

Chemical Composition Distribution of Biosynthesized Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) Determined by Temperature Rising Elution Fractionation

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The chemical composition distributions (CCDs) of biosynthesized poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), P(3HB-*co*-3HV), samples with different average compositions were determined by temperature rising elution fractionation (TREF). P(3HB-*co*-3HV) samples were supplied by Sigma–Aldrich Co. Inc. According to catalog data, the 3HV contents were about 8, 14, and 24 mol%, respectively. The TREF condition was as follows: the sample concentration was 0.3% dissolved in acetonitrile at 75 °C; the column temperature was slowly (2 °C/h) decreased from 75 °C to 0 °C to crystallize the sample. The fractions were eluted with acetonitrile by stepwise increasing the temperature by 5 °C.

The chemical compositions of the original samples and the fractions were determined by ^{1}H NMR. Cumulative CCD curves were constructed from the fractionation data for the respective samples. It became clear that these samples had bimodal CCDs with two discrete peaks, that is, one part in the wide composition range around 10–15 mol% of 3HV contents and another part at different compositions.

Microbial copolyesters are attracting attention for their special properties of environmental biodegradability and biocompatibility. Especially poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), P(3HB-*co*-3HV), was produced commercially under the trade name BIOPOL®. Their chemical composition can be controlled by the fermentation conditions. Wide ranges of properties of the copolyesters have been intensively studied, such as the mechanism of biosyntheses, microstructure, thermal behaviors, mechanical properties, and enzymatic degradation process. The copolyesters have high stereoregularity and crystallizability. It was reported that P(3HB-*co*-3HV) crystallized either in the P(3HB) or P(3HV) crystal lattice for 3HV contents lower or higher than ca. 40 mol%, respectively.[1,2] Also, the coexistence of both crystal phases and the pseudoeutectic behavior in a narrow composition range around 41 mol% of 3HV content were reported.[3,4] Therefore, the chemical composition distribution (CCD) is a very important molecular characteristic for the copolyesters. However, only a few papers on CCD have so far been published.

Mitomo et al.[5,6] and Yoshie et al.[7] reported, respectively, that microbial P(3HB-*co*-3HV) samples were fractionated according to the chemical compositions by a precipitation fractionation method, and that the samples had broad CCDs. However, precipitation fractionation is not so effective in the fractionation of crystalline polymers. Crystallizable polymers are precipitated as the gel phase with physical crosslinking from the solution by the addition of a precipitant. Since the process is irreversible, and is affected by molecular weight distribution, both the reproducibility and efficiency of the fractionation method are low. On the other hand, it is well known[8,9] that the temperature rising elution fractionation (TREF) is very effective in the compositional fractionation of crystalline copolymers. In TREF, a polymer is dissolved in a solvent at high temperature. The solution is added into an inert support material in a column at a controlled temperature. The mixture is then slowly cooled, allowing polymer molecules to crystallize on the support according to their crystallizability. After crystallization, the temperature is raised continuously and/or stepwise with the solvent flowing. Components crystallized with difficulty are eluted first (at lower temperatures). As the temperature rises, those components crystallized more easily are eluted. The influence of the molecular weight distribution on the fractionation is usually negligible for molecular weights larger than about ten thousand.[10,11]

In the present work, we fractionated samples of microbial P(3HB-*co*-3HV) supplied commercially, according to their chemical compositions by the TREF method, and constructed CCD curves from the fractionation data. It was clarified that the CCD curves had two peaks for all samples.

Experiment

P(3HB-*co*-3HV) Samples. P(3HB-*co*-3HV) samples were supplied by Sigma-Aldrich Co. Inc. According to catalog data, the 3HV contents are about 8 mol% (Lot. No. KN18102DF), 14 mol% (Lot. No. KG06009DZ), and 24 mol% (Lot. No. KG17722CN), respectively. The respective samples were purified before fractionation. The samples were dissolved in chloroform. The solution was filtrated by a poly(tetrafluoroethylene) membrane filter with a pore diameter of 0.5 μm. The filtrated sample solutions were poured into excess methanol, and the precipitated samples were washed with fresh methanol and dried under vacuum at room temperature. The commercially supplied solvents

Table 1. Purified Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) Samples

Sample Code	Average Contents of 3HV units/mol%[a)]	Mp[b)] ($\times 10^{-5}$)	Sample Lot. No.
HV8	9.0	4.37	Aldrich Lot. No. KN18102DF
HV14	15.5	1.12	Aldrich Lot. No. KG06009DZ
HV24	23.1	1.21	Aldrich Lot. No. KG17722CN

a) Determined by ^{1}H NMR (270 MHz, $CDCl_3$).
b) Molecular weight at the peak position estimated by SEC corresponding to standard polystyrene.

were purified by conventional methods.

Temperature Rising Elution Fractionation (TREF). The TREF system was composed of a glass column with a water jacket and a temperature-control device. The column was 1.8 cm in inner diameter and 60 cm in effective length, and filled with glass beads of 0.1 mm on the average diameter as the support material.

The fractionation procedure was as follows: about 0.5 g of the sample was first dissolved in 167 cm^3 of acetonitrile (polymer concentration was about 0.3%) at 75 °C. The column temperature was kept at 75 °C. The sample solution was charged into the column by replacing pure acetonitrile used for packing the glass beads into the column. The column temperature was slowly (2 °C/h) decreased from 75 °C to 0 °C to deposit the polymer sample. Then, the sample solution was slowly replaced with pure acetonitrile at 0 °C. The solution eluted from the column was collected as the fraction. Then, the column was heated to 5 °C and kept at this temperature for 30 min. The next fraction was eluted in a similar manner. The procedure was repeated to 75 °C by stepwise increasing the temperature in 5 °C steps. The polymer in the fraction was concentrated by evaporation and precipitated with excess methanol.

Characterization of Samples. The molecular weights corresponding to standard polystyrene of the purified original samples were estimated by size-exclusion chromatography (SEC). The SEC measurement was carried out using two TSK gel GMH_{HR}-M columns (Tosoh) and chloroform as an eluent. The column temperature was 30 °C, the flow rate was 1.0 cm^3/min., the injection volume was 0.02 cm^3, and the sample concentration was 2.0 mg/cm^3. Samples were detected by an evaporative light-scattering detector (ELSD Mk II, Varex).

The average compositions of the samples and the fractions were determined by ^{1}H NMR at 270 MHz (JEOL EX270) in $CDCl_3$.

Results and Discussion

Table 1 gives the 3HV contents and molecular weights corresponding to standard polystyrene for purified P(3HB-*co*-3HV) samples. These samples were characterized by the TREF.

The fractionation results are summarized in Tables 2–4. The sample recoveries were fairly good. The source of the recovery over 100% for sample HV14 is supposed to be a fine powder of the glass beads contained in the fractions of the sample. The average 3HV contents calculated from the fractionation data agreed with the values of the original samples within the experimental error, respectively.

The fractions were eluted in the order of the 3HV contents on the whole for the respective samples. It was reported that microbial P(3HB-*co*-3HV) crystallizes either in a P(3HB) or a P(3HV) crystal lattice for 3HV contents lower or higher than

Table 2. Results of Fractionation of HV8

Fraction No.	Fractionation Temperature / °C	Yield / g	Weight Fraction	3HV Contents / mol%
1	0	0.0322	0.071	14.6
2	5	0.0052	0.012	13.4
3	10	0.0003	0.001	8.5
4	15	0.0015	0.003	5.3
5	20	0.0004	0.001	5.1
6	25	0.0008	0.002	10.9
7	30	0.0026	0.006	11.0
8	35	0.0053	0.012	11.6
9	40	0.0095	0.021	12.3
10	45	0.0126	0.028	11.2
11	50	0.0316	0.070	9.0
12	55	0.0482	0.107	6.5
13	60	0.0926	0.206	6.2
14	65	0.0975	0.216	5.6
15	70	0.0973	0.216	4.1
16	75	0.0130	0.029	5.6

The recovery of the sample was 90.8 wt%.
3HV Content: Origin 9.0 mol%, Average of fractions 6.9 mol%.

Table 3. Results of Fractionation of HV14

Fraction No.	Fractionation Temperature / °C	Yield / g	Weight Fraction	3HV Contents / mol%
1	0	0.1581	0.311	22.3
2	5	0.0154	0.030	23.3
3	10	0.0006	0.001	13.7
4	15	0.0004	0.001	11.7
5	20	0.0019	0.004	11.2
6	25	0.0017	0.003	14.3
7	30	0.0071	0.014	15.8
8	35	0.0127	0.025	16.0
9	40	0.0211	0.042	13.0
10	45	0.0352	0.069	12.9
11	50	0.0680	0.134	12.2
12	55	0.0461	0.091	10.2
13	60	0.0792	0.156	9.2
14	65	0.0453	0.089	8.0
15	70	0.0118	0.023	7.0
16	75	0.0030	0.006	7.7

The recovery of the sample was 105.7 wt%.
3HV Content: Origin 15.5 mol%, Average of fractions 14.7 mol%.

Table 4. Results of Fractionation of HV24

Fraction No.	Fractionation Temperature / °C	Yield / g	Weight Fraction	3HV Contents / mol%
1	0	0.3202	0.673	24.4
2	5	0.0619	0.130	23.8
3	10	0.0068	0.014	25.3
4	15	0.0008	0.002	13.5
5	20	0.0009	0.002	18.5
6	25	0.0013	0.003	12.8
7	30	0.0025	0.005	16.3
8	35	0.0055	0.012	16.4
9	40	0.0169	0.035	15.8
10	45	0.0247	0.052	13.9
11	50	0.0130	0.027	14.9
12	55	0.0058	0.012	12.6
13	60	0.0027	0.006	13.6
14	65	0.0023	0.005	10.7
15	70	0.0061	0.013	7.9
16	75	0.0047	0.010	7.5

The recovery of the sample was 93.9 wt%.
3HV Content: Origin 23.1 mol%, Average of fractions 22.4 mol%.

ca. 40 mol%, respectively.[1,2] As is clear from the fractionation data, the 3HV contents of all fractions are much lower than 40 mol% for the respective samples. All components of the samples should crystallize in the P(3HB) crystal lattice. Since the relative crystallizability of the component decreases as the 3HV content increases in this region, the elution orders in TREF conform to the 3HV contents. On the other hand, it was reported that the mixture of the present copolyesters with different compositions showed an isomorphous crystalline phase, or two crystalline phases from the miscible melt, when the mixture was crystallized at 80 °C in bulk.[12] If such co-crystallization occurs in the TREF, the fractionation efficiency must decline. Therefore, a situation where the degree of supercooling is very high for the components with different compositions at the same time as in the case mentioned above should be avoided, though the samples were crystallized from very dilute solutions in the present case. Therefore, the cooling rate was very low, as mentioned in the "Experiment" section.

Remarkable reversals of the 3HV content were observed for a few fractions of a small amount in the respective samples (Fraction Nos. 3–5 in HV8 sample, Nos. 3–5 in HV14 sample, Nos. 4 and 6 in HV24 sample). This may have been caused by the effect of the molecular weight distribution, though the molecular weights of the fractions could not be measured because of the very small amounts. It is well known that the effect of the molecular weight on the melting point is very small compared with that of the chemical composition, except for the region of very low molecular weight. These fractions are supposed to be components of very low molecular weight and low 3HV content. However, the present fractionations may not be affected by the molecular weight as a whole without the fractions mentioned above, since the samples have high molecular weights of the order of 10^5, corresponding to the standard polystyrene by SEC.

The CCDs were constructed from fractionation data in the form of the cumulative distribution, plotting the cumulative weight fraction versus the 3HV content, as shown in Figs. 1–3. For the HV8 sample shown in Fig. 1, the CCD was composed of two parts, that is, the main part in a narrow composition range of 4–7 mol% of 3HV content, and the secondary part in a wide range of 9–15 mol%. For the HV14 sample, as shown in Fig. 2, the CCD was composed of the main part in a wide composition range of 7–16 mol%, and the secondary part around 23 mol%. For the HV24 sample, as shown in Fig. 3, the CCD was composed of the main part round 24 mol%, and a small part in a wide range of 7–17 mol%. Since the weight fractions of the first fractions eluted at 0 °C are fairly large, respectively, for the samples of HV14 and HV24, it is not obvi-

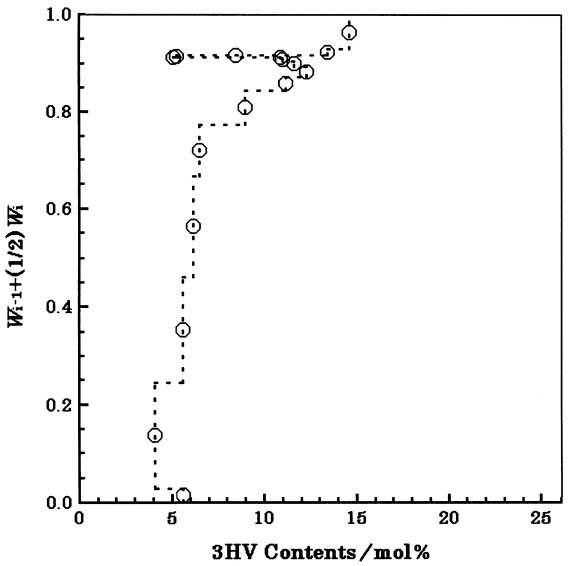

Fig. 1. Cumulative chemical composition distribution of HV8.

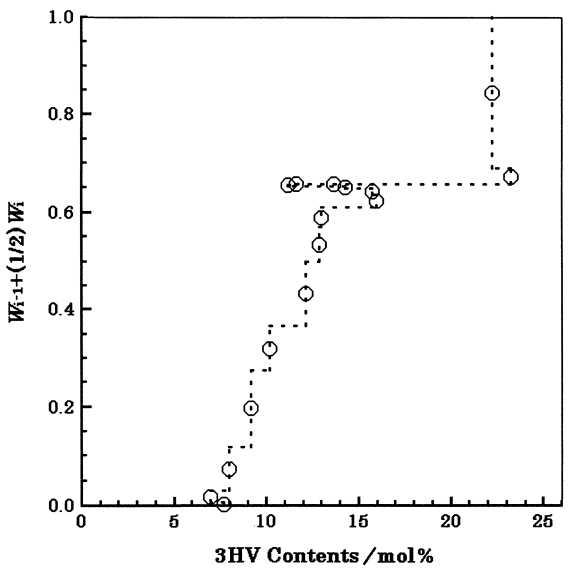

Fig. 2. Cumulative chemical composition distribution of HV14.

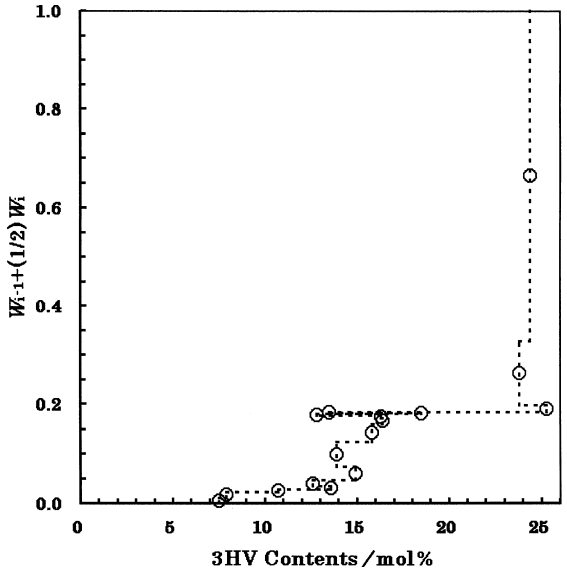

Fig. 3. Cumulative chemical composition distribution of HV24.

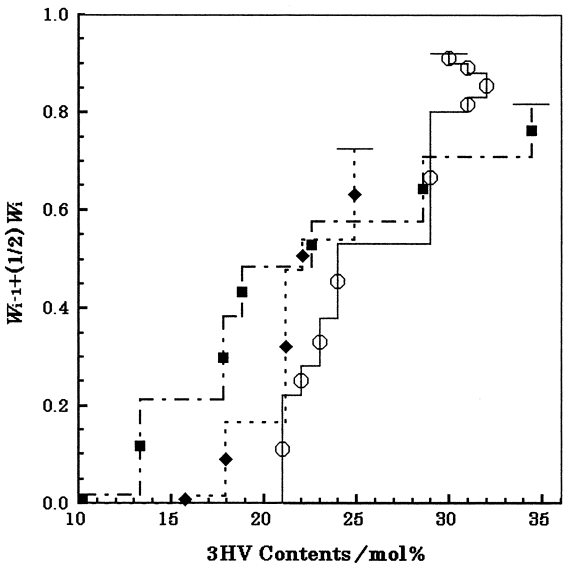

Fig. 4. Cumulative chemical composition distributions reconstructed from the data of Mitomo et al.[6] and Yoshie et al.[7]
Wi; weight fraction of fraction *i* to the charged amount.
○; Mitimo 3HV-24%, ◆; Yoshie 3HV-19.4%, ■; Yoshie 3HV-21.8%

ous whether the second parts of the CCDs are sharp or not. However, it is difficult to think that the second part has a very broad CCD, since the 3HV content of the first fraction is near to the values of the second and third fractions for both samples. All of the samples have bimodal CCDs with two discrete peaks, that is, one part in a wide composition range around 10–15 mol%, and the other part (maybe in a narrow composition range) at different compositions.

Mitomo et al.[6] fractionated the bacterial P(3HB-*co*-3HV) [3HV content 24 mol%; Aldrich Chemical Co. Inc.] by precipitation fractionation with an acetone/H_2O system. Although they showed no CCD curve in their paper, the cumulative CCD curve was reconstructed from the data of Table 2 in their paper,[6] as shown in Fig. 4 (○). It appears that the sample had also bimodal CCD in accordance with the present results. Yoshie et al.[7] fractionated the bacterial P(3HB-*co*-3HV) samples [3HV contents 19.4 and 21.8 mol%; Aldrich Chemical Co. Inc.] into several fractions by a precipitation method using a mixed solvent of chloroform/heptane. They plotted the mass fractions at the compositions of the respective fractions as a histogram, and concluded that the samples had broad composition distributions or composition distributions with many peaks. However, it is appropriate for the data treatment of a preparative fractionation to plot the cumulative weight fractionations versus the chemical compositions in a similar way to Schultz's method,[13] which is used to express the molecular weight distributions from the fractionation data, as pointed out by Fuchs,[14,15] since the width of the chemical composition range of each fraction is unknown. We thus reconstructed the cumulative CCDs from the fractionation data of Table 1 in their paper[7] in accordance with the method, as shown in Fig. 4 (■ and ◆). From the plots, it appears that the samples have continuous broad CCDs. However, the bimodal CCDs are not necessarily denied for their samples, if taking into account that the recoveries were low (72.5% and 81.7%) and the fractionation efficiency of the precipitation method of crystalline polymers is not high, as mentioned in the "Introduction" section. The low efficiency of the compositional fractionation can make continuous broad CCD to appear from a binary mixture of samples with different compositions and broad molecular weight distributions. Therefore, our results are consistent with the result of Mitomo et al., and not necessarily in contradiction to the results of Yoshie et al.

The bimodal CCDs obtained in the present work may prove that the present commercial samples of bacterial P(3HB-*co*-3HV) were mixtures of two samples prepared in different batches.

However, the bimodal CCD must have a close relationship to the mechanism of the biosynthesis, if the sample is a product of one batch. There have been several reports concerning the mechanism of biosynthesis for the bacterial poly(hydroxyalkanoate)s (PHA). In the case of biosynthesis by *Ralstonia eutropha* (supposed to produce the present samples), roughly speaking, the pathway for the biosynthesis of PHA may be divided into two steps: the derivation of monomeric species (acetoacetyl-CoA and/or β-oxovaleryl-CoA) from carbon sources, and copolymerization of the species by the function of the reductase (NADPH dependent acetoacetyl-CoA reductase) and synthase (PHA synthase).[16] However, it is difficult to suppose that the bimodal CCDs with two peaks were caused by a compositional drift of monomeric species during the course of cultivation. It was reported that liner low-density polyethylene (LLDPE) prepared by Ziegler–Natta catalyst and poly(styrene-*co*-acrylonitrile) prepared by radiation-induced copolymerization had bimodal CCDs, respectively. In the case of LLDPE,[17] the bimodal CCD determined by TREF was consistent with a two-site polymerization model, while in the latter case[18] the bimodal CCD was caused by the coexistence of radical and anionic propagation mechanisms. In the present case, the bimodal CCD may involve the mechanism of the latter step in the

biosynthesis pathway.

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