4038 *Macromolecules* **2009**, *42*, 4038–4046
DOI: 10.1021/ma900427z

Macromolecules
ARTICLE

End-Group Analysis of Bacterially Produced Poly(3-hydroxybutyrate): Discovery of Succinate as the Polymerization Starter

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Received February 25, 2009; Revised Manuscript Received April 30, 2009

ABSTRACT: The end groups of bacterial poly(3-hydroxybutyrate) (PHB) were analyzed by ^{1}H and ^{31}P NMR spectroscopy and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). A PHB component having a molecular weight around 200 000 Da was broken down into low-molecular-weight oligomers by acid-catalyzed partial hydrolysis. MALDI-TOF MS showed that one series of fragments contain a common terminal group that originated from the primer used for initiating in vivo polymerization. Analysis of the fragments proved that the terminal group is succinate. On the basis of this result, a plausible mechanism is proposed for enzymatic polymerization in microbial PHB synthesis.

Introduction

Poly(3-hydroxybutyrate) (PHB), which is produced by a large number of bacteria, has been developed as a biodegradable polymer that can readily be assimilated in a natural environment.[1,2] Recently, significant attention has been paid to its bio-based nature because it can be generated from renewable bioresources such as polysaccharides instead of petroleum-based feedstock. However, application of PHB has been limited until now because of its poor processability and its insufficient polymer properties. It would be worthwhile to improve the structure and properties of PHB, preferably during the process of its biosynthesis.[3]

The biosynthesis of PHB has been well established with *Ralstonia eutropha*.[4−9] According to a proposed biosynthetic mechanism, two acetyl-CoA molecules are first condensed into acetoacetyl-CoA, and in the next step the acetoacetyl-CoA is reduced to 3-hydroxybutyryl-CoA (3HB-CoA). Next, 3HB-CoA is polymerized into PHB and CoA is released by the action of PHA synthase (*phaC*). In this polymerization, the initiation step ought to involve a reaction of 3HB-CoA with an appropriate starter (X) that can react with the hydroxyl group of 3HB-CoA as schematized by eqs 1a−1c.[7]

$$\mathrm{E} + \mathrm{X} \rightarrow \mathrm{E{-}X} \tag{1a}$$

$$\mathrm{E{-}X} + \mathrm{3HB{-}CoA} \rightarrow \mathrm{E{-}3HB{-}X} + \mathrm{CoA{-}H} \tag{1b}$$

$$\mathrm{E{-}3HB{-}X} + \mathrm{3HB{-}CoA} \rightarrow \mathrm{E{-}3HB{-}3HB{-}X} + \mathrm{CoA{-}H} \tag{1c}$$

where E is the free *phaC* and E−X is a complex of *phaC* and X. Once initiated, the polymer chain can propagate by repeating the reaction of (1c), having a terminal group of X in addition to a carboxyl terminal derived by hydrolysis of its thiolate complex with the enzyme *phaC*. Although this synthetic process has been studied in detail, few ideas have been proposed until now on what is the starter X in this polymerization.[10] Analysis of the terminal groups of PHB is therefore essential to fully understand the polymer structure and the polymerization mechanism, and identification of the polymerization starter is the first approach to control the molecular weight of PHB more precisely.

In this study, a PHB sample having a molecular weight as low as possible was prepared by incubating *R. eutropha*, and its end groups were analyzed by several spectroscopic methods including mass spectroscopy (MS). On the basis of the discovery of a specific end group, succinate, we propose a plausible mechanism for enzymatic initiation and polymerization in microbial synthesis of PHB.

Experimental Section

Microorganisms. *R. eutropha* H16 (ATCC 17699) was provided by Riken Bioresource Center, Japan Collection of Microorganisms (Riken BRC-JCM).

Biosynthesis of PHB. *R. eutropha* H16 (ATCC 17699) was cultivated in a fermenter by the two-step shake-flask fermentation method. In the first step, the bacteria were grown in a nutrient broth medium with shaking (120 rpm) at 30 °C for 20 h. Then, in the second step, the bacteria were transferred to an equal volume of an N-limited medium (NH_4Cl: 0.50 g/L; Na_2HPO_4: 3.3 g/L; KH_2PO_4: 2.8 g/L; $MgSO_4$: 0.12 g/L) containing glucose (3 wt %) as the carbon source and 100 μL of a micro mineral solution ($FeCl_3 \cdot 6H_2O$: 70 mmol/L; $MnCl_2 \cdot 4H_2O$: 2 mmol/L; $CuSO_4 \cdot 5H_2O$: 1 mmol/L; $ZnSO_4 \cdot 7H_2O$: 3 mmol/L; $CaCl_2 \cdot H_2O$: 7 mmol/L) and incubated at 30 °C for 72 h. The resulting bacterial cells were centrifuged, washed with N-limited medium, lyophilized, and extracted with chloroform. The chloroform extract was filtered and precipitated into a 10-fold volume of methanol. The white precipitates obtained were filtered and dried in vacuo.

Partial Hydrolysis of PHB. A PHB sample (10 mg) was dissolved in chloroform (2 mL), to which 0.5 mL of 0.5 N aqueous HCl was added. The heterogeneous mixture was mechanically stirred at room temperature for 72 h to hydrolyze the PHB. The chloroform solution was then separated and reprecipitated into a 10-fold volume of diethyl ether. After centrifuging, the supernatant was evaporated to obtain 1−2 mg

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 Published on Web 05/29/2009

of the PHB oligomer. About 7 mg of the reprecipitated polymer was recovered.

End-Capping of PHB. A reference solution was first prepared by dissolving an accurately weighed quantity of bisphenol A (BPA: 10 mg) in pyridine and subsequently bringing the total volume to 5 mL. A PHB sample (50 mg) was dissolved in deuterated chloroform (1.0 mL), and 4 mg of chromium acetylacetonate was added. This polymer solution (0.5 mL) and the reference solution (0.05 mL) were transferred into a 5 mm NMR tube. Subsequently, an excess of 2-chloro-4,4,5,5-tetramethyldioxaphospholane (CTMDP) was introduced into the NMR tube, followed by shaking. The mixture was left overnight and subjected to the ^{31}P NMR spectroscopy on the following day. The delay between acquisitions in the ^{31}P NMR measurement was set between 30 and 40 s as described previously.[10,11] The Bisphenol A (also derivatized with CTMDP) provided a chemical shift reference (138.38 ppm) and a quantification reference with which the absolute number of end groups in the polymers could be calculated.

Measurements. The ordinary gel permeation chromatography (GPC) was conducted on a Shimadzu analyzer system consisting of a LC-10ADvp HPLC pump and a RID-6A refractive index detector. A polystyrene gel column of Tosoh TSK gel GMR$_{HR}$-M was used with chloroform as the eluent at 35 °C. The number-average (M_n) and weight-average (M_w) molecular weights were calibrated using polystyrene standards. To determine the absolute molecular weights (M_W) by multiple angle laser light scattering (MALLS), GPC was run on another system composed of a Shodex GPC SYSTEM-21, a light scattering detector (Wyatt Technology DAWN DSP Light Scattering Instrument), and an ASTRA A/D converter for Windows. A polystyrene gel column of Tosoh TSK gel GMR$_{HR}$-M was used. The mobile phase was chloroform flowing at a rate of 1.0 mL/min at 35 °C. ^{1}H NMR spectra were recorded on a Bruker-500 ARX NMR spectrometer operated at 500 MHz. Each sample was dissolved in deuterated chloroform containing 0.03 vol % tetramethylsilane (TMS) as the internal standard. ^{31}P NMR spectra were also recorded on a Bruker-500 ARX NMR spectrometer operated at 202.3 MHz for the ^{31}P nucleus. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed using an Autoflex III mass spectrometer (Bruker Daltonics) equipped with the reflector and lift modes using 2,5-dihydroxybenzoic acid (DHB) as the matrix and sodium trifluoroacetate (NaTFA) as the salt to support particular ions.

Results and Discussion

Microbial Production of PHB and Its Partial Hydrolysis. Ordinary two-step fermentation was employed for microbial production of PHB; the first step was for cell growth of *R. eutropha*, and the second was to accumulate high levels of polymer using the N-limited medium. The cultured cells were finally harvested after 72 h, and the resultant PHB was extracted from the cells with chloroform. The PHB thus obtained had a relatively lower M_n (1.2×10^5 Da) and a wider dispersity of 2.7 in M_w/M_n as analyzed by GPC. Much higher M_n values (3.0×10^5 Da) were generally reported for a previous PHB syntheses that utilized *R. eutropha*.[4] The present low M_n may be attributed to the use of newly grown bacteria, for which metabolic reactions are more highly activated and in which partial degradation of PHB was induced by the PHB depolymerases in the cells. The M_n attained, however, was not enough low for end-group analysis by ^{1}H NMR spectroscopy. Therefore, this PHB sample was subjected to acid-catalyzed partial hydrolysis in a bilayer system (PHB in $CHCl_3$/HCl(aq)). The products of this partial hydrolysis were separated into two fractions by reprecipitation ($CHCl_3$/dietyl ether); the lower oligomers having a M_n of 800 Da and M_w/M_n = 1.7 were isolated

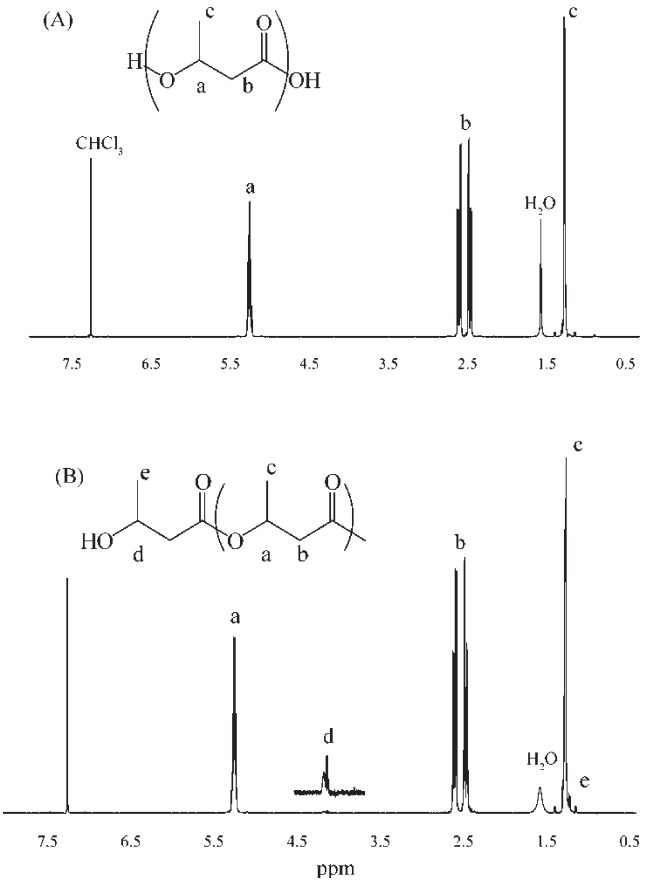
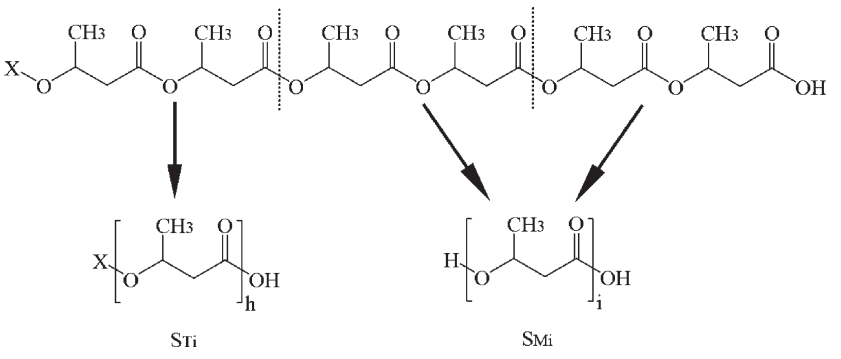


Figure 1. Typical 500 MHz ^{1}H NMR spectra of (A) the original PHB and (B) the PHB oligomers ($CDCl_3$).

Scheme 1. Oligomers Cut Out from the Terminal (S_{Ti}) and Middle (S_{Mi}) Chains of PHB by the Partial Hydrolysis



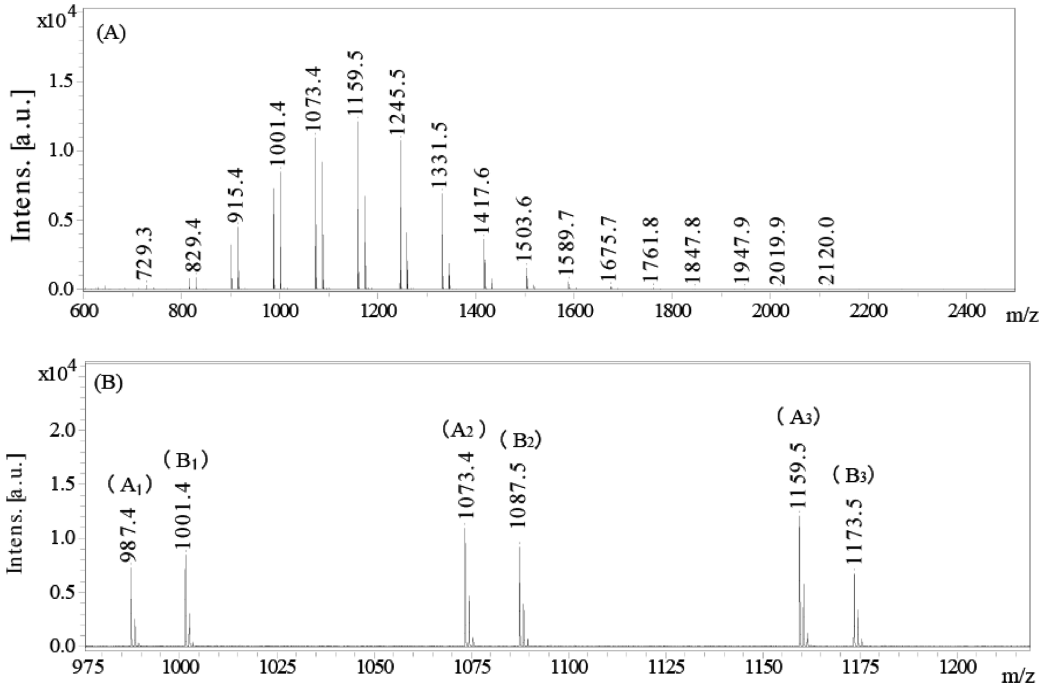


Figure 2. MALDI-TOF MS spectra of the PHB oligomers obtained by partial hydrolysis in positive reflector mode (A) and the enlarged portion around m/z = 975−1200 (B).

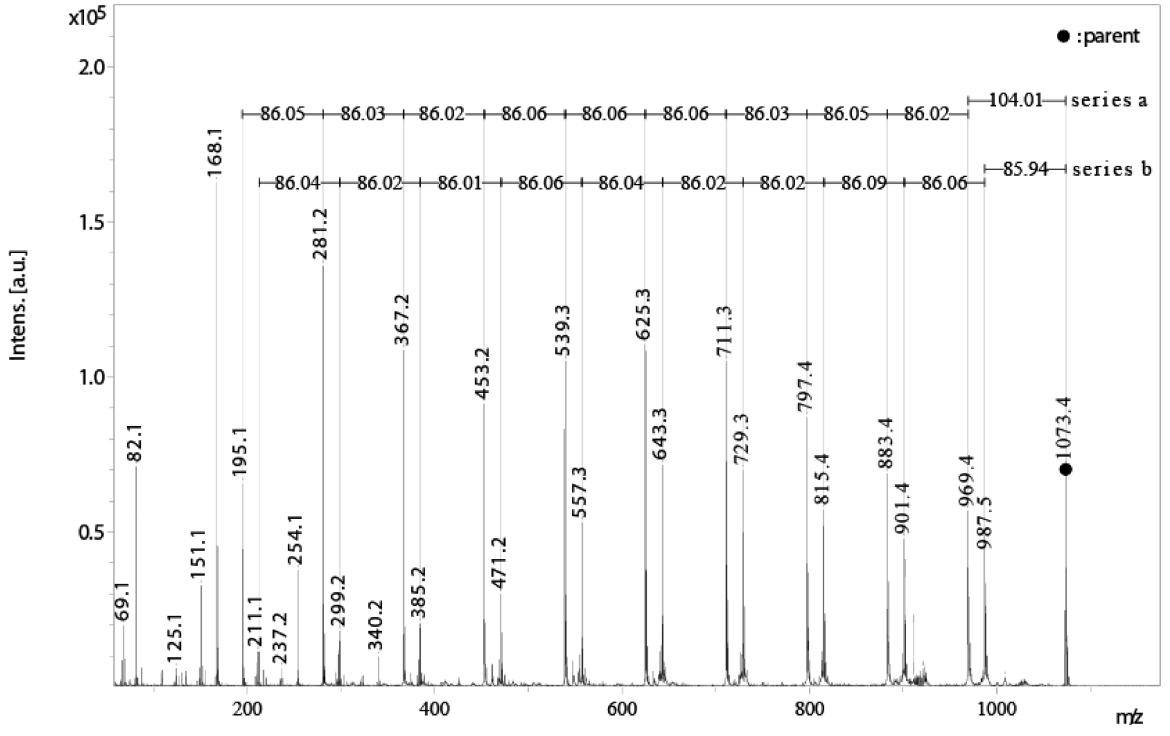


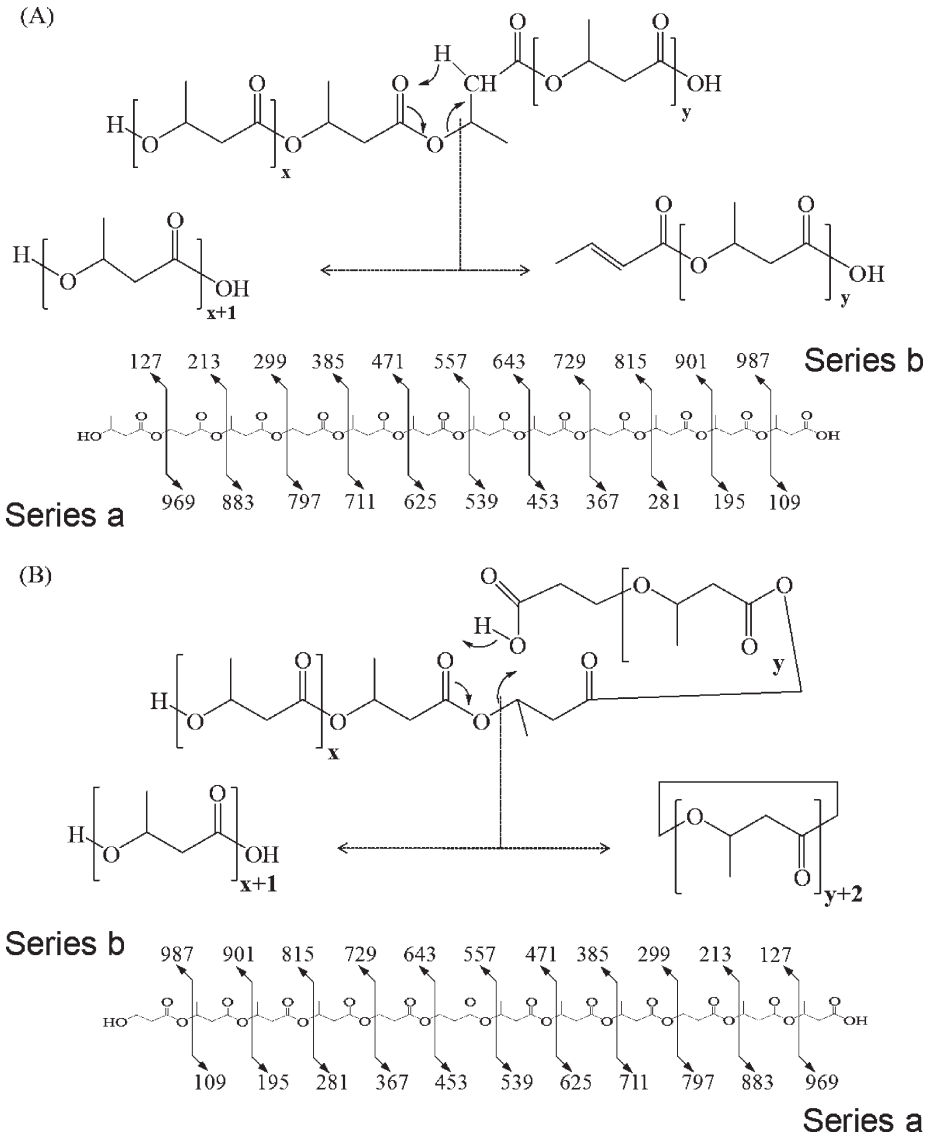
Figure 3. MALDI-TOF/TOF-MS/MS spectrum in positive lift mode of species A_2 having m/z = 1073.4.

from the supernatant, and the higher oligomers having M_n of 2500 Da and M_w/M_n = 2.3 were obtained as precipitates.

Figure 1 compares the ^{1}H NMR spectra of (A) the original PHB and (B) its lower oligomers (M_n = 800). The large signals a, b, and c in both spectra were assigned to the three types of protons in the 3-hydroxybutyryl (3HB) units. The original PHB shows no terminal signal, while the oligomers show small signals at δ 4.1 (d) and 1.2 ppm (e) due to the methyne and methyl protons of the hydroxyl 3HB terminal groups.[12−15] On the basis of these data, the oligomers (S_{Mi}) are cut out mainly from the middle segments and possess both hydroxyl and carboxyl end groups, being different from those (S_{Ti}) cut out of the terminal segments in the hydroxyl side which ought to contain the original end groups, as shown in Scheme 1. The molar ratio of S_{Ti} to S_{Mi} is estimated to be 1/150 (= 800/120 000), assuming that S_{Mi} and S_{Ti} cut out from the PHB (M_n = 120 000 Da) have a similar M_n (800 Da). Therefore, the analysis of S_{Ti} is even more difficult.

MALDI-TOF MS Analysis. Figure 2A shows a typical MALDI-TOF MS spectrum in the positive reflector mode of the lower oligomers obtained by partial hydrolysis.

Scheme 2. Reaction Mechanisms of (A) the β-Hydrogen Transfer and (B) Ester Bond Cleavage and the Resultant Mass Numbers of the Sodiated Product Ions Formed from a Sodiated Precursor Ion at m/z = 1073.4



The spectrum extends from 600 to 2000 in m/z, showing a distribution of ions in singly charged sodium adduct form.[14,16−18] There are two sets of peaks showing a peak-to-peak mass increment of 86 m/z, which corresponds to the molecular weight of the 3HB repeating unit. Each peak of the higher molecular ion set is larger than the corresponding peak of the lower molecular ion set by 14 m/z.

Figure 2B displays an enlarged portion of the spectrum around 900−1200 m/z. Here, the lower and higher molecular ions are labeled A_1−A_3 and B_1−B_3, respectively, corresponding to the sodium adducts of the PHB oligomers having different end groups. For structural assignment of these ions, MS/MS experiments were performed for the parent positive ions shown at m/z = 1073.4 (A_2) and m/z = 1087.5 (B_2), which were selected from the respective ion sets.

Figure 3 shows the MALDI-TOF/TOF-MS/MS spectrum obtained by fragmentation of the ion A_2 (m/z = 1073.4) in positive lift mode. Two series of fragment ions (series a and b) are present in the mass range of m/z = 60−1100. In each series the mass differences between the neighboring peaks are equal to 86 m/z. Since A_2 (m/z = 1073) includes a sodium ion, the real mass of the PHB oligomer consisting of A_2 is 1050, being exactly equal to that of 12 3HB units (1032) plus water (18). Therefore, this PHB oligomer is assumed to be a 12-mer having both hydroxyl and carboxyl ends. As reported by Adamus,[19] two fragmentation pathways can be deduced as shown in Scheme 2. Most of the secondary ion peaks are related with the fragments formed by (A) the β-hydrogen transfer and (B) ester bond cleavage of the 12-mer. In the β-hydrogen transfer the rearranged hydrogen is attached to the carbonyl oxygen, and this leads to cleavage of the −O−CH⟨ bond resulting in formation of two ions: one bearing a carboxylic acid end group (series a) and the other terminating with a crotonate group (series b).[19−22] The repeated ester bond cleavages are driven by the backbiting reaction of the carboxyl terminus of the PHB chain, leading to formation of two ions: one having the macrocyclic ester structure (series a) and the other terminating with a hydroxyl end group (series b).[16,23−26] When these secondary fragmentations occur, the sodium cation remains on one or the other of the two fragments, resulting in different relative strengths between the two complementary ion series. In the secondary fragmentations of the 12-mer, however, the mass numbers of both series of fragments formed by the two different fragmentations are incidentally identical, and only two series of fragment ions are therefore shown in Figure 3.

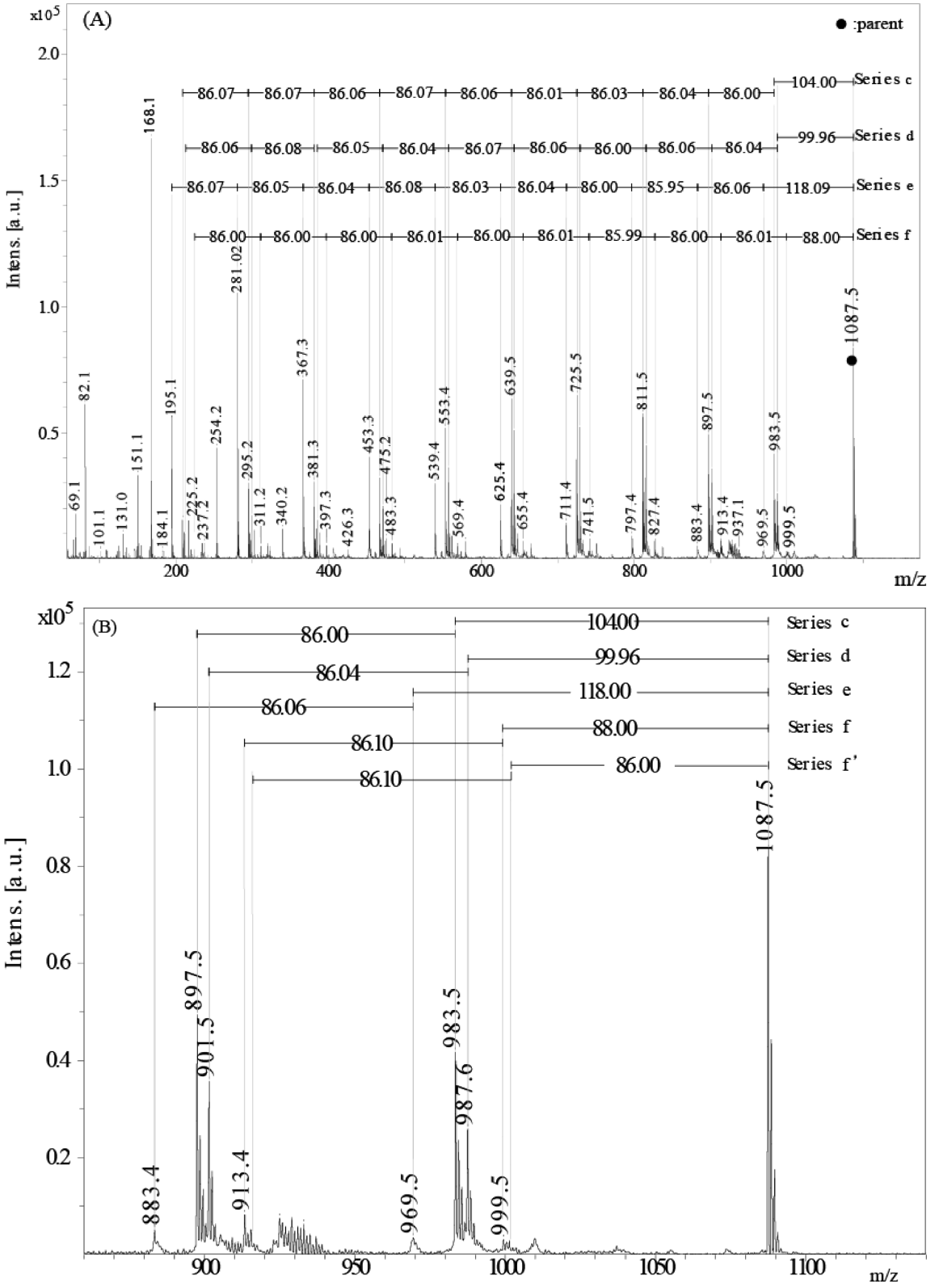
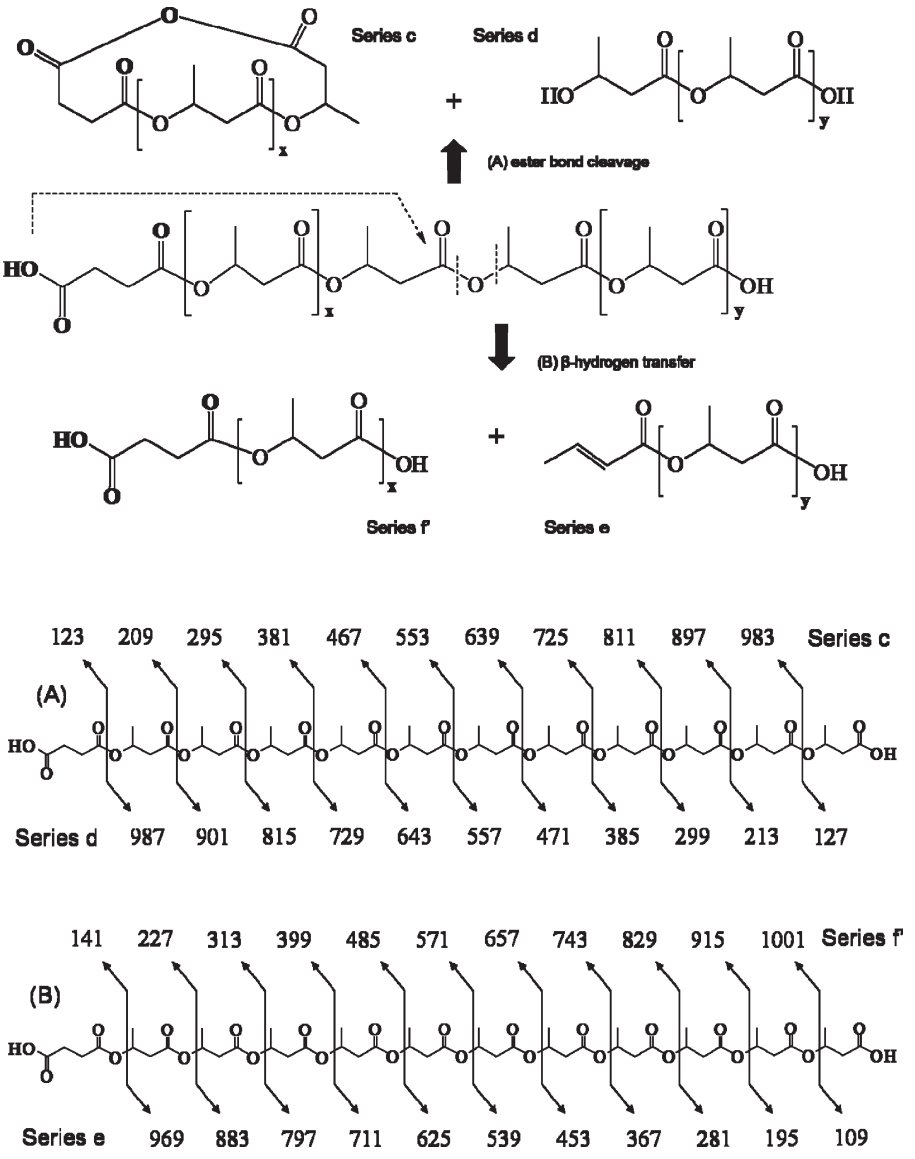


Figure 4. MALDI-TOF/TOF-MS/MS spectrum of species B_2 having m/z = 1087.5 in positive lift mode (A) and enlarged portion around m/z = 960−1100 (B).

Figure 4A shows the MALDI-TOF/TOF-MS/MS spectrum obtained by fragmentation of the ion B_2 (m/z = 1087.5) in positive lift mode. Since B_2 (m/z = 1087) includes the sodium ion, the real mass of the PHB oligomer consisting of B_2 is 1064. This oligomer ought to have an end group other than a hydroxyl when it is terminated by a carboxyl at the other end. Four series of fragment ions (series c, d, e, and f) were found in the range m/z = 60−1100. These fragment ions should occur by the aforementioned two fragmentation mechanisms shown in Scheme 3.[20,23−25] The fragment ions derived by the ester bond cleavage can be detected more clearly as series c and d in a complementary manner. In this mechanism, one fragment terminating with a hydroxyl end group (series d: ordinary PHB oligomers) is formed together

Scheme 3. Mass Numbers of the Sodiated Product Ions Formed by (A) Ester Bond Cleavage and (B) the β-Hydrogen Transfer of a Sodiated Precursor Ion at m/z = 1087.5



with its counterpart having macrocyclic anhydride structure (series c), assuming that the OH end of PHB is capped by another carboxyl group to induce the backbiting reaction. In series c, therefore, the molecular masses of the fragments are larger than those of the corresponding PHB oligomers by 100 in m/z. In series d, on the other hand, the fragment ion at m/z = 987.6 corresponds to the oligomer ion formed by losing a mass of 100, which corresponds to the molecular mass of the terminal group introduced to the OH end of PHB. Contrarily, the fragment ions derived by the β-hydrogen transfer mechanism are detected as series e in which the peaks are stronger in the lower mass number region (ca. m/z = 625, 539, 453, etc.). Their complementary fragment ions (series f′), however, are hardly detected in this spectrum. Its enlarged spectrum, shown in Figure 4B, exhibits very small peaks at 1001 m/z and 915 m/z, corresponding to the fragment ions of series f′. These fragments, once formed, may readily be involved in the ester bond cleavage driven by the carboxyl terminus capping the OH end of PHB as mentioned above for series c and d. In series e, the fragment ion at m/z = 969.4 corresponds to the oligomer ion formed by the loss of an end group having a mass number of 118. The alternative ester bond cleavage driven by the carboxyl terminus of PHB should also give the fragments having mass numbers identical with those formed by the β-hydrogen transfer (series e and f′), as observed in the fragmentation of the ion A_2. The weak intensities of these fragments suggest that the carboxyl backbiting reaction from the OH terminal side to form cyclic anhydrides should be preferred to that from the COOH side to form cyclic esters in the second fragmentation.

From these data we lead to a conclusion that the fragments derived from the OH side of PHB are capped with an end group having a mass number of 100 or 118 (100 plus H_2O). Analysis of these mass numbers tells that the OH group of PHB is succinate for which one carboxyl group is bonded with the PHB hydroxyl terminal, leaving the other carboxyl free. This assignment is well supported by the detection of the fragment ions series f. As mentioned above, fragmentation of the parent ion B_2 by the β-hydrogen transfer mechanism ought to generate the fragments of series f′ having mass numbers of $1087-86n$ (n = 1−11). The fragment ions of series f (225, 311, 397, 483, 569, 655, 741, 827, 913, 999) have mass numbers smaller than the corresponding mass

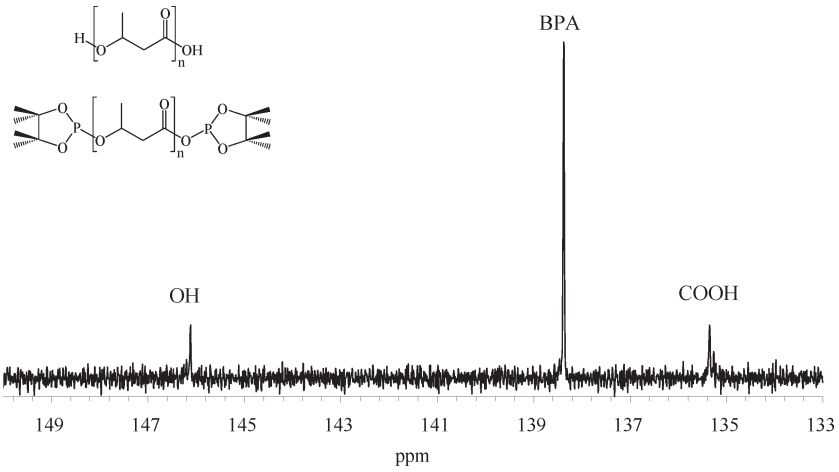
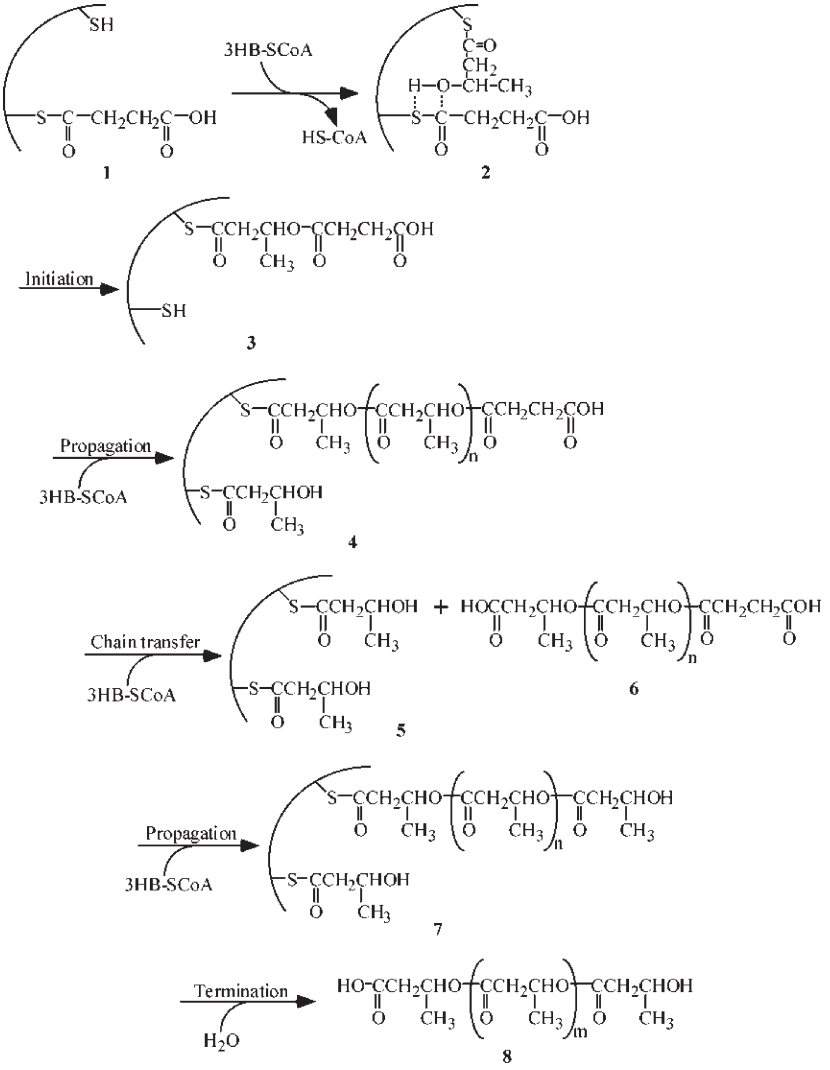


Figure 5. ^{31}P NMR spectrum of the PHB derivatized with CTMDP in reference to bisphenol A (BPA). The two peaks corresponding to the hydroxyl and carboxyl groups bonded to CTMDP are noted as OH and COOH, respectively.

Scheme 4. A Plausible Mechanism for the Enzymatic Initiation and Propagation during Microbial PHB Synthesis with a Succinate Starter



numbers of series f′ by 2. Those ions can reasonably be assigned to oligomeric ions of PHB having a fumarate group, which is derived from the succinate by dehydrogenation during the process of secondary fragmentation. Other small signals observed in Figure 4 will be assigned in further studies.

The present study verified that the end group X is succinate and that succinic acid should be used as the starter in biosynthesis of PHB. The dicarboxyl-terminated structure of the terminal oligomers (S_{Ti}) may contribute to an increased anionic nature that should favor their ionization in the MS measurement. For this reason, relatively strong ion peaks

Table 1. Molecular Weights of PHB Estimated by Different Methods

	M_n, from OH	M_n, from COOH	M_n, averaged	M_n	M_w
^{31}P NMR	172 000	106 000	139 000		
GPC				120000	326000
MALLS					258000

could be detected as compared with those from the middle oligomers (S_{Mi}).

End-Group Analysis. It was difficult to directly detect the proton and carbon signals of the two methylenes in the succinic acid terminus by the ^{1}H and ^{13}C NMR spectra of the PHB sample, probably because of the presence of impurities showing signals around the magnetic fields where the required signals should appear. The PHB sample, therefore, was derivatized with CTMDP and subjected to ^{31}P NMR spectroscopy. Figure 5 shows a typical ^{31}P NMR spectrum of the CTMDP−PHB. The ^{31}P NMR signals arising from the derivatized hydroxyl and carboxyl groups can be clearly identified and quantified. Among the three main peaks, the one at δ 135.36 ppm is assigned to the derivatized carboxyl end groups of PHB. Since the chemical shift of this peak is not sensitive to chemical structure, carboxyl groups should all show signals at similar magnetic field strengths. The peak at δ 138.38 ppm (represented as BPA) is due to the derivatized hydroxyls of bisphenol A, which was used as an internal standard. The downfield signal at δ 146.18 ppm is attributed to the terminal secondary hydroxyl group of PHB. The present detection of both COOH and OH terminal groups may be compatible with the 3HB sequential structure of PHB. However, the integral ratio of OH/COOH is less than unity 0.62/1. In other words, the quantity of COOH is significantly larger than that of OH, supporting the data indicating that some of the OH terminals ought to be capped by succinate, as revealed by the MALDI-TOF MS analyses.

In reference to the BPA signal, the quantities of OH and COOH groups were determined and used for estimating the M_n value of the PHB sample. Table 1 compares the molecular weights measured using different methods. The M_n value estimated from the OH quantity is much larger than that estimated from the COOH quantity. Their average value should correspond to the real M_n, considering that both terminals of the polymer chain should have either OH or COOH. However, the M_w value estimated by MALLS is slightly smaller than the M_w determined by GPC, and the real M_n value is close the M_n value determined by GPC. At least one-third of the hydroxyl terminals ought to be capped by succinate groups which must be the starters of polymerization in vivo.

Mechanism of Polymerization. The above finding revealed that the first PHB polymers formed are capped by succinate residues. These succinates must be derived from the primers that are responsible for initiating the in vivo polymerization. On the basis of this fact, we propose a plausible mechanism for the enzymatic initiation and propagation during microbial PHB synthesis, as shown in Scheme 4.

Previously, Ballard et al.[27] and Doi et al.[4] proposed an active-site model for the PHB synthase consisting of two thiol groups: one binding the incoming 3HB monomer and the other binding the propagating polymer chain. Condensation occurs through a four-membered transition state, leaving one of the thiol groups vacant for binding the next.[4−6,27,28] With this structure in mind, we propose a model (**1**) in which the active site of the PHB synthase is a capped with a succinate. Probably, succinyl-CoA generated in the metabolic TCA cycle is used for this capping. Then, the PHB synthesis is initiated by the condensation reaction of 3HB-CoA with the succinyl thiolate (**2** → **3**) which is followed by chain propagation to form the succinyl-terminated PHB chain (**4**). During this propagation chain transfer reaction is induced with 3HB-CoA monomer to produce a succinyl-terminated PHB (**6**) together with an initiating species (**5**), from which chain propagation restarted to form a hydroxyl-terminated PHB chain (**7**). The following termination gives the hydroxyl-terminated PHB (**8**). The ratio of the succinyl- to hydroxyl-terminated PHB depends on how often the chain transfer may happen. According to the OH/COOH ratio of 0.62/1.0 revealed by the end-group analysis, the succinyl to hydroxyl ratio happened to be 24/76 due to higher frequency of chain transfer, giving the relatively low molecular weight of the polymer.

Conclusions

The MALDI-TOF MS of the oligomers obtained by partial hydrolysis of PHB revealed the presence of a succinate residue on the hydroxyl termini of PHB. The ^{31}P NMR analysis of the CTMDP-derivatized terminal groups of PHB also supported the presence of more carboxyl terminals than hydroxyl terminals. Therefore, succinate should be the primer that initiates polymerization in vivo. On the basis of this fact, a plausible mechanism is proposed for enzymatic initiation and polymerization during microbial PHB synthesis.

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