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Sequence Distribution Dependence on Molar Mass in Microbial Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolyesters

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Abstract

The comonomer composition distribution (CCD) of natural aliphatic polyhydroxyalkanoate copolyesters has a strong influence on their morphology and thus on their properties. Using ^{13}C NMR spectroscopy, based on dyad and triad analysis, we examined the composition and CCD dependence on molar mass in two non-random poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)s (PHBV) with different comonomer ratios. For this purpose the copolyesters were fractionated using preparative size exclusion chromatography and their fractions were analyzed according to composition, CCD and thermal properties. The PHBV copolyester with a higher content of hydroxyvalerate comonomer units (HV, 27.1%) showed a more pronounced dependence of composition and CCD on molar mass than the PHBV with a lower content of HV units (12.9%), which showed very little variation. Both PHBV copolyesters and their fractions showed multiple melting peaks in their DSC curves.

Keywords: Polyhydroxyalkanoate; poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolyesters; microstructure; comonomer composition distribution; NMR; molar mass

1. Introduction

Polyhydroxyalkanoates (PHA) are thermoplastic aliphatic polyesters produced by microorganisms as energy storage materials.[1–4] Lately, they have received considerable attention due to their biodegradability and the possibility of using a number of different renewable and waste resources as the feedstock in their production, thus making them good candidates for truly sustainable plastics. PHA allow a high degree of structural and property variation through the incorporation of different comonomers, i.e. hydroxyacids with different alkyl side chains. By changing PHA chemical composition we can vary properties such as melting temperature or degree of crystallization that have great importance for processing and other applications. Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV, Figure 1) is the most common of the PHA copolymers, as a number of microorganisms can produce it without the need for special substrates.

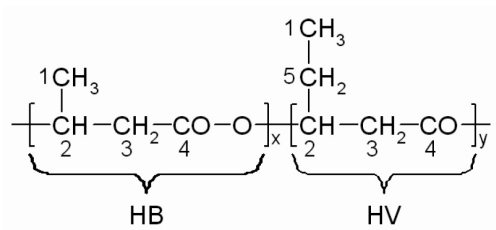

Figure 1. Schematic structure of PHBV copolyesters.

Comonomer composition distribution (CCD), the distribution of comonomers along the copolymer chain, is of particular interest in PHA copolymers since it strongly influences the morphology of the copolymer and its properties in solution, melt, and particularly in solid form. CCD can be determined by ^{13}C NMR spectroscopy by analyzing the sequence distribution of two or three adjacent monomers-dyad and triad distribution-or by mass spectroscopy.[5–9] The dyad sequence distribution is defined by the parameter *D* calculated as:

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

where F_{XY} indicates the relative fraction of the XY dyad sequence (X and Y denote the individual comonomers). The D value for statistically random copolymers is 1.0, while "blocky" and alternating copolymers have D values either much larger than 1 or very close to 0, respectively. The term "blocky" indicates a true block copolymer, a mixture of copolymers with different compositions, or a mixture of poly-3-hydroxybutyrate (PHB) and poly-3-hydroxyvalerate (PHV) homopolymers, meaning that additional information must be available in order to be able to distinguish between these options. The parameter D is not very sensitive to the broadness of CCD, while it is sensitive to bimodal or multimodal (very large D) CCD.[6,8] PHBV copolymers of microbial origin have reported D values either close to 1 or much larger than 1.[5–9] The latter copolymers have a bimodal (or multimodal) CCD and more than one melting peak in their DSC thermograms and are considered to be mixtures of two or more random copolymers with different comonomer compositions. It has been shown that copolymers with D close to 1, which are considered to be random copolymers, have broad CCD.

More precise information on CCD can be obtained by analyzing the triad sequence distribution.[5,7,9,10–14] The degree of randomness in the copolymer chain can be obtained by calculating the coefficient R (see footnote to Table 2),[14] which has a value of 1 for a completely random distribution of two comonomer units in the copolymer chain, and 0 for a diblock copolymer. Details of triad distribution determination in PHBV can be found in the literature.[5,7,9,10–14]

CCD studies of PHA copolymers reported in the literature were carried out on samples subjected to fractionation according to composition based on solubility differences of copolymers with different comonomer contents in solvent/non-solvent mixtures.[15–23] The obtained fractions had a narrower CCD than the original non-fractionated sample. Results of fractionation followed by CCD analysis revealed that bacterial PHA copolyesters have broad and/or multimodal CCD. Using fractions with narrow CCD the authors were able to study the relation between comonomer unit composition in PHA copolyesters and their physical and thermal properties, as well as their morphology and biodegradability. The fractionation of PHA copolyesters by the solvent/non-solvent precipitation technique is assumed to be based mainly on the difference in comonomer composition due to the distinctive polarities of pendant chains at the β site of either comonomer, which define copolymer solubility. The effect of copolyester molar mass and molar mass distribution on the fractionation behavior was believed to be insignificant. In our earlier work with Kowalczuk and Adamus[9] we determined the microstructure of PHBV samples by NMR and mass spectrometry showing that both methods give results that are in good agreement.

In this report we extend our earlier work by a study of the comonomer composition distribution dependence on molar mass which has not yet been systematically examined and is largely believed to be insignificant. For this purpose two non-random PHBV copolyesters with different content and distribution of comonomer units were fractionated according to molar mass by preparative size exclusion chromatography (SEC) followed by NMR-based dyad and triad distribution analysis. To our knowledge this work represents the first attempt at correlating detailed sequence distribution with molar mass distribution in PHBV copolyesters.

2. Experimental

2. 1. Materials

PHBV with 12.9 mol % of 3-hydroxyvalerate units (HV) (denoted as PHBV-12.9) was biosynthesized using *Ralstonia eutropha* fed on hydrolyzed sucrose and was supplied by Biocycle, PHB Industrial S/A, Brazil. PHBV copolymer with 27.1 mol % HV (denoted as PHBV-27.1) was provided by the Institute of Biotechnology and Bioprocess Engineering, Graz University of Technology (Graz, Austria). The copolyester was biosynthesized by an osmophilic bacterium fed with hydrolyzed whey. The content of HV comonomer units in the individual PHBV copolymer was determined by ^{1}H NMR spectrometry. Before the measurements the powders of copolyester samples were dried to a constant weight at room temperature under vacuum and stored in a desiccator prior to use. All solvents used in chromatographic measurements were of analytical grade and were used without additional purification.

Size exclusion chromatography coupled to multi angle light scattering photometer as a detector (SEC-MALS). Sample solutions for SEC-MALS measurements were prepared by dissolving the samples at 60 °C for 1 h in acid-free chloroform (J. T. Baker) at a concentration of 1×10^{-3} g mL^{-1}.[24] The molar mass averages (MMA) and molar mass distributions (MMD) of non-fractionated samples and fractions were determined according to the procedure described in our previous paper.[24]

2. 2. Preparative SEC

Sample solutions for preparative SEC were prepared by dissolving the PHBV samples in acid free chloroform at 60 °C for 3 h at concentration of 5×10^{-2} g mL^{-1}. The fractionation of PHBV copolymers was carried out in chloroform using a PLgel 10 μm Mixed-D preparative column (300 mm length and 25 mm i.d.; effective molar mass range of the column: 200 g mol^{-1} to 400,000 g mol^{-1}; Polymer Laboratories). The mass injected onto the column was 0.1 g and the flow rate of the solvent was 2.3 mL min^{-1}. At the outlet of the column three fractions of

each PHBV sample were collected. After solvent evaporation, the collected fractions were dried in a vacuum oven at room temperature for several days.

2. 3. NMR Spectrometry

Solution ^{1}H and ^{13}C NMR spectra of PHBV samples and fractions were collected on a Varian Inova 600 MHz spectrometer in the pulse Fourier Transform mode. The 600 MHz ^{1}H NMR spectra were recorded at 30 °C using 5 × 10^{-3} g mL^{-1} PHBV solutions in $CDCl_3$ (6.0 s pulse repetition; 8 kHz spectral width; 80 scans). The 150 MHz ^{13}C proton-noise decoupled NMR spectra were recorded at 30 °C using 25 × 10^{-3} g mL^{-1} PHBV solutions in $CDCl_3$ (5.0 s pulse repetition; 30 kHz spectral width; 15,000 scans). Tetramethylsilane (Me_4Si, δ = 0) was used as an internal chemical shift standard.

2. 4. Thermal Characterization

Sample films used for the DSC measurements were prepared by casting PHBV chloroform solutions onto glass Petri dishes. The solvent was allowed to evaporate under vacuum at room temperature. So obtained films were melted at 180 °C and subsequently isothermally crystallized at 30 °C for 1 month. Thermal analyses on so prepared samples were then carried out using a differential scanning calorimeter (DSC) Perkin-Elmer Pyris 1 under a nitrogen atmosphere. The isothermally crystallized samples were heated from –50 °C to +200 °C (first heating scan) at a heating rate of 50 °C min^{-1}. The peak melting temperatures (T_m) were determined from the obtained DSC curves. For more details concerning on the techniques used consult our previous work.[9]

3. Results and Discussion

The sequence distribution of 3-hydroxyvalerate (HV) and 3-hydroxybutyrate (HB) comonomers in PHBV copolyesters and their fractions was determined from proton-noise decoupled ^{13}C NMR spectra from the relative peak intensities of the HB and HV carbonyl (4HB and 4HV in Figure 1) and HV methylene carbon resonances (5HV in Figure 1).

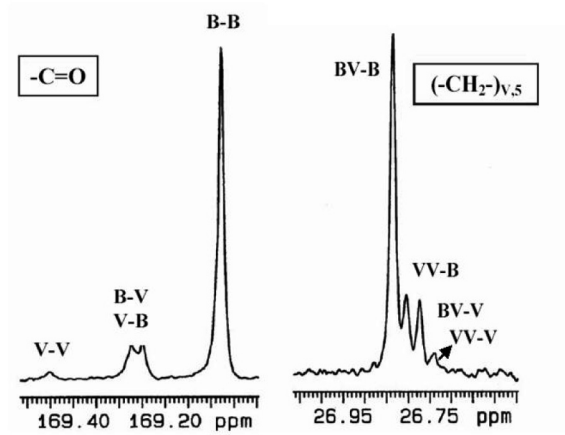

Figure 2. Splitting of carbonyl (4HB, 4HV) and methylene carbon resonances (5HV) in the ^{13}C NMR spectra of PHBV copolyesters.

These signals are split into several peaks due to the sensitivity of carbon nuclei to different sequences of HB and HV units (Figure 2). The carbonyl region showed four peaks, arising from different dyad sequences (VV, VB, BV, BB) of connecting HB and HV units. The triad sequences were determined from the resonance of the HV side-chain methylene carbon (5HV, Figure 1) composed of four peaks assigned to HV-centered triad sequences: VVV, BVV, VVB, and BVB. Relative intensities of ^{13}C resonances were determined from the peak areas. The compositional, dyad, and HV-centered triad mole fractions are collected in Table 1.

Based on the dyad and triad mole fractions we calculated the parameters *D* and *R*, respectively, to get information on the CCD. Calculated values are shown in Table 2. *D* values of 1.94 and 8.10, and *R* values of 0.93 and 0.58 were obtained for original non-fractionated PHBV-12.9 and PHBV-27.1 copolymers, respectively. Within the

Table 1. Mass fractions after fractionation (mol %) and experimental mole fractions of individual structural units (F_B, F_V), dyad and triad sequence distributions of HB and HV monomeric units for non-fractionated 12.9 and 27.1 mol % PHBV copolymers and their fractions (F1, F2, F3).[a]

PHBV	m %	F_B[b]	F_V[b]	F_{BB}	F_{BV}	F_{VB}	F_{VV}	F_{VVV}	F_{BVV}	F_{VVB}	F_{BVB}
12.9 %	100	0.871	0.129	0.771	0.101	0.102	0.026	0.006	0.019	0.020	0.084
F1-12.9	33.3	0.880	0.120	0.791	0.089	0.099	0.021	0.005	0.0175	0.0195	0.078
F2-12.9	33.3	0.870	0.130	0.769	0.101	0.106	0.024	0.006	0.019	0.021	0.084
F3-12.9	33.3	0.865	0.135	0.763	0.102	0.108	0.027	0.006	0.021	0.023	0.085
27.1 %	100	0.729	0.271	0.616	0.113	0.109	0.162	0.115	0.042	0.040	0.074
F1-27.1	20	0.751	0.249	0.628	0.123	0.106	0.143	0.091	0.0465	0.035	0.0765
F2-27.1	40	0.749	0.251	0.640	0.109	0.111	0.140	0.101	0.038	0.035	0.077
F3-27.1	40	0.708	0.292	0.596	0.112	0.113	0.179	0.130	0.044	0.044	0.074

[a] F_X, F_{XY}, F_{XYZ} indicate mole fractions of sequence X, XY, and XYZ, respectively, where X, Y, Z is V or B representing HV and HB comonomer units, respectively. [b] Determined from ^{1}H NMR spectra.

experimental uncertainty of the measured dyad and triad fractions (±3 mol %), the obtained *D* parameters were higher, and *R* parameters lower than 1, thus indicating that both non-fractionated PHBV samples exhibit a blocky sequence distribution. The deviation of *D* and *R* values from 1 was considerably higher for the PHBV-27.1 than for the PHBV-12.9 copolymer indicating that the former copolymer has a much more pronounced blocky character than the latter one.

Both PHBV copolyesters were fractionated according to molar mass into three fractions (F1, F2, and F3) using preparative SEC. Each fraction of PHBV-12.9 contained approximately 1/3 of the entire sample mass, whereas the fraction F1-27.1 of PHBV-27.1 contained approx. 20 wt %, and the fractions F2-27.1 and F3-27.1 about 40 wt % each, of the original sample. The original samples, as well as all collected fractions, were analyzed by SEC-MALS to obtain their molar mass averages (MMA) and molar mass distributions (MMD). The results are presented in Table 3. All samples gave chromatograms with a single peak. The MMA of the collected fractions of both PHBV samples decrease from fraction F1 to fraction F3, whereas the polydispersity indices (PDI = $\overline{M}_w/\overline{M}_n$) of the fractions of each PHBV sample were slightly lower compared to those of original non-fractionated samples.

Table 2. Parameters *D* and *R*, experimental number average sequence length of HV units (L_V^E), number average sequence length of randomly distributed HV units in copolymer (L_V^R) and ratio between the concentration of HB and HV units (*k*) for non-fractionated 12.9 and 27.1 mol % PHBV copolymers and their fractions (F1, F2, F3).

PHBV	D [a]	R [b]	L_V^E [c]	L_V^R [d]	k [e]
12.9 %	1.94	0.93	1.24	1.15	6.75
F1	1.89	0.93	1.23	1.14	7.33
F2	1.72	0.93	1.24	1.15	6.69
F3	1.87	0.93	1.25	1.16	6.41
27.1 %	8.10	0.58	2.38	1.37	2.69
F1	6.89	0.60	2.23	1.33	3.02
F2	7.41	0.59	2.24	1.34	2.98
F3	8.43	0.57	2.47	1.41	2.42[d]

[a] $D = F_{BB}F_{VV} / F_{BV}F_{VB}$,
[b] $R = L_B^R / L_B^E = L_V^R / L_V^E$
[c] $L_V^E = (F_{VVV} + F_{VVB} + F_{BVV} + F_{BVB}) / (F_{BVB} + F_{VVB})$
[d] $L_V^R = (k + 1)/k$
[e] k = [HB]/[HV]

For each collected fraction we determined the comonomer composition and sequence distribution as described above. The results are shown in Tables 1 and 2. The mole portions of individual structural units (F_B, F_V) in the fractions (F1, F2, F3) of PHBV-12.9 show only a slight increase in the content of HV units with decreasing molar mass (F_V: from 12.0 in F1-12.9 to 13.5 mol % in F3-12.9). A similar result is seen in the dyad and triad distribution: although differences in the values are not very large there is a gradual increase in dyads and triads that contain HV sequences. For example, in going from F1-12.9 to F3-12.9, F_{VV} increased from 0.021 to 0.027 while F_{BB} decreased from 0.791 to 0.763. The same trend is seen in the triad fractions. The calculated *D* and *R* parameters, as well as the number average HV sequence length, show very little (*D*, L_V^E) or no (*R*) differences between the fractions (Table 2). The value of the parameter *D* ranged from 1.72 to 1.89, however little meaning can be assigned to this relatively small change. The parameter *R* had a constant value of 0.93, which is the same as that of the original non-fractionated PHBV-12.9 sample.

Table 3. SEC-MALS results for non-fractionated 27.1 and 12.9 mol % PHBV copolymers and their fractions (F1, F2, F3).

PHBV	$\overline{M}_n \cdot 10^5$ g mol^{-1}	$\overline{M}_w \cdot 10^5$ g mol^{-1}	$\overline{M}_w/\overline{M}_n$
[a] 12.9 mol %	0.738	1.562	2.12
F1-12.9	0.992	1.899	1.91
F2-12.9	0.661	1.332	2.01
F3-12.9	0.561	1.178	2.10
[a] 27.1 mol %	0.454	1.635	3.60
F1-27.1	0.808	2.500	3.06
F2-27.1	0.671	1.960	2.92
F3-27.1	0.339	1.196	3.53

[a] Non-fractionated sample.

The same analysis of PHBV-27.1 copolymer fractions showed a more pronounced dependence of these parameters on molar mass. The content of HV comonomer units (F_V) changed from 24.9 in F1-27.1 to 29.2 mol % in F3-27.1. The altered composition is again reflected in the relative content of the dyad and triad fractions. For example, from F1-27.1 to F3-27.1 F_{VV} increased from 0.143 to 0.179 while F_{BB} decreased from 0.628 to 0.596. Among the triad fractions the change is most pronounced for F_{VVV} where the value changed from 0.091 in F1-27.1 to 0.130 in F3-27.1. Consequently, changes in the *D* and *R* parameters and number average sequence length are more pronounced. With decreasing molar mass *D* increased from 6.89 in F1-27.1 to 8.43 in F3-27.1, whereas *R* decreased from 0.60 in F1-27.1 to 0.57 in F3-27.1.

Since *D* and *R* values of PHBV-12.9 fractions showed no significant dependence on molar mass with all values comparable to the original sample, we conclude that this copolymer sample has a broad CCD or that it consists of a mixture of random PHBV copolymers with different compositions and similar molar mass characteristics.

On the other hand, PHBV-27.1 and its fractions showed much larger *D* and lower *R* values than those of PHBV-12.9 and its fractions, signifying a more "blocky" character of the copolymer. More importantly this sample exhibits a much more pronounced dependence of *D* and *R* values on the molar mass. In addition, the increase in HV content from F1-27.1 to F3-27.1 was larger (from 24.9 mol % to 29.2 mol %) and the molar mass distribution of

this sample was broader (PDI = 3.6) than in the case of PHBV-12.9 (HV content: 12.0 mol % in F1-12.9, 13.5 mol % in F3-12.9, PDI = 2.1). These results indicate that the PHBV-27.1 copolymer is most probably a mixture of several copolymers in which the HV content and the block character of the comonomer distribution increase more extensively with decreasing molar mass. This result is likely a consequence of the fact that in the case of PHBV copolymers of lower HV content the HV units are effectively "diluted" by the majority comonomer (HB), whereas in PHBV samples of higher HV content a higher level of blockiness can be supported by the higher HV content. The effect of the higher HV content on CCD can be most clearly seen in the values of dyad F_{VV} and triad F_{VVV} relative mole fractions: $F_{VV} = 0.026$ and $F_{VVV} = 0.006$ for PHBV-12.9 and $F_{VV} = 0.162$ and $F_{VVV} = 0.115$ for PHBV-27.1.

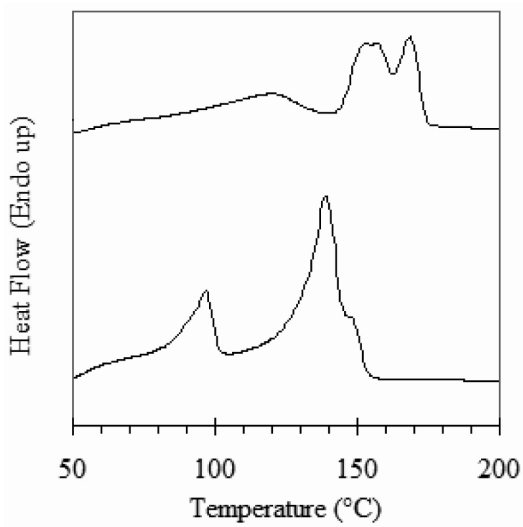

Figure 3. DSC melting curves of non-fractionated 12.9 mol % (top DSC trace) and 27.1 mol % PHBV copolymers (bottom DSC trace).

The influence of CCD and molar mass on the thermal properties of PHBV copolyesters was investigated by DSC measurements. The results are shown in Table 4. The DSC melting curves of both non-fractionated PHBV copolyesters showed complex multiple melting (Figure 3). Considering that the melting points of the mixture composed of random PHBV copolyesters of different chemical composition are the same as those of the individual copolyesters constituting the mixture before mixing,[7] the melting peaks in the DSC trace of the mixture can be directly assigned to constituting PHBV copolyesters. PHBV-12.9 copolymer showed three melting peaks at approximately 170, 150, and 121 °C. According to the results of Doi et al.,[7] they should correspond to copolymers with composition between few to approximately 20 mol % HV units. On the other hand, the DSC melting trace of PHBV-27.1 showed three melting peaks at approximately 100, 137, and 149 °C (Figure 3), corresponding to copolymer compositions of approximately 27, and between 11 and 13 mol % HV units.[7] Since the crystallization behavior of this PHBV copolyester indicated the presence of a significant amount of PHBV copolyesters with lower HV contents than the average composition determined by ^{1}H NMR it appears that the crystalline phase is composed primarily of chains with relatively low HV content, whereas chains with higher HV content remain in the amorphous phase. Due to this and the probability that at a heating rate of 50 °C min^{-1} PHBV copolyesters can still partially recrystallize during heating, leading to additional melting peaks as a consequence of crystal rearrangement, we conclude that melting curves of PHBV copolymers are not adequate to infer the copolymer CCD.

Table 4. Melting temperatures (Tmx) of non-fractionated 12.9 and 27.1 mol % PHBV copolymers and their fractions (F1, F2, F3).

PHBV	T_{m1} °C	T_{m2} °C	T_{m3} °C
[a] 2.9 mol %	169.6	149.6	121.2
F1-12.9	169.8	151.4	123.1
F2-12.9	168.9	146.2	120.9
F3-12.9	167.0	143.3	120.0
[a] 27.1 mol %	148.7	136.6	100.4
F1-27.1	149.3	139.2	101.3
F2-27.1	148.4	136.2	100.1
F3-27.1	147.5	136.0	100.1

[a] Non-fractionated sample.

Similar to non-fractionated PHBV samples, the fractions of both PHBV samples also showed complex melting. The number of peaks in DSC thermograms of the fractions was the same as in the DSC traces of the original samples, only the intensity of individual peaks was found to be somewhat different and T_{mx} values at the apex of the peaks decreased slightly with decreasing molar mass (Table 4). From these results it is clear that fractionation according to molar mass was not sufficiently selective to observe significant effects on T_m values.

A number of authors have fractionated PHA copolyesters by the solvent/non-solvent precipitation technique into several fractions with a narrower CCD compared to the original non-fractionated sample.[15–23] It is generally accepted that such fractionation is caused by the different solubility of the copolymers with different comonomer ratios and that the copolymer molar mass has negligible effect on the fractionation. In this work we show that CCD of PHBV copolyesters can change with molar mass, while the effect of CCD on the copolyester hydrodynamic volume, according to which the macromolecules are separated in SEC, was neglected. In fact, copolymer fractionation by solubility in the precipitation technique or by hydrodynamic volume in SEC depends not solely on comonomer composition and molar mass, respectively, but on both of these parameters. Therefore, for

unambiguous characterization of PHA copolymers we will need to employ a two-dimensional separation system in which we will be able to separate copolymer macromolecules according to their composition and molar mass independently. Our results will likely need to be confirmed with investigations of other high HV content PHBV, also taking into account that the choice of microorganisms producing PHBV and fermentation conditions (such as feed composition and feeding rate) could easily affect CCD. Only on the basis of more results it will be possible to evaluate the general significance of CCD variation with molar mass.

4. Conclusions

Fractionation of the non-random PHBV copolyester with a high HV unit content according to molar mass showed that with decreasing molar mass both the content of HV comonomer units as well as the block character of the copolymer increase. On the other hand, the non-random PHBV sample with a low HV unit content showed neither a change in composition nor in composition distribution with molar mass. The results of the effect of microstructure on copolyester melting behavior indicate that PHBV samples with complex composition distribution showed multiple melting peaks in their DSC curves.

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Povzetek

Distribucija sestave komonomerov (CCD) v naravnih alifatskih polihidroksialkanoatnih kopoliestrih ima velik vpliv na morfologijo in s tem na lastnosti teh kopolimerov. Z uporabo ^{13}C NMR spektroskopije na osnovi diadne in triadne analize smo ugotovili odvisnost sestave in CCD od molske mase v dveh nenaključnih poli(3-hidroksibutirat-*ko*-3-hidroksivalerat)ih (PHBV) z različnimi vsebnostmi komonomerov. V ta nemen smo kopoliestre frakcionirali s preparativno izključitveno kromatografijo in analizirali sestavo, CCD in termične lastnosti frakcij. PHBV kopoliester z višjo vsebnostjo hidroksivaleratnih komonomernih enot (HV, 27.1 %) je pokazal večjo odvisnost sestave in CCD od molske mase kot PHBV z nižjo vsebnostjo HV enot (12.9 %), pri katerem je bila odvisnost majhna. Oba PHBV kopoliestra in njihove frakcije so imeli v DSC termogramih več tališč.