplying level of comonomers in the form of CoA is more critical than the substrate specificity of PHA synthase in PHA synthesis (24).

The local sequence-dependent degradation suggests that the depolymerization step is the rate-determining one in intracellular PHA degradation. In other words, the depolymerization rate is governed by the specificity of the enzyme toward the local monomer sequence such as dyad, triad, and tetrad. At present, we do not know anything about the mechanism by which the local sequences are recognized and how they are processed in the catalytic site. Such a local sequence dependence only suggests that the H. pseudoflava intracellular PHA depolymerases are not the processive enzymes that cleave the polymer chain sequentially. In addition, from the lack of intracellular degradability of P(4HB) or 4HB-rich P(3HB-co-4HB) polymer chain sequentially. In addition, from the lack of intracellular depolymerases are not the processive enzymes that cleave the backbone and/or the oxidation position located three bonds away from the carbonyl is essential for the hydrolysis reaction by the depolymerase (11). In conclusion, if any two different monomers are copolymerized in a cell and the $^{13}$C NMR signals of the copolymer synthesized exhibit splittings because of the neighboring monomer units, the relative specificity of the intracellular depolymerase against the local sequences could be determined by analyzing PHA before and after degradation using $^{13}$C NMR spectroscopy without purification of the enzyme and native substrates.