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BIOSYNTHESIS AND CHARACTERIZATION OF BACTERIAL POLY(3-HYDROXYBUTYRATE-co-3-HYDROXYPROPIONATE)

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ABSTRACT

A new copolyester of 3-hydroxybutyrate (3HB) and 3-hydroxypropionate (3HP) was produced by Alcaligenes eutrophus in a nitrogen-free culture solution of 3-hydroxypropionic acid, 1,5-pentanediol, or 1,7-heptanediol. The copolymer composition varied from 0 to 7 mol% 3HP, depending on the type of carbon substrates supplied. The bacterial copolyesters were characterized by ^{1}H and ^{13}C NMR, gel permeation chromatography (GPC), and differential scanning calorimetry (DSC). The copolymers were shown to have a random sequence distribution of 3HB and 3HP units by analysis of the 125-MHz ^{13}C NMR spectra. The melting temperatures (Tm) decreased from 178 °C to 150 °C as the 3HP fraction increased up to 7 mol%. It has been found that the presence of 3HP units in polyesters accelerates the rate of enzymatic degradation.

INTRODUCTION

A variety of bacteria accumulate an optically active poly(3-hydroxybutyrate) within cells as a reserve material [1]. Recently, copolyesters incorporating 3-hydroxyalkanoate units other than 3-hydroxybutyrate have been produced by several bacterial strains. A copolyester of 3-hydroxybutyrate (C_4) and 3-hydroxyvalerate (C_5) has been produced by Alcaligenes eutrophus from propionic acid [2, 3] or pentanoic acid [4]. Pseudomonas oleovorans has been found to produce copolyesters cotaining 3-hydroxyalkanoate units of C_6 to C_{12} from alkanes [5] and alkanoic acids [6]. In addition, A. eutrophus has been shown to produce a copolyester of 3-hydroxybutyrate and 4-hydroxybutyrate,

when 4-hydroxybutyric acid, γ-butyrolactone, or 1,4-butanediol was used as the carbon source [7-9]. These bacterial copolyesters have attracted much attention as environmentally degradable thermoplastics for a wide range of agricultural, marine, and medical applications [10,11].

In this communication we report the biosynthesis of a new copolyester of 3-hydroxybutyrate and 3-hydroxypropionate by A. eutrophus. In addition, thermal properties and biodegradability of the copolyester have been studied.

EXPERIMENTAL

Biopolymer Synthesis

A. eutrophus (ATCC17699) was first grown at 30 °C in a nutrient-rich medium (1 l) containing 10 g/l of yeast extract, 10 g/l of polypeptone, 5 g/l of meat extract, and 5 g/l of $(NH_4)_2SO_4$. The cells were harvested by centrifugation after 24 h and washed with water. Under these culture conditions an accumulation of polyesters in the cells was not observed. To promote polyester synthesis, about 3-4 g quantities of the washed cells were transferred into nitrogen-free mineral media (1 l, pH 7.5) containing different carbon sources [12]. The cells were cultivated in the media at 30 °C, harvested by centrifugation, washed with water, and finally dried under vacuum at room temperature. Polyesters were extracted from dried cells with hot chloroform in a Soxhlet apparatus and purified by reprecipitation with hexane.

Analytical Procedures

The solution ^1H and ^{13}C NMR spectra of polyesters in chloroform were recorded on a JEOL GX-500 spectrometer. The 500-MHz ^1H NMR spectra were recorded at 27 °C on a $CDCl_3$ solution of polyester (3 mg/ml) with 3.8 μs pulse width (π/4 pulse angle), 7.3 s pulse repetition, 4500 Hz spectral width, 32 K data points, and 128 accumulations. The 125-MHz ^{13}C NMR spectra were recorded at 27 °C on a $CDCl_3$ solution of polyester (25 mg/ml) with 6.0 μs pulse width (π/4 pulse angle), 2.0 s pulse repetition, 38000 Hz spectral width, 32 K data points, and 8347 accumulations. Tetramethylsilane (Me_4Si) was used as an internal chemical shift standard.

All molecular weight data were obtained at 40 °C by using a Shimadzu 6A GPC system and a 6A refractive index detector with a Shodex 80 M column. Chloroform was used as eluant at a flow rate of 0.5 ml/min, and sample concentration of 1.0 mg/ml was used. Polystyrene

standards with a low polydispersity were used to make a calibration curve.

The melting temperatures of polyester samples were recorded on a Seiko DSC-10. The as-isolated powder samples of 3 mg were encapsulated in aluminum pans heated at 10 °C/min up to 200 °C. The heat of fusion of indium (6.80 cal/g) was used as a calorimetric calibration.

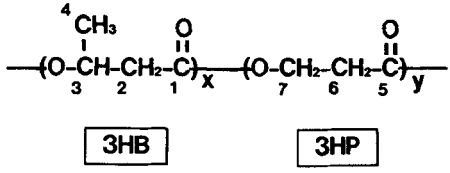
Enzymatic Degradation

The extracellular P(3HB) depolymerase was purified to electrophoretic homogeneity from A. faecalis T_1 as described in a previous paper [11]. The enzymatic degradation of polyester films by the extracellular P(3HB) depolymerase was carried out at 37 °C in a 0.1 M phosphate buffer (pH 7.5). Polyester films (initial weights; 4.0-8.0 mg, and initial film dimensions; 10x10 mm in size and 0.03-0.06 mm thick) were placed in small bottles containing 1.0 ml of buffer. The films were prepared by conventional solvent-casting techniques from chloroform solutions of polyesters. The reaction was started by the addition of 5 µl aqueous solution of P(3HB) depolymerase (3 µg). The reaction solution was incubated at 37±0.1 °C with shaking. Samples were periodically removed, washed with water, and dried to constant weight in vacuo.

RESULTS AND DISCUSSION

Biosynthesis of P(3HB-co-3HP)

The copolyesters of 3-hydroxybutyrate and 3-hydroxypropionate, P(3HB-co-3HP), were produced in A. eutrophus by using 3-hydroxypropionic acid, 1,5-pentanediol, or 1,7-heptanediol as the carbon source.



In this paper, the carbon species in the 3HB units are referred to as CO (1), CH_2 (2), CH (3), and CH_3 (4), respectively. The carbon species in the 3HP units are denoted by CO (5), CH_2 (6), and CH_2 (7). The mole fractions of 3HB and 3HP units in the copolyesters were determined from the ^1H NMR spectra.

TABLE 1

Production of Copolyesters of 3-Hydroxybutyrate (3HB) and 3-Hydroxypropionate (3HP) by Alcaligenes eutrophus at 30 °C

Sample	Carbon source[a],	g	Cultivation time, h	Cell dry wt., g	Polyester content[b], wt %	Polyester composition[c], mol%		Molecular weight[d]	
						3HB	3HP	$10^{-3}\overline{Mn}$	$\overline{Mw}/\overline{Mn}$
1	$HO(CH_2)_2COOH$	5	48	4.1	6	96	4	773	1.8
2		10	48	4.1	4	94	6	n.d.	n.d.
3		15	48	5.1	23	96	4	1400	1.6
4		20	48	4.3	12	93	7	964	1.7
5		30	48	3.9	1	95	5	586	2.5
6	$HO(CH_2)_5OH$	10	48	5.2	34	97	3	212	2.6
7		15	48	5.6	42	97	3	80	2.7
8		20	24	4.1	1	99	1	36	6.5
9		20	48	5.5	28	97	3	82	2.1
10		20	72	6.8	40	97	3	90	2.0
11		20	96	3.4	28	97	3	43	2.5
12	$HO(CH_2)_7OH$	5	48	5.5	13	96	4	183	2.6
13		10	48	5.2	10	98	2	135	2.4

[a] Carbon source in nitrogen-free culture media (1.0 l , pH =7.5).

[b] Polyester content in dry cells.

[c] Determined from ^1H NMR spectra.

[d] Determined by GPC.

Table 1 gives the result of P(3HB-co-3HP) biosynthesis by A. eutrophus from different carbon substrates. The compositions of copolyester samples varied from 1 to 7 mol% 3HP, depending on the type of carbon sources. When 1,5-pentanediol was used as the carbon source, the polyester contents in dry cells were as high as 30-40 wt%.

Figure 1 shows a typical 500-MHz ^{1}H NMR spectrum of P(3HB-co-3HP); sample 1, together with the chemical shift assignments. In addition to the well-characterized resonances of methyl (4), methylene (2), and methine (3) protons in 3HB unit [13], a triplet methylene proton resonance (7) appears at 4.33 ppm. The resonance of the other methylene proton (6) overlaps with the methylene resonance (2) in 3HB unit. The compositions of copolymer samples were determined from the relative areas of methine proton resonance (3) and methylene proton resonance (7) in two different units.

Figure 2 shows a typical 125-MHz ^{13}C NMR spectrum of P(3HB-co-3HP); sample 2, together with the chemical shift assignment for each ^{13}C resonance. In addition to the well-characterized resonances of carbons (1)-(4) in 3HB unit [13], three carbon resonances (6), (7), and (5) in 3HP unit appear at 33.84, 60.00, and 169.78 ppm, respectively. The carbonyl resonances at 169.1-169.8 ppm were clearly resolved into three peaks, arising from the different diad sequences of connecting the 3HB and 3HP units. The peak at 169.12 ppm is assignable to the carbonyl resonance in the 3HB*-3HB sequence, since its chemical shift is consistent with that (169.14 ppm) of the carbonyl resonance of P(3HB) homopolymer. The peak at 169.60 ppm is assigned to the carbonyl resonance in the 3HB*-3HP sequence, and the other peak at 169.78 ppm is assigned to the carbonyl resonance in the 3HP*-3HB sequence. The carbonyl resonance in the 3HP*-3HP sequence was not detectable since its intensity was too low. The diad sequence distribution of 3HB and 3HP units in the copolyester sample 2 was ; F_{BB} =0.89, F_{BP} =0.05, F_{PB} =0.06, and F_{PP} =0.00, determined from the well resolved peaks of carbonyl resonances.

The diad sequence distribution of 3HB and 3HP units were compared with the Bernoullian statistics applicable to a statistically random copolymerization. In the Bernoullian model, the mole fraction F_{ij} of diad sequence ij can be expressed with the mole fractions F_i and F_j of i and j units as ; $F_{ij}=F_iF_j$. The calculated diad fractions (F_{BB} =0.88, F_{BP} =0.06, F_{PB} =0.06, and F_{PP} =0.00) of sample 2 (F_B =0.94 and F_P =0.06) are in good agreement with the observed values. Therefore, it may be cocluded that the sequence distribution of 3HB and 3HP units is statistically random.

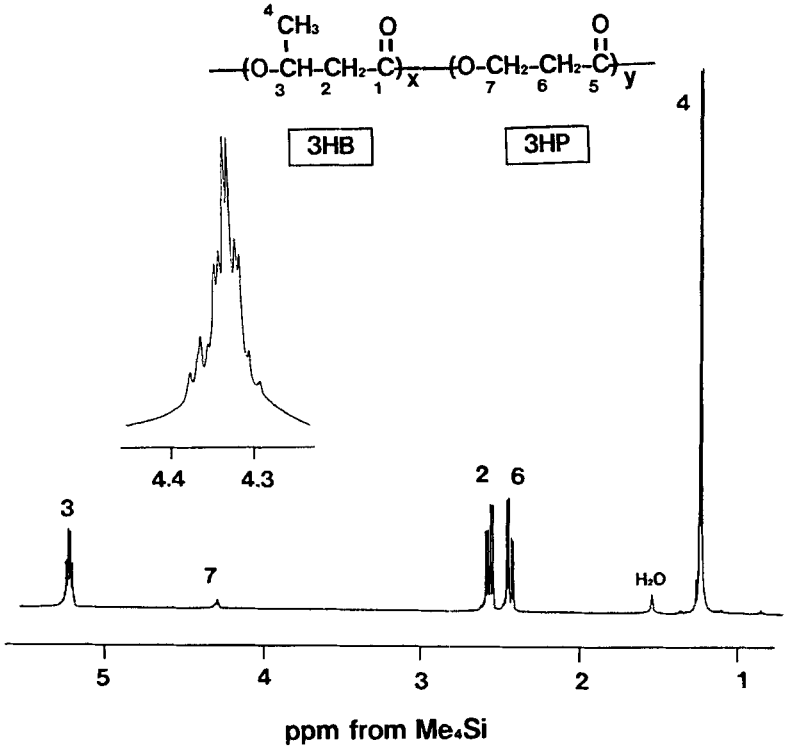


FIG. 1. 500-MHz ^{1}H NMR spectrum of sample 1 (3HP= 4 mol%) in $CDCl_3$.

Figure 3 shows a schematic pathway of P(3HB-co-3HP) biosynthesis in A. eutrophus. When 3-hydroxypropionic acid is used as the carbon source, 3-hydroxypropionyl-coenzyme A (CoA) is first formed in the cells. Most of 3-hydroxypropionyl-CoA is then metabolized into acetyl-CoA, and D(-)-3-hydroxybutyryl-CoA is formed from acetyl-CoA via acetoacetyl-CoA [12]. A random copolyester of 3HB and 3HP units may be produced by the copolymerization of D(-)-3-hydroxybutyryl-CoA with 3-hydroxypropionyl-CoA under the action of P(3HB) synthase. When 1,5-pentanediol or 1,7-heptanediol is used as the carbon source, 3-hydroxypropionyl-CoA may be formed via an oxidation cycle. As a result, copolyesters containing 3HP units are formed from these carbon sources.

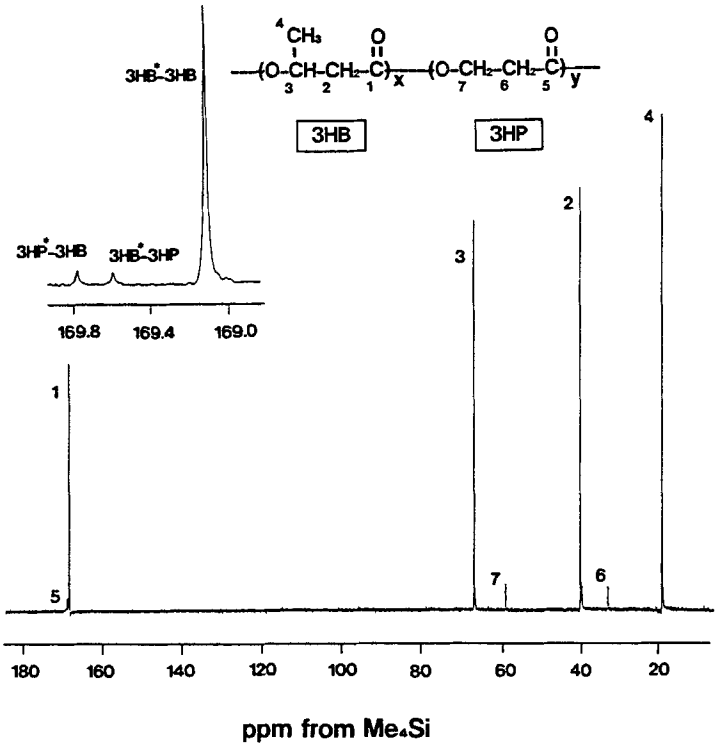


FIG. 2. 125-MHz ^{13}C NMR spectrum of sample 2 (3HP= 6 mol%) in $CDCl_3$

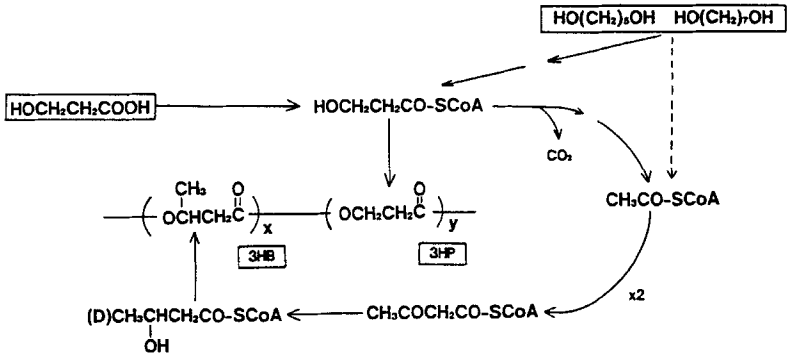


FIG. 3. Schematic pathway of P(3HB-co-3HP) biosynthesis

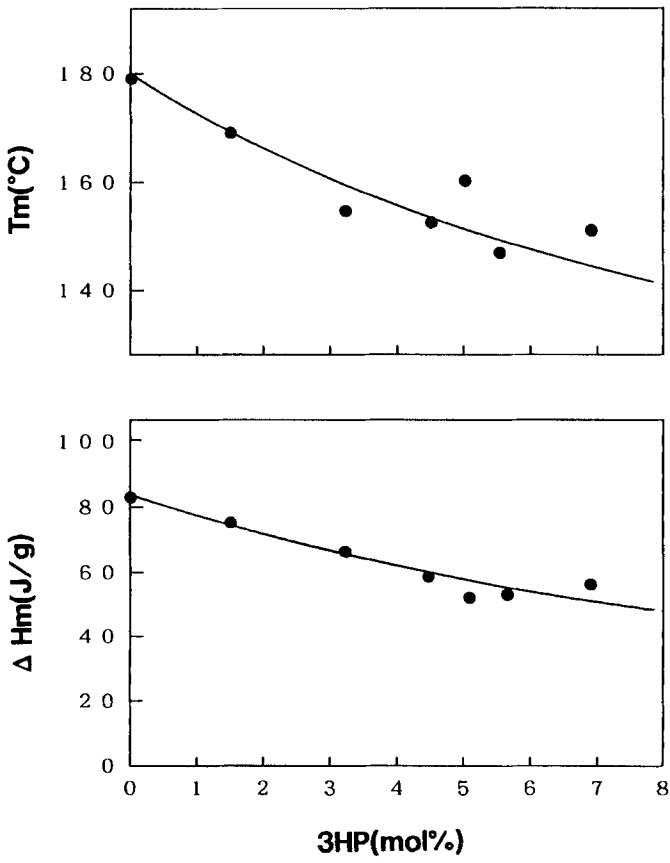


FIG. 4. Melting temperature (Tm) and enthalpy of fusion (ΔHm) versus composition curves for P(3HB-co-3HP) samples. Samples were heated at 10 °C/min.

Thermal Properties and Biodegradability

Figure 4 shows the melting temperatures (Tm) and the ehthalpies of fusion (ΔHm) of P(3HB-co-3HP) samples in powder form. The Tm value of P(3HB) homopolymer was 178 °C. The Tm values of P(3HB-co-3HP) samples decreased to 150 °C as the 3HP fraction was increased up to 7 mol%. The ΔHm values decreased gradually with an increase in the 3HP fraction, indicating that the crystallinity of copolymers decreases with the 3HP fraction.

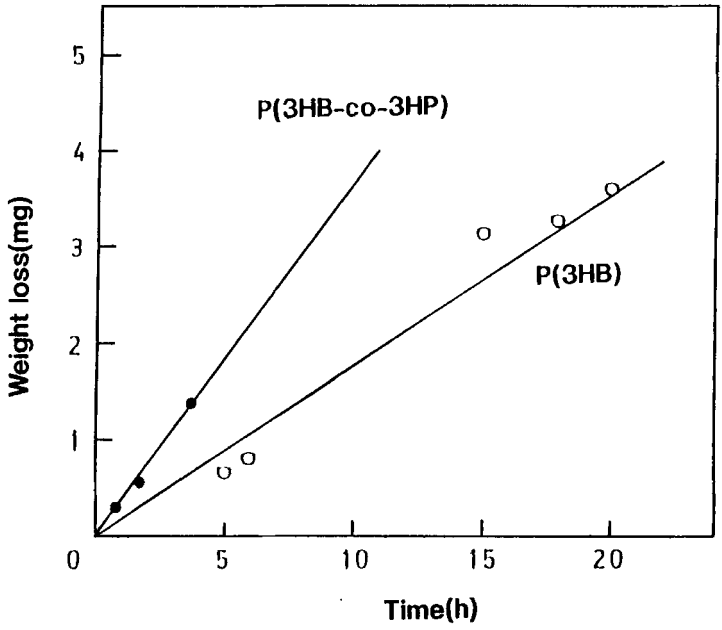


FIG. 5. Enzymatic degradation (erosion) profiles on solution-cast films of P(3HB) and P(3HB-co-4%3HP) samples in the aqueous solution of P(3HB) depolymerase at 37 °C at pH 7.4 . (o): P(3HB) ($\overline{Mn}$= 768000); (●): P(3HB-co-4%3HP) ($\overline{Mn}$= 773000).

The biodegradability of P(3HB-co-3HP) polyester films was studied at 37 °C and pH 7.5 in the aqueous solution of the extracellular P(3HB) depolymerase purified from A. faecalis. In this study, two samples of P(3HB) ($\overline{Mn}$ =768000) and P(3HB-co-4%3HP) ($\overline{Mn}$ =773000) were used.

Figure 5 shows the weight loss (erosion) profiles of polyester films as a function of degradation time. The weight of film erosion increased proportionally to time. The erosion rate of P(3HB-co-4%3HP) film by P(3HB) depolymerase is faster than the rate of P(3HB) erosion. Thus, the presence of 3HP units in the P(3HB) sequence accelerated the rate of enzymatic degradation.

REFERENCES

[1] E.A.Dawes and P.J.Senior, Adv. Microbiol. Physiol., 10, 135 (1973)
[2] P.A.Holmes, Phys. Technol., 16, 32 (1985)
[3] Y.Doi, M.Kunioka, Y.Nakamura, and K.Soga, Macromolecules, 20, 2988 (1987)
[4] Y.Doi, A.Tamaki, M.Kunioka, and K.Soga, Appl. Microbiol. Biotechnol., 28, 330 (1988)
[5] R.G.Lageveen, G.W.Huisman, H.Preusting, P.Ketelear, G.Eggink, and B.Witholt, Appl. Environ. Microbiol., 54, 2924 (1988)
[6] H.Brandl, R.A.Gross, R.W.Lenz, and R.C.Fuller, Appl. Environ. Microbiol., 54, 1977 (1988)
[7] M.Kunioka, Y.Nakamura, and Y.Doi, Polymer Commun., 29, 174 (1988)
[8] Y.Doi, M.Kunioka, Y.Nakamura, and K.Soga, Macromolecules, 21, 2722 (1988)
[9] M.Kunioka, Y.Kawaguchi, and Y.Doi, Appl. Microbiol. Biotechnol., 30, 569 (1989)
[10] M.Kunioka, A.Tamaki, and Y.Doi, Macromolecules, 22, 694 (1989)
[11] Y.Doi, Y.Kanesawa, M.Kunioka, and T.Saito, Macromolecules, 23, 26 (1990)
[12] Y.Doi, Y.Kawaguchi, Y.Nakamura, and M.Kunioka, Appl. Environ. Microbiol., 55, 2932 (1989)
[13] Y.Doi, M.Kunioka, Y.Nakamura, and K.Soga, Macromolecules, 19, 2860 (1986)