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Sequence Distribution and Fragmentation Studies of Bacterial Copolyester Macromolecules: Characterization of PHBV Macroinitiator by Electrospray Ion-Trap Multistage Mass Spectrometry

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ABSTRACT: Sequence distribution and chemical structure of mass-selected macromolecules of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), PHBV, biopolyester macroinitiator obtained by partial alkaline depolymerization of natural PHBV containing 5 mol % of HV units was determined by electrospray ionization multistage mass spectrometry (ESI-MS^n). On the basis of the ESI-MS^n studies of PHBV individual oligomers, the microstructure of this bacterial copolyester was assessed, starting from dimer up to the oligomer containing 22 repeat units, and the results obtained were compared with those described previously for other "soft" ionization MS techniques.

Introduction

Among environmentally friendly polymeric materials the bacterial origin poly(hydroxyalkanoate)s (PHA), which are high-molecular-weight polyesters produced by a wide range of microorganisms as intracellular carbon and energy reserve materials, constitute an interesting group of polymers due to their susceptibility to hydrolytic and enzymatic biodegradation.[1] One of the key members of these materials is the copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) produced under the trade name Biopol. The microstructure of high-molecular-weight bacterial PHBV has been determined by ^{13}C NMR based on diad and triad analysis.[2] The significant information on PHBV copolymer microstructure was provided by mass spectrometry. For this purpose the theoretical mass spectra of PHBV copolymers containing various compositions of HB and HV units were generated and compared with those experimentally acquired with the aid of such "soft" MS ionization techniques as matrix-assisted laser desorption−ionization (MALDI),[3] fast atom bombardment (FAB),[4] and desorption−chemical ionization (DCI).[5] It was found that intensities in FAB and DCI mass spectra of PHBV with various compositions of HB and HV units reflect the relative amounts of oligomers produced in limited degradation of these copolymers via partial methanolysis or pyrolysis.[6,7] On the basis of this assumption, the Bernoullian sequence distribution of PHBV copolymers was demonstrated up to the hexamer level with the aid of FAB-MS,[4] up to the nonamer level with the aid of negative-ion DCI-MS[5] and on the level of hexamer up to hexadecamer by MALDI-TOF in the linear mode.[3]

Structural studies of polyester resins by elecrospray mass spectrometry (ESI-MS) as well as comparison of ESI-MS with MALDI-TOF and GPC for their characterization have been reported recently.[8,9] The electrospray ionization multistage mass spectrometry (ESI-MS^n) has been successfully applied for verification of chemical homogeneity and the end group analysis of synthetic poly[(*R*,*S*)-3-hydroxybutyrate], a-PHB, as well as the oligomeric products of its biodegradation.[10−14] The implementation of multistage mass spectrometry significantly improves the MS analysis of the detailed structure of individual chains of a-PHB bearing various end groups including bioactive ones,[12] thus demonstrating the utility of this technique in the studies of the well-defined homopolyester macromolecules. However, to the best of our knowledge, the characterization of PHA copolymers with the aid of ESI-MS^n technique has not been described until now.

Complex polymeric materials such as block or graft copolymers may be synthesized by means of macrointermediates suitable for further specific chemical reactions.[15] Monofunctional macroinitiators can be derived from natural PHA using the recently reported by Doi controlled depolymerization of natural PHB via partial saponification catalyzed by KOH/18-crown-6 in the $CHCl_3/H_2O$ system.[14,16]

In this report we present for the first time the results of evaluation of the subtle structure of macroinitiator, obtained by the described above partial depolymerization of natural PHBV containing 5 mol % of HV units, thus showing utility of the ESI-MS^n technique for analysis of a sequence distribution of HB and HV repeat units as well as the chemical structure of the individual

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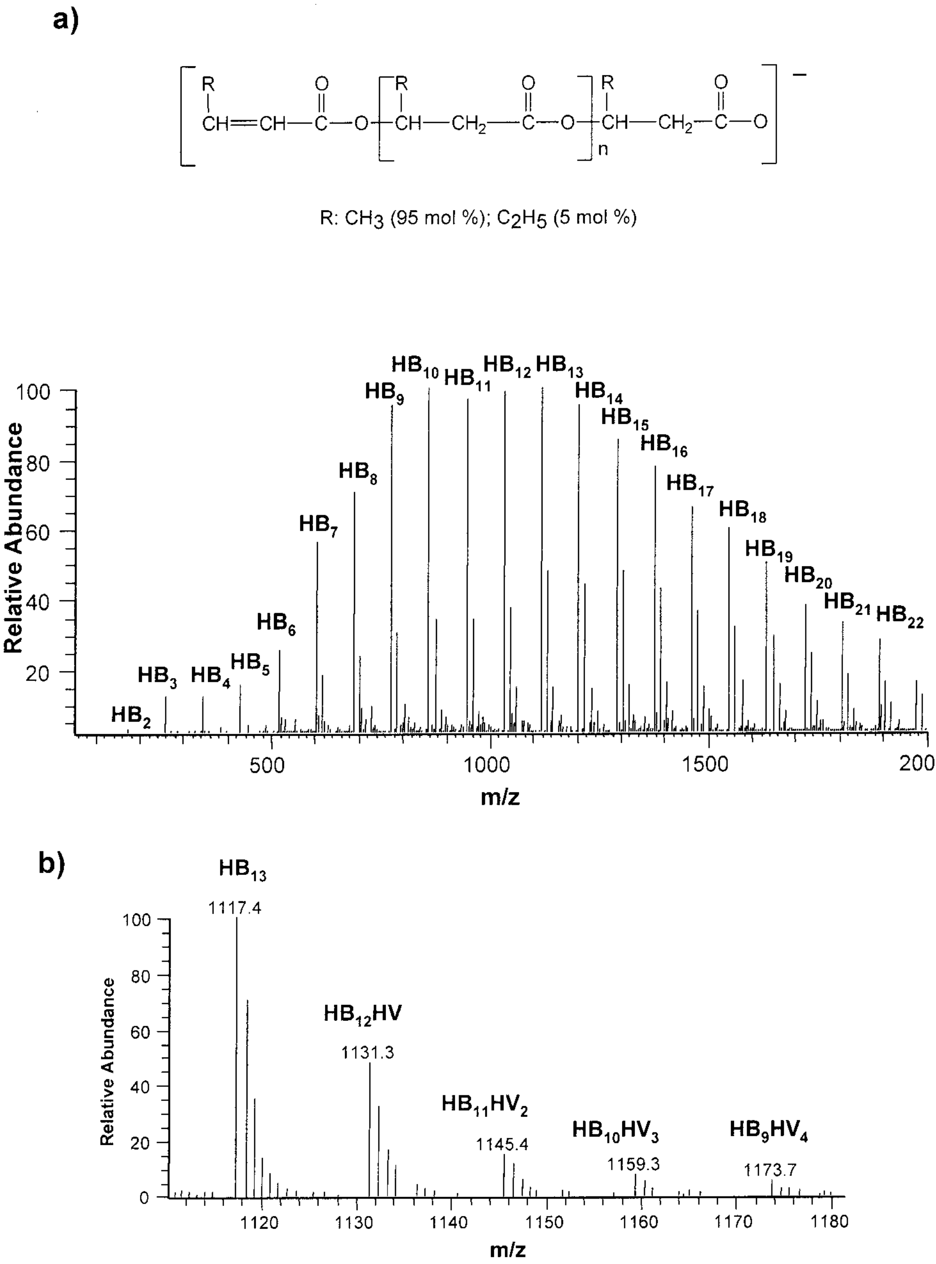


Figure 1. (a) ESI-MS spectrum (negative ion mode) of PHBV macroinitaitor. (b) Expanded ESI-MS spectrum of PHBV 13-mer oligomers containing various amounts of HV units.

mass-selected copolyester macromolecules including their end groups.

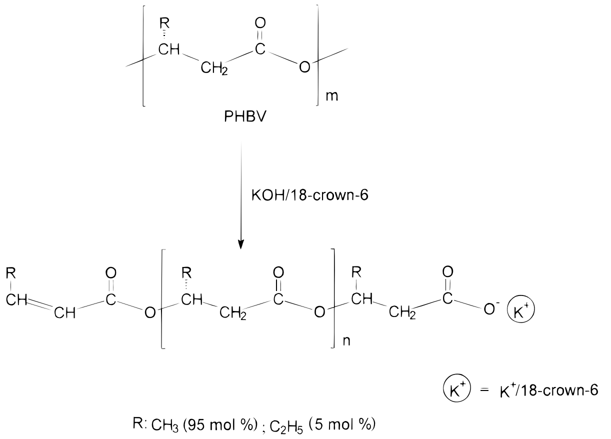
Experimental Section

Materials. The bacterial poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), PHBV (number-average molecular weight M_n = 125 000 and polydispersity $M_w/M_n = 3.7$), was supplied by Aldrich. The sample of commercial PHBV was purified by reprecipitation in hexane from chloroform solution and dried under vacuum at room temperature. The composition of natural PHBV estimated by ^1H NMR was equal to 95% of HB units and determined by GC-MS analysis of the corresponding methyl esters formed after acidic methanolysis of this copolymer, carried out as described in ref 17, was equal to 94.8 mol % of methyl 3-hydroxybutyrate and 5.2 mol % of methyl 3-hydroxyvalerate.

PHBV Macroinitiator. PHBV macroinitiator was obtained via partial saponification of bacterial PHBV (dissolved in chloroform) carried out at the temperature of 35 °C in the presence of 5 N aqueous solution of KOH containing 18-crown-6 complexing agent.[14] The macroinitiator selected for ESI-MS^n studies was additionally characterized by GPC as well as ^1H and ^{39}K NMR spectrometry. The estimated number-average molecular weight of macroinitiator was equal to $M_n = 1300$ and its polydispersity to $M_w/M_n = 1.7$. The ^1H NMR analysis revealed the presence of characteristic signals corresponding to the protons of HB (95 mol %) and HV (5 mol %) repeating units and also the signals attributed to the crotonate end group[18] as well as the singlet of the protons of 18-crown-6. In the ^{39}K NMR spectrum the presence of characteristic broad singlet at $\delta = 20$ ppm, corresponding to the crowned K^+, was observed.[18]

GPC Analysis. Number-average molecular weight and polydispersity index M_w/M_n of commercial PHBV were estimated by GPC experiments conducted in THF solution at 35 °C, at a flow rate of 1 mL/min using a Spectra-Physics 8800 solvent delivery system with two Mixed C Styragel columns in series and a Shodex SE 61 refractive index detector. A volume of 10 μL of sample solutions in $CHCl_3$ (concentration 2% w/v) was injected. Polystyrene standards with low polydispersity were used to generate a calibration curve. Number-average molecular weight and polydispersity index (M_w/M_n)

Scheme 1



of PHBV macroinitiator were estimated by GPC experiments carried out under the same conditions with the aid of a PLgel 3 μm MIXED-E ultrahigh-efficiency column.

NMR Analysis. The NMR spectra were recorded using a Varian VCR-300 multinuclear magnetic resonance spectrometer. The ^{1}H NMR spectra were run in $CDCl_3$ using tetramethylsilane (TMS) as an internal standard. The ^{39}K NMR spectra were taken in $CDCl_3$, chemical shifts being referenced to an aqueous solution of potassium fluoride used as an external standard.

ESI-MS^n Experiments. Electrospray mass spectrometry analysis was performed using a Finnigan LCQ ion trap mass spectrometer (Finnigan, San Jose, CA). The sample of PHBV macroinitiator was dissolved in the chloroform−methanol system (10:1 v/v), and the solution was introduced to the ESI source by continuous infusion by means of the instrument syringe pump with the rate at 3 μL/min. The ESI source was operated at 4.25 kV, and the capillary heater was set to 200 °C. For ESI-MS^n experiments mass-selected mono-isotopic parent ions were isolated in the trap and collisionally activated with 33% ejection radio-frequency amplitude at standard He pressure. The experiments were performed in the negative-ion mode.

Results and Discussions

Controlled depolymerization of natural PHBV catalyzed by KOH/18-crown-6 complex, due to the partial saponification of ester linkages and elimination reaction,[19] leads to the formation of macroinitiator containing carboxylic and olefinic end groups as revealed by NMR and ESI-MS analysis performed in negative-ion mode (Scheme 1, Figure 1a).

The ESI-MS spectrum of PHBV macroinitiator consists of the clusters of singly charged negative ions of the general structure shown in Figure 1 that are derived from PHBV after loss of the potassiated crown ether moiety. The anions were MS separated due to their different degrees of oligomerization (from dimer up to 22-mer) and composition. The mass difference between the *m*/*z* values of molecular ions in each cluster is equal to 14 Da, which corresponds to the difference between molecular mass of individual HV and HB units of macroinitiator (Figure 1b).

The most abundant anion in this spectrum (located at *m*/*z* 1117.4; Figure 1a) was assigned to the PHB 13-mer $[CH(CH_3){=}CHC(O)O[CH(CH_3)CH_2C(O)O]_{11}CH(CH_3)CH_2C(O)O]^-$ (1117.17 Da). The MS^2 spectrum of this parent anion (Figure 2) shows fragmentation at the carboxylic side of PHB.[12] The set of fragment anions at *m*/*z* 1031, 945, 859, 773, 687, 601, 515, 429, and 343 is formed due to successive loss of crotonic acid (Scheme 2). The same mechanism was observed in further fragmentation experiments performed for selected fragment anions. The anion *m*/*z* 601 in MS^3 experiment forms the dimer *m*/*z* 171, which in MS^4 experiment forms finally the crotonic acid anion (Figure 2).

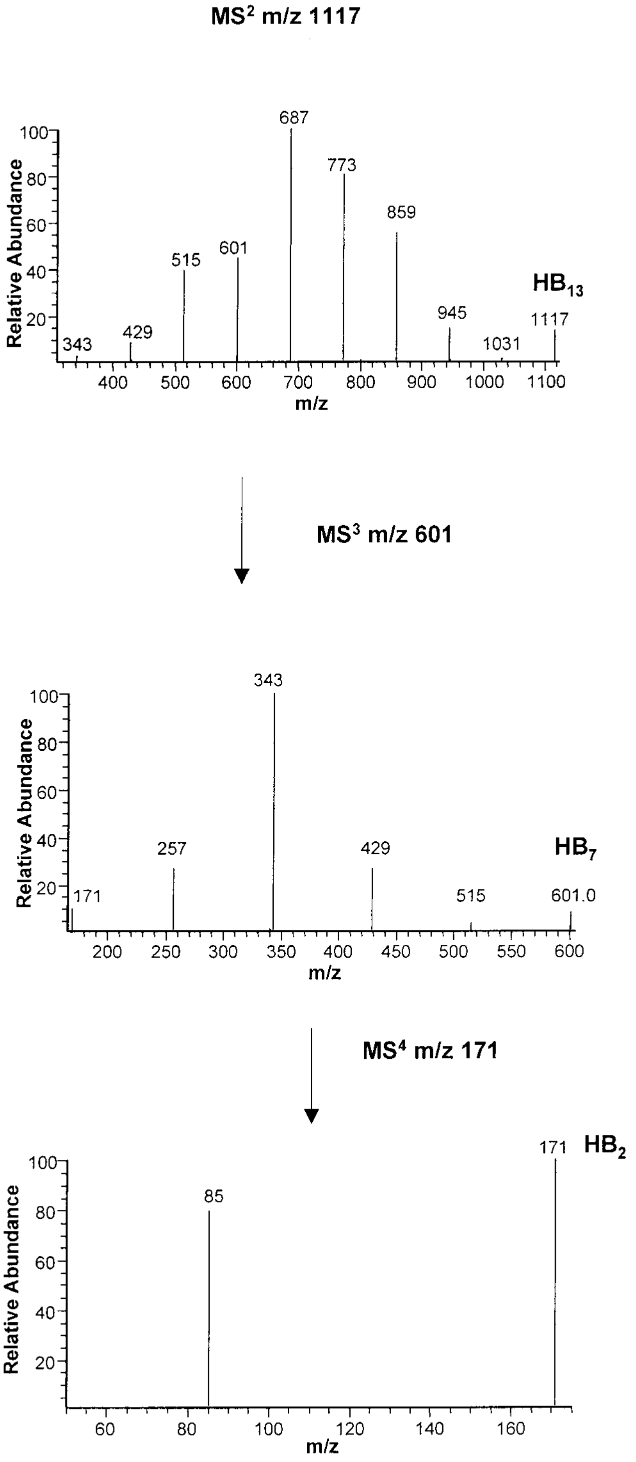


Figure 2. Sequential fragmentation spectrum MS^n (negative ion mode) obtained for selected PHB 13-mer parent ion *m*/*z* 1117.

The intensities of the detected anions of PHBV of various HV composition (Figure 1) have been used for estimation of the sequence distribution of the copolymer,[5] and the estimated values have been compared with the theoretical ones calculated for random copoly-

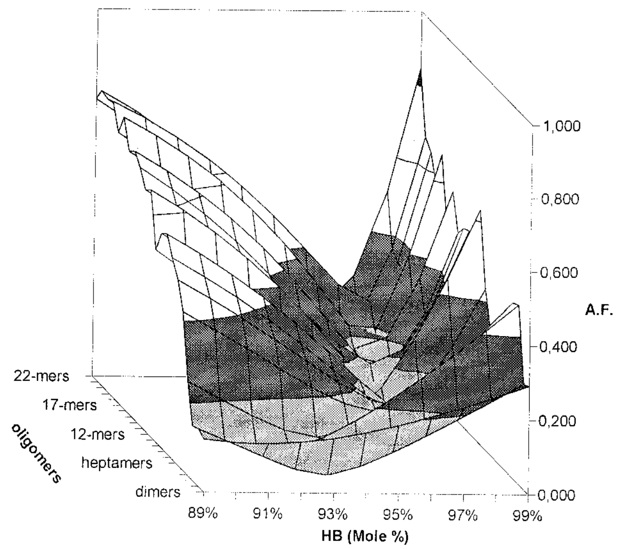
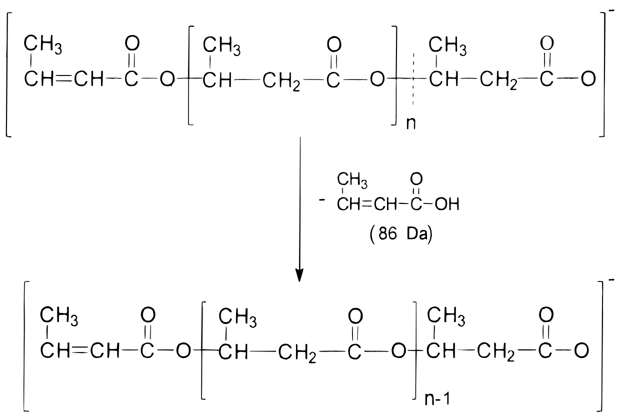


Figure 3. Agreement factor (AF) versus HB content corresponding to individual PHBV oligomers.

Scheme 2



mers of similar compositions (in the region of 4 mol % up to 6 mol % of HV units) according to the formula based on Bernoullian statistics:

$$P_{x,y} = \binom{x+y}{y} P_{\mathrm{B}}^{x}\, P_{\mathrm{V}}^{y}$$

where P_B and P_V are the molar fractions of HB and HV in the copolymer.

The differences between observed and calculated values have been expressed in terms of error by means of the Hamilton agreement factor (AF):[4]

$$\mathrm{AF} = [\textstyle\sum (I_{\mathrm{exp,i}} - I_{\mathrm{calcd,i}})^2 / \sum I_{\mathrm{exp,i}}{}^2]^{1/2}$$

where $I_{exp,i}$ and $I_{calcd,i}$ are the normalized experimental and calculated abundance of partial depolymerization products. For details of the results concerned with the ESI-MS experimental and calculated relative amounts of HB and HV units in PHBV macroinitiator oligomers, see the Supporting Information.

The agreement between the estimated and calculated values was a function of the P_B/P_V ratio. The best fit of the composition value estimated from the results of ESI-MS analysis was found for the composition ratio around 95/5 (HB/HV) calculated for the random copolymer (Figure 3). This result is in good agreement with that of NMR and GC-MS analysis of natural PHBV copolymer presented in the Experimental Section.

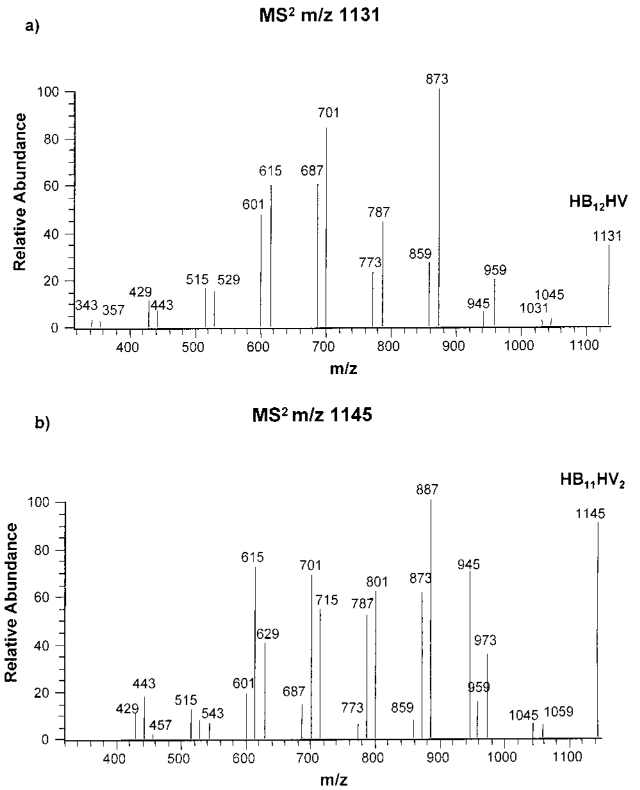


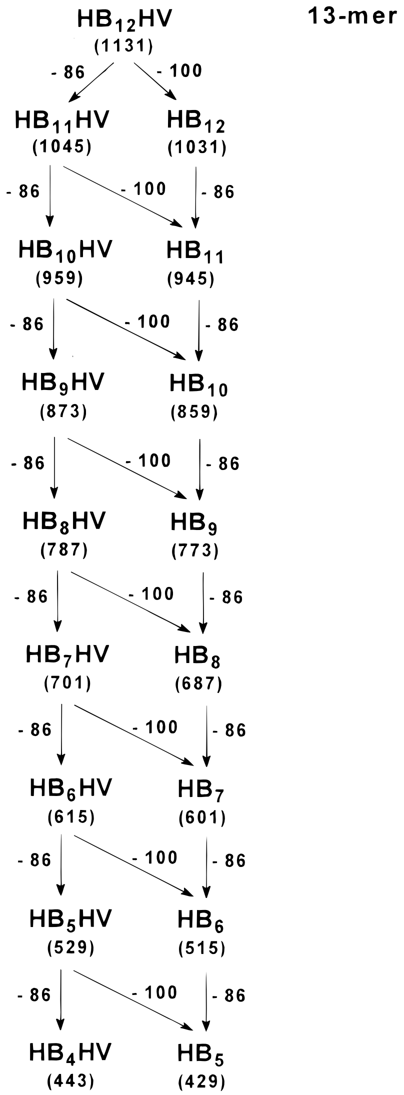
Figure 4. MS² fragmentation spectra (negative ion mode) obtained for (a) selected PHBV parent ion *m/z* 1131 and (b) selected PHBV parent ion *m/z* 1145.

The random composition of comonomer units in the copolymer studied has been visualized for the first time by the fragmentation experiments. The results of MS² fragmentation studies of the parent anions selected from the 13-mer cluster depicted in Figure 1b and contained in the macromolecular chain one HV unit (*m/z* 1131.3; $HB_{12}HV$) and two HV units (*m/z* 1145.4; $HB_{11}HV_2$) are presented in Figure 4.

The MS² fragmentation experiment performed for selected parent anion of $HB_{12}HV$ (*m/z* 1131, Figure 4a) induces the set of clusters containing two fragment anions, with the difference of 14 Da. The fragment anion at *m/z* 1045 corresponds to $HB_{11}HV$ oligomer, formed due to the loss of crotonic acid (86 Da), and the fragment ion at *m/z* 1031 corresponds to HB_{12} oligomer formed by the expulsion of 2-pentenoic acid (100 Da) from the carboxylic side of the $HB_{12}HV$ parent anion; the former one was observed also in the MS² spectrum of HB_{13} parent ion (Figure 2). The fragment ion *m/z* 959 ($HB_{10}HV$) is formed due to expulsion of crotonic acid from fragment anion *m/z* 1045 ($HB_{11}HV$). However, the fragment anion *m/z* 945 (HB_{11}) may by formed from fragment anion *m/z* 1031 (HB_{12}) due to the loss of crotonic acid or from the fragment ion *m/z* 1045 ($HB_{11}HV$) by the expulsion of 2-pentenoic acid. Thus, the following fragmentation pathway may illustrate the result of MS² experiment of parent ion *m/z* 1131 ($HB_{12}HV$) (Scheme 3).

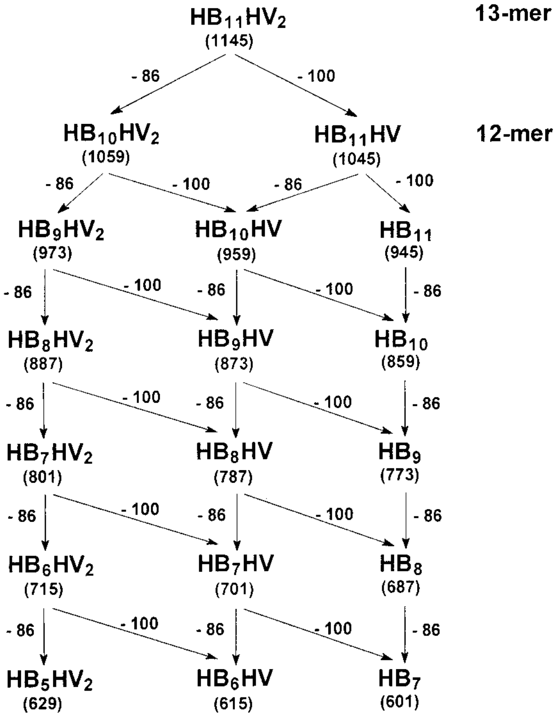
Such a fragmentation pathway indicates clearly that the parent anion *m/z* 1131 (Figure 4a) corresponds to the mixture of macromolecules of the general formula $HB_{12}HV$ with the random location of one HV unit along their chains, as revealed by the MS² experiment.

Scheme 3



The results presented in Figure 4b of the MS^2 fragmentation experiment of the parent anion *m/z* 1145 ($HB_{11}HV_2$) indicate that this parent ion corresponds to the mixture of macromolecules containing two randomly positioned HV units along their chains. According to the fragmentation pathway presented in Scheme 4, at the first stage two fragment ions are formed: $HB_{10}HV_2$ and $HB_{11}HV$ (Figure 4a; *m/z* 1059 and 1045). Then, the set of clusters containing three fragment ions of the same degree of oligomerization but different content of HV units are produced due to the successive loss of crotonic or 2-pentenoic acid. In particular, in the cluster of fragment ions corresponding to heptamers (Figure 4b; *m/z* 629, 615, and 601) the anion *m/z* 629 (HB_5HV_2) is formed from the fragment anion *m/z* 715 (HB_6HV_2) due to the loss of crotonic acid, and due to the loss of 2-pentenoic acid from the fragment ion *m/z* 715, the fragment anion *m/z* 615 (HB_6HV) is formed. This fragment anion may be also formed from the fragment anion *m/z* 701 (HB_7HV) by the expulsion of crotonic acid. The last in the heptamers cluster fragment ion *m/z* 601 (HB_7) may by formed from the fragment ion *m/z* 701 (HB_7HV) by the expulsion of 2-pentenoic acid or from the fragment anion *m/z* 687 (HB_8) due to the loss of crotonic acid.

Scheme 4



Conclusions

The results of the present study indicate that as a complement to the previously reported “soft” ionization MS techniques used for the studies of PHA copolymers, the ESI-MS technique enables determination of sequence distribution of comonomeric units in the natural PHBV copolyester starting from dimer up to the oligomer containing 22 repeat units. Moreover, the random distribution of comonomers in the mass-selected PHBV macromolecules can be shown by the ESI-MS^n fragmentation experiments performed in negative-ion mode, thus demonstrating the utility of this technique for the analysis of individual macromolecules of the macroinitiator obtained thereof.

The characterization of “tailored” random-block copolymers prepared with the aid of this well-defined macroinitiator as well as their biodegradation will be published soon.

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Supporting Information Available: Table containing the ESI-MS experimental and calculated relative amounts of HB and HV units in PHBV macroinitiator oligomers. This material is available free of charge via the Internet at http://pubs.acs.org.

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