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Structural changes of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) fractionated with acetone–water solution

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Biosynthesized poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(3HB-*co*-3HV)) copolymers with 3HV mole fraction of 36 and 61% were fractionated by fractional precipitation methods using a solution of acetone–water. Both samples were mixtures of random copolymers having a wide variety of 3HV composition. The melting points of the fractionated samples showed pseudoeutectic changes with minimum breaking point at ca. 40 mol% 3HV. The fractionated samples whose 3HV composition range was from 36 to 56 mol% showed a single melting peak and the coexistence of both P(3HB) and P(3HV) crystal phases. The crystal lattice of the copolymer was of either P(3HB) or P(3HV) type depending on whether the 3HV composition was lower or higher than this range, respectively. The crystal phase transition of isodimorphism for P(3HB-*co*-3HV), therefore, occurred in a broad composition range from 36 to 56 mol% 3HV, which corresponded to the pseudoeutectic composition range. The amorphous density estimated by density measurement decreased linearly as the 3HV composition increased, showing a breaking point at ca. 30 mol% 3HV. The crystalline density estimated by the X-ray method was obtained over the whole range of 3HV composition. Crystallinity estimated by the density method was almost parallel to that evaluated by the X-ray method.

(Keywords: fractionation; random copolymers; pseudoeutectic)

INTRODUCTION

Poly(3-hydroxybutyrate) (P(3HB)) and its copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(3HB-*co*-3HV)) can be isolated from the cells of many types of bacteria by solvent extraction[1,2]. The products are highly pure because of their microbial origin without catalysts. It has been reported that a structural characteristic of P(3HB-*co*-3HV) is isodimorphism, i.e. the copolymer crystallizes in either a P(3HB) or P(3HV) crystal lattice depending on whether the 3HV composition is lower or higher than around 40 mol%, respectively[3,4]. Recently, it has been reported that the coexistence of both crystal phases and pseudoeutectic behaviour occur in a narrow composition range, i.e. at a composition of around 32 mol% for synthetic P(3HB-*co*-3HV)[5] or 41 mol% for bacterial P(3HB-*co*-3HV)[6,7]. Cocrystallization of these copolymers has been interpreted theoretically with a thermodynamic treatment[8–10].

In the present study, continuing to the preceding paper[11], the composition range of crystal phase transition, where both P(3HB) and P(3HV) crystal phases coexist, was investigated for samples fractionated with a solution of acetone–water. The copolymers used were those of 3HV composition outside the pseudoeutectic composition range of 41–55 mol% as reported previously[11]. The melting points and molecular weights of these fractionated samples were measured. The melting points of P(3HB-*co*-3HV) were plotted against the thus refined 3HV composition. Amorphous and crystalline densities of P(3HB-*co*-3HV) were determined by density and X-ray scattering methods, respectively. Crystallinity was estimated from the density measurement and compared with that estimated from the X-ray method.

EXPERIMENTAL

Samples

P(3HB-*co*-3HV) copolymers containing 36 and 61 mol% 3HV (hereinafter these are called samples I and II, respectively) were mainly used, which were isolated from *Alcaligenes eutrophus* (ATCC 17699). The

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microorganism was first grown at 30°C in nutrient-rich medium (100 ml) containing 1 g yeast extract, 1 g polypeptone, 0.5 g meat extract and 0.5 g $(NH_4)_2SO_4$. The cells were harvested after 24 h and washed with water. At this stage, accumulation of polyesters in the cells was not observed. To promote polyester synthesis about 0.4 g quantities of the washed cells were transferred into a nitrogen-free medium[2] containing 2 g in total of two carbon sources, i.e. butyric and valeric acids, in various ratios. The cells were cultivated in these media (100 ml, pH 7.0) for 48 h at 30°C, harvested by centrifugation, washed with acetone, and finally dried under vacuum at room temperature. Polyesters were extracted from the dried cells with hot chloroform in a Soxhlet apparatus, and purified by reprecipitation with hexane[2]. P(3HB-*co*-3HV) containing, for example, 61 mol% 3HV units is hereafter abbreviated as P(3HB-*co*-61% 3HV) for the one containing 61% 3HV.

Fractionation

The fractionation was performed by dissolving the samples (0.3 g) with hot acetone (30 ml), and keeping them at 8°C for 20 h followed by centrifugation to isolate the precipitate. In the next step, the solution was diluted with water to a concentration of 95% acetone, dissolved completely and kept at the same condition followed by isolation. The same procedure was repeated lowering the concentration in 2.5 or 5% steps.

Analytical procedures

The melting behaviour of 3 mg sample was studied by using a Perkin-Elmer DSC-7 differential scanning calorimeter (d.s.c.) at a heating rate of 10°C min^{-1} under nitrogen flow. The melting peak temperature after being corrected for heating-rate dependence was defined as the melting point T_m with accuracy within ±0.1°C. The temperature scale was calibrated with high-purity standards.

The wide-angle X-ray scattering (WAXS) pattern was recorded photographically using a cylindrical vacuum camera (50 mm diameter) by the method of rotating the sample. Nickel-filtered Cu Kα X-ray beams from a Rigaku-Denki Rotaunit RU-200 were used. The diffraction pattern was obtained from the microdensitometer trace along the equatorial direction.

The ^1H noise-decoupled 67.5 MHz ^{13}C n.m.r. spectra of the fractionated samples were recorded at 30°C in $CDCl_3$ (20 g l^{-1}) on a JEOL GSX-270 spectrometer with 3s pulse repetition, 15 000 Hz spectral width, and 64 K data points, similar as those of another report[10].

The g.p.c. chromatograms were recorded with an HLC-802A high-performance liquid chromatograph (Tosoh Co. Ltd) at 38°C equipped with a series of four columns of TSK-gel and an RI-8 differential refractometer. The eluent was chloroform with a flow rate of 1 ml min^{-1} and polymer concentration was 2–3 mg ml^{-1}. The number-average molecular weight $\bar{M}_n$ was calibrated using mainly six PHB samples having different $\bar{M}_n$ values (from 4.29×10^3 to 5.64×10^5 g mol^{-1}) evaluated by g.p.c. and a low-angle laser-light scattering system.

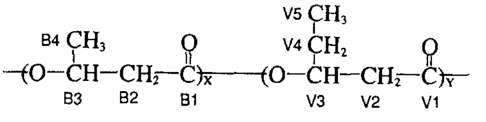
The sample density was measured at 25°C for 24 h using a density gradient column containing mixtures of toluene and carbon tetrachloride as the column liquid. The fractionated samples used were films once molten at 185°C and kept at room temperature for at least several weeks so as to crystallize fully.

The amorphous samples were obtained by melting at 185°C for 2 min and immediately quenching into liquid nitrogen. The densities of the amorphous samples were measured at 25°C by flotation in aqueous KBr solutions[12]. The solutions were prepared to cover the range of densities required in 0.001 g cm^{-3} steps. Once the quenched sample warms to room temperature, the density began to increase after about 1 min in the most rapid case. Therefore, all measurements of amorphous densities were made within 1 min so as to prevent the onset of crystallization.

RESULTS AND DISCUSSION

*Characterization of fractionated P(3HB-*co*-3HV)*

Typical d.s.c. heating curves for the fractionated sample II are shown in *Figure 1*. The unfractionated sample and that precipitated at 100% acetone show two broad melting peaks at temperatures of 80–95 and 166°C, respectively. The melting peak at 166°C corresponds to the copolymer that is extremely rich in 3HB units, while that at 80–90°C corresponds to the typical copolymer. This implies that these samples are mixtures of several copolymers having various compositions. The sample precipitated at 90% acetone solution shows a single sharp peak at 83°C, and samples precipitated at concentrations below 90% show a single peak at 70–80°C. Melting peaks became broader and lower as the concentration of acetone decreased.


$$-\!\!(O-\overset{CH_3}{CH}-CH_2-\overset{O}{\overset{\|}{C}})_X\!\!-\!\!(O-\overset{\overset{CH_3}{CH_2}}{CH}-CH_2-\overset{O}{\overset{\|}{C}})_Y\!\!-$$

P(3HB-*co*-3HV)

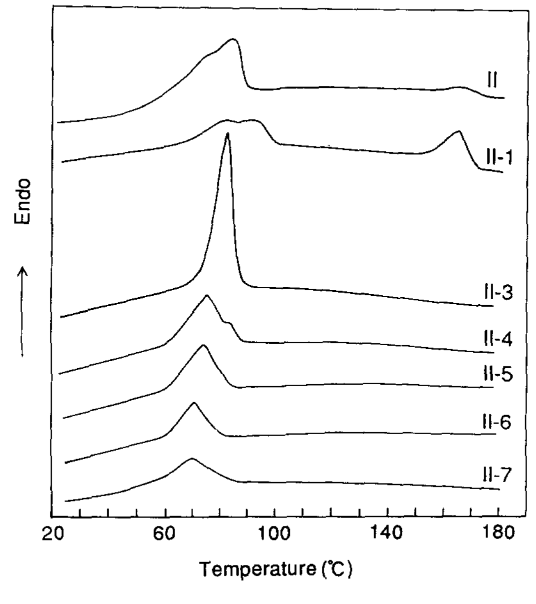


Figure 1 Typical d.s.c. heating curves for the fractionated P(3HB-*co*-61% 3HV) (sample II): samples II fractionated in the acetone concentration ranges of 100% (II-1), 95–90% (II-3), 90–87.5% (II-4), 87.5–85% (II-5), 85–82.5% (II-6) and 82.5–80% (II-7) as denoted in *Table 1*

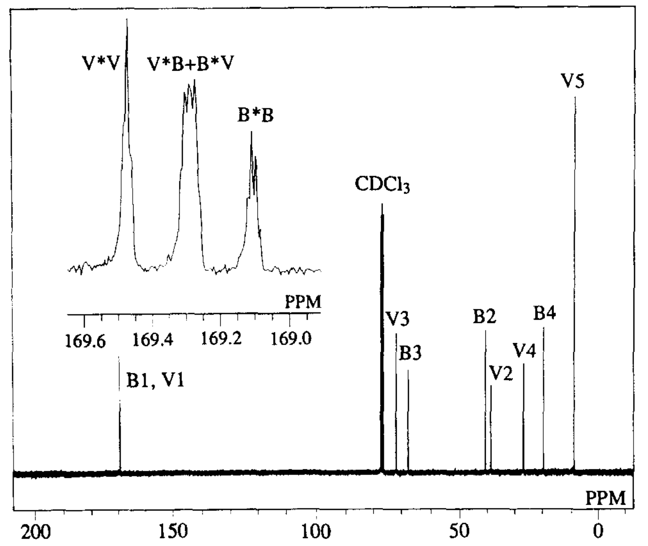


Figure 2 Typical ^{13}C n.m.r. spectrum of sample II-6. Enlarged views of peaks B1 and V1 are shown in the inset

A typical ^{13}C n.m.r. spectrum of sample II precipitated at acetone concentration of 82.5% (II-6) is shown in *Figure 2*. All the carbon resonances of P(3HB-*co*-3HV) are split into multiplets owing to diad and/or 3HV-centred triad comonomer sequences. The assignment of these signals has been reported already[13,14].

Magnified peaks B1 and V1 are also shown in *Figure 2* inset. In order to determine the sequence distribution, it is convenient to use the parameter D proposed by Kamiya *et al.*[15]:

$$D = (F_{VV}F_{BB})/(F_{VB}F_{BV}) \qquad (1)$$

where F_{XY} represents the mole fraction of the XY sequence estimated from the ^{13}C n.m.r. spectrum, and the subscripts B and V represent the 3HB and 3HV units, respectively. The peak V*B + B*V at 169.23 ppm was divided into F_{BV} and F_{VB} in the same ratio as estimated from peaks B2 and V2, which split into multiplets due to the diad comonomer sequences (and from peaks V2 and V4 due to the triad ones), respectively, as demonstrated by Kamiya *et al.*[15] If a copolymer is statistically random, D is close to 1, and it is larger or smaller than 1 for a block or an alternating copolymer, respectively. For example, D value for the fractionated sample II-6 is 1.0 (the error involved in this procedure is within ±2%), which implies a typical random copolymer.

The results of fractionation of both samples I and II are listed in *Table 1*. Both samples were mainly precipitated at acetone concentrations of 100% and around 85% and a very little fraction was precipitated at concentrations below 80%. The composition of 3HV component estimated from the ^1H n.m.r. spectrum is listed in *Table 1*. That varied over a wide range from ca. 20 to 80% 3HV, showing that both samples are mixtures of several copolymers having different compositions. Except for the samples I-1 and II-1, the 3HV composition of the precipitate decreased from the higher value as the acetone concentration decreased. The samples I-1 and II-1 are still mixtures of copolymers of various compositions, and both show T_m of 166°C, which corresponds to T_m of 3HB-rich (maybe around 5% 3HV) copolymer fraction. The unfractionated sample I hardly shows the peak at 166°C, implying that this component is too small to be detected and diffuses too broadly to crystallize. Fractionation with differences in the 3HV composition was mainly observed until the concentration of 85%. Fractionation at concentration below 85% was performed with differences both in the 3HV composition and in the molecular weight, i.e. $\bar{M}_n$ and polydispersity ($\bar{M}_w/\bar{M}_n$) values decreased as the acetone concentration decreased. All precipitates are random copolymers having narrow 3HV compositions (except for the samples I-1 and II-1) because the D values are close to 1.

Figure 3 shows typical WAXS patterns of the samples I, I-3, II and II-5. Samples I and II show typical P(3HB) and P(3HV) crystal phases, respectively. The diffraction peaks denoted B(0 2 0), B(1 1 0) and B(0 0 2) in *Figure 3* were assigned to the (0 2 0), (1 1 0) and (0 0 2) planes of the P(3HB) lattice, and those denoted V(1 1 0), V(0 2 0) and V(2 1 1) were assigned to those planes of the P(3HV) lattice. In contrast, the fractionated samples I-3 and II-5 show the mixed patterns of P(3HB) and P(3HV) crystal phases very similar to those samples reported pre-

Table 1 Fractionation and characterization of samples I and II

Sample	Conc. of aq. acetone (%)	Fraction (wt%)	HV[a] (mol%)	T_m (°C)	$\bar{M}_n \times 10^{-3}$ (g ml^{-1})	$\bar{M}_w/\bar{M}_n$	D	Crystal lattice
I			36	82, 92	568	4.48	1.2	PHB
I-1	100	28	19	166	279	4.32	1.4	PHB
I-2	90	2	55	80	288	5.72	1.1	PHB, PHV
I-3	87.5	10	40	76	722	3.93	1.1	PHB, PHV
I-4	85	41	36	82	552	5.82	1.1	PHB, PHV
I-5	82.5	9	33	86	323	2.98	1.0	PHB
I-6	80	4	30	87	166	3.30	1.1	PHB
I-7	70	3	31	87	103	2.37	1.1	PHB
II			61	86, 166	263	12.68	1.7	PHV
II-1	100	13	28	82, 93, 166	161	3.29	7.9	PHB
II-2	95	6	78	85	140	5.27	1.3	PHV
II-3	90	10	71	83	414	6.40	1.0	PHV
II-4	87.5	22	67	76, 85	313	6.27	1.0	PHV
II-5	85	32	56	74	263	4.64	1.0	PHB, PHV
II-6	82.5	7	55	72	125	4.52	1.0	PHB, PHV
II-7	80	6	55	72	119	4.16	1.0	PHB, PHV
II-8	75	4	54	71	114	3.86	1.0	PHB, PHV

[a] The HV mol% was estimated from ^1H n.m.r. spectrum

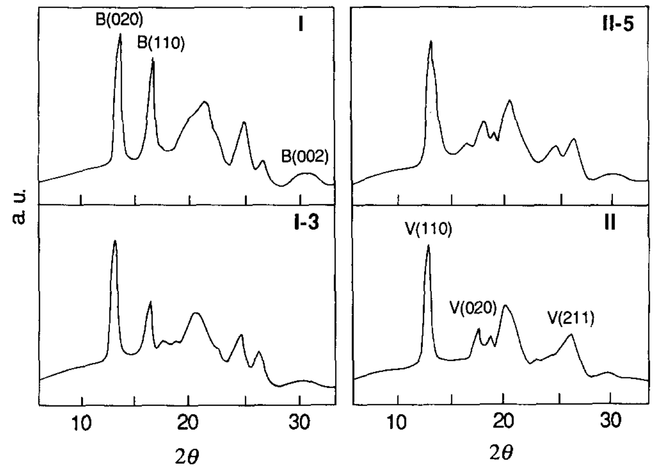


Figure 3 Typical WAXS patterns of fractionated samples I, I-3, II and II-5

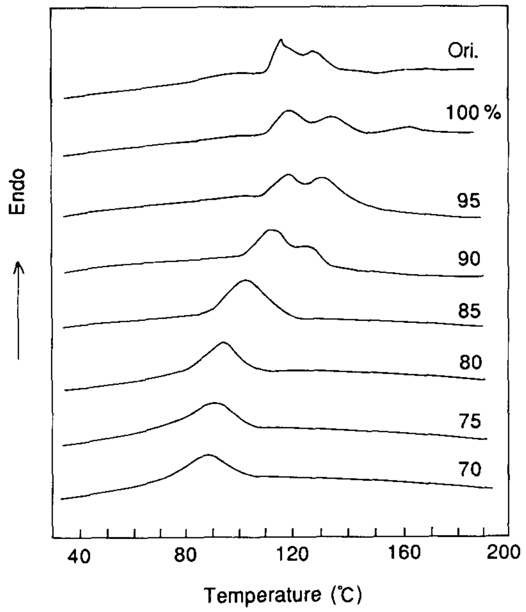


Figure 4 D.s.c. heating curves of P(3HB-*co*-24% 3HV) samples fractionated with various acetone concentrations as indicated

viously[11]. The characteristic diffraction peaks of the P(3HV) crystal phase increased in their intensities as the 3HV composition increased.

It seems very common that most copolymers obtained by biosynthesis are a mixture of several copolymers having different compositions, because there are various changeable factors during the cultivation that result in a wide composition range of copolymers. Therefore, a typical commercial P(3HB-*co*-24% 3HV) copolymer (Aldrich Chemical Co. Inc.) was chosen to demonstrate this fractionation. Their d.s.c. heating curves are shown in *Figure 4*. Melting peaks of the fractionated samples shifted to lower temperatures as the acetone concentration decreased.

Table 2 lists the fractionation and characterization of P(3HB-*co*-24% 3HV). The 3HV composition estimated from the ^{1}H n.m.r. spectrum increased from 21% to 32% as the acetone concentration decreased up to 80%. The sample fractionated with acetone concentration below 80% frequently showed rather lower 3HV composition and much lower $\bar{M}_n$ value, reflecting that the fractionation was performed with differences in both the composition and molecular weight, i.e. $\bar{M}_n$ and $\bar{M}_w/\bar{M}_n$ decreased as the acetone concentration decreased. The D values were close to 1 except for the sample fractionated at 100% acetone, implying that they are typical random copolymers.

Melting points versus *composition*

Figure 5 shows plots of the melting points of all fractionated P(3HB-*co*-3HV) copolymers having D values close to 1 against thus refined 3HV) composition. In the case when multiple melting peaks were obtained, T_m of the major melting peak was used for the plot, e.g. T_m of 76°C for sample II-4. The result is similar to other reports obtained for unfractionated samples[3,4]. *Figure 5* is very similar to the result reported by Kamiya *et al.*[15], which was the plot of T_m of the copolymer having D value less than 1.5. The minimum T_m is observed at ca. 40% 3HV, which is the intermediate point of pseudo-eutectic or transition composition range from the P(3HB) to the P(3HV) crystal lattice[11]. The 3HV composition of 36–56% is the transition range of crystal phases in which crystal phases of both P(3HB) and P(3HV) are observed. Recently, Orts *et al.* have reported that T_m of bacterial P(3HB-*co*-3HV) is usually lower than that of synthetic P(3HB-*co*-3HV)[8].

The melting-point depression for complete co-unit exclusion is represented by the Flory random copolymer equation[16]:

$$\frac{1}{T^\circ_{\mathrm{m,AB}}} - \frac{1}{T^\circ_{\mathrm{m,A}}} = -\left(\frac{R}{\Delta H_u}\right) \ln X_A \qquad (2)$$

Table 2 Fractionation and characterization of P(3HB-*co*-24% 3HV)

Conc. of aq. acetone (%)	Fraction (wt%)	HV[a] (mol%)	T_m (°C)	$\bar{M}_n \times 10^{-3}$ (g mol^{-1})	$\bar{M}_w/\bar{M}_n$	D
		24	118, 129	316	5.54	1.2
100	22	21	119, 136	337	3.32	1.5
95	6	22	119, 136	255	3.22	1.1
90	10	23	114, 129	214	2.78	1.0
87.5	15	24	112	188	2.61	1.0
85	27	29	104	178	2.41	1.0
82.5	3	31	94	70.1	1.93	1.0
80	5	32	93	56.2	1.74	1.0
75	2	31	92	20.8	1.57	1.0
70	2	30	89	14.2	1.49	1.0

[a] The HV mol% was estimated from ^{1}H n.m.r. spectrum

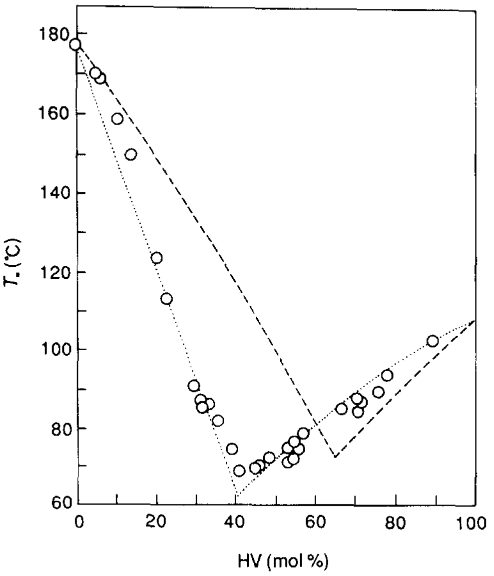


Figure 5 Plots of melting points of the fractionated copolymers P(3HB-*co*-3HV) having D values close to 1 *versus* the HV composition

where X_A is molar fraction of the A component, ΔH_u is the heat of fusion and R is the gas constant. Using the observed melting point of homopolymers instead of the equilibrium $T^{\circ}_{m,AB}$, we can approximately draw the calculated $T^{\circ}_{m,AB}$ curve as shown by the dashed line in *Figure 5*. Here, $T^{\circ}_{m,A}$ and ΔH_u were assumed to be 177°C and 14.4 J g^{-1} for P(3HV) homopolymer and 110°C and 13.1 J g^{-1} for P(3HB) homopolymer[5,17], respectively. The dashed line clearly underestimates the melting-point depression in the range below 40% 3HV and overestimates it in the range above 40%. If one is allowed to apply the Flory equation to describe the observed T_m as shown with the dotted line, the ΔH_u value of P(3HB) crystal phase of the copolymer (below 40% 3HV) derived is 5.6 kJ mol^{-1}, and ΔH_u of P(3HV) crystal phase of the copolymer (above 40% 3HV) is 20.1 kJ mol^{-1}. The former value is close to the reported value of 6.7 kJ mol^{-1} in the composition range below 30 mol% 3HV[3].

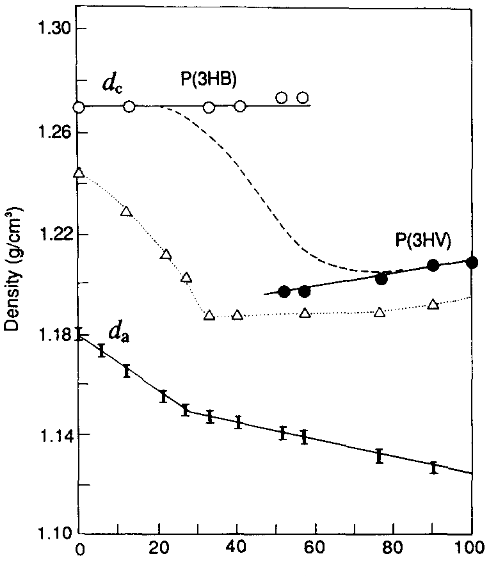


Figure 6 Plots of amorphous density d_a and crystalline density d_c *versus* the HV composition. The d_c values of P(3HB) crystal phase (○) and P(3HV) crystal phase (●) are shown. Sample densities are plotted as the dotted curve (△)

Recently, Orts *et al.*[8,17] pointed out that for isodimorphic systems the Flory exclusion model does not correctly predict the melting-point depression, but the Sanchez–Eby inclusion model[18,19] provides a good fit. However, such an attempt is not carried out here because a few factors are unknown, which will be discussed in the subsequent paper.

*Density and crystallinity of P(3HB-*co*-3HV)*

Figure 6 shows amorphous densities d_a measured by the flotation method. The d_a decreased linearly as the 3HV composition increased. However, there is a break point at a 3HV composition of ca. 30%, and the first depression line is very close to that reported by Barham *et al.*[12] (0–24% 3HV), while the second line (above 30% 3HV), not reported hitherto, decreased more gently than the former. The d_a of P(3HV) is evaluated as 1.125, which is obtained from the extrapolation of the curve. The curve implies that the extension of molecular packing due to the larger side group is not uniform over the whole composition, i.e. the extension is less sensitive in the range above 30% 3HV.

Crystalline densities (d_c) estimated from lattice indices measured by WAXS patterns were plotted against the refined 3HV composition. Here, we assume that the 3HV composition is equivalent to that in crystalline regions though there are several reports that the 3HV component is partially excluded from the 3HB crystal lattice because of the larger side group[7,12,20]. The d_c of P(3HB) crystal lattice remained almost constant, while d_c of P(3HV) crystal lattice decreased as the 3HB component increased. The copolymer in the range of crystal phase transition shows the two d_c values of P(3HB) and P(3HV) crystal phases. Therefore, we can approximately estimate d_c of the copolymer as the arithmetic mean value using the ratio of X-ray diffraction intensities of P(3HB) and P(3HV) crystal components (i.e. the ratio of intensities of B(1 1 0) and V(0 2 0) after normalization with the diffraction intensity of each crystal for P(3HB) and the copolymer of 90% 3HV). The result is shown as the dashed curve, assuming that the transition range is wider than 36–56% 3HV because at the outside of this

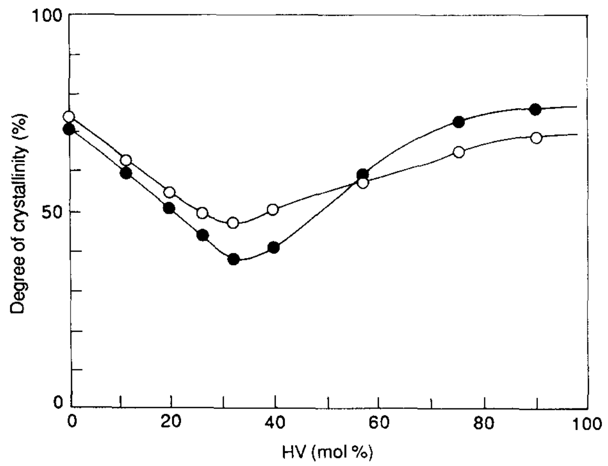


Figure 7 Plots of crystallinities estimated from density (●) and X-ray (○) methods against the HV composition

range the diffraction of the minor crystal component is too weak to be detected by WAXS measurement. Sample densities measured by a density gradient column are plotted in *Figure 6* (dotted curve). The minimum values were observed in the range of 30–40% 3HV.

Figure 7 shows degree of crystallinity X (%) estimated from the density measurement using the following equation:

$$X = [d_c(d - d_a)/d(d_c - d_a)] \times 100 \qquad (3)$$

where d is the density of the sample. The X value decreased steeply from around 70% to 40% as the 3HV composition increased from 0 to 30%. One of the authors[21] has previously reported X values of 81 and 39% for P(3HB) and P(3HB-*co*-28% 3HV) samples unfractionated and solvent-cast, assuming d_c and d_a of both samples to be 1.260 and 1.174 g cm^{-3}, respectively. In the range above 30% 3HV, X increased steeply as the 3HV composition increased up to 75% and levelled off to around 75% over the range of 75–100% 3HV. For comparison, degree of crystallinity estimated from the X-ray method is also plotted, which is very close to the other report[6]. Both X values changed similarly, i.e. the minimum values were observed in the range of 30–40% 3HV, though the variation of X value from density is larger than that from X-ray method. Bluhm *et al.*[3] and Doi *et al.*[4] have reported that degree of crystallinity from X-ray diffraction for unfractionated and solvent-cast P(3HB-*co*-3HV) samples is almost constant throughout wide ranges of 0–45% and 0–95% 3HV, respectively. The difference of above results with our results may be caused by the differences in sample origin and crystallization conditions and so on. The range of 30–40% 3HV, where the minimum X is observed, closely corresponds to the pseudoeutectic region. It is considered that, in this region, the 3HB co-unit is prevented most from crystallizing with the 3HV co-unit; however, in the region of 75–100% 3HV, the 3HV co-unit is rather promoted to crystallize by the presence of the 3HB co-unit of 0–25%, presumably playing a role of diluent or lubricating agent.

CONCLUSIONS

Microbial P(3HB-*co*-3HV) copolymers were fractionated by fractional precipitation methods using a solution of acetone–water. Fractionation mainly took place with a difference in the 3HV composition until the acetone concentration diluted to 85%, converging to the composition of 30–40% 3HV from lower or higher composition. Both copolymers were mixtures of random copolymers having a wide range of 3HV composition. At a concentration below 85%, fractionation took place with differences in both the 3HV composition and molecular weight. Crystal phase transition of isodimorphism for P(3HB-*co*-3HV) occurred in a broad range of 36–56% 3HV, which corresponds to the pseudoeutectic composition range. A plot of T_m was obtained against the refined 3HV composition. The minimum T_m was observed at ca. 40% 3HV. The amorphous and crystalline densities of the fractionated samples were measured, from which the degree of crystallinity estimated from the density method was calculated, which is almost parallel to the crystallinity estimated from the X-ray method.

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