Aliphatic Polyesters for Advanced Technologies – Structural Characterization of Biopolyesters with the Aid of Mass Spectrometry

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Summary: Application of the ESI- mass spectrometry technique for the structural studies of biopoliesters is reported. The structural studies were performed on two selected commercially available biopolyesters i.e. poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) and poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBH), contained up to 87% of PHB repeat units, with the aid of ESI-MS analysis of the respective low molar mass oligomers. The molecular architecture of individual macromolecules, including the chemical structure of the end groups, composition and sequence distribution were determined based on mass-resolved signals of ESI-mass spectra of PHBH and PHBV oligomers. The general utility of MS characterization of biopoliester macromolecules at the molecular level has been demonstrated and supported by NMR analysis.

Keywords: biopolymers; ESI-MS analysis; mass spectrometry; PHBH; PHBV

Introduction

Polyhydroxyalkanoates (PHAs) are thermoplastic aliphatic polyesters produced by microorganisms as energy storage materials. They represent an interesting group of biodegradable polymers produced from renewable resources which have functional properties that are quite suitable for commercial applications, particularly in the fields of health, environment, food, agriculture and agro-industries as environmentally friendly materials.[1,2] PHAs have attracted both research and industrial attentions because of their inherent properties of biodegradability and biocompatibility.[3–7]

Poly(3-hydroxybutyrate) (PHB) was a first isolated biopolyester unfortunately of high crystallinity and thus limited in its commercial application. In order to improve the physical properties of biopolyesters (PHA) considerable efforts have been directed to the production of PHAs with lower melting point, lower crystallinity, and high ductility by incorporation into their polymer chains the repeat units containing longer alkyl chains both as a side group as well as the constituents of the main chain. Incorporation of repeat units containing long alkyl side chain, like 3-hydroxyvalerate 3-hydroxyhexanoate and 3-hydroxyoctanoate leads to considerable change of bulk and solution properties of PHA.[8,9]

The fundamental knowledge of the structure-properties relationship of biodegradable materials constitutes the key to their successful exploitation. Therefore, the applications of biodegradable (co)polyesters with defined molecular architecture and properties requires unambiguous information about their structure.

The physical properties and microstructure of high-molecular-weight bacterial PHAs has been determined by FT-IR and NMR spectroscopy.[10–14] The microstructure of high-molecular-weight PHBV biopolyester has been determined by ^{13}C NMR

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based on resolved dyads or triads analysis.[15,16] However, for more advanced studies, the correct assignment of the ^{13}C NMR resonance signals, correlated with NMR theory, is required. In order to solve this problem two-dimensional NMR experiments are performed together with time-consuming syntheses of model compounds.

Mass spectrometry of polymers is a rapidly growing field recently. Matrix assisted laser desorption ionization, (MALDI), and electrospray ionisation, (ESI), are suitable techniques for the ionisation of biomacromolecules because they permit the formation of ions from such macromolecules, with little or no fragmentation during ionisation process.[17]

The present work demonstrates utility of ESI mass spectrometry for the structural studies of selected biopolyesters i.e. poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) and poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBH) produced in the commercial scale.

Materials and Methods

Materials

The PHBH biopolyester with average HH content of 13 mol % kindly supplied by P&G USA. The PHBV with 13.0 mol% of HV units was provided by the Institute of Biotechnology and Bioprocess Engineering, Graz University of Technology (Graz, Austria). Biopolyesters were purified by reprecipitation in hexane from chloroform solution and dried under vacuum at room temperature.

ESI-MS^n Characterization

Preparation of Oligomers

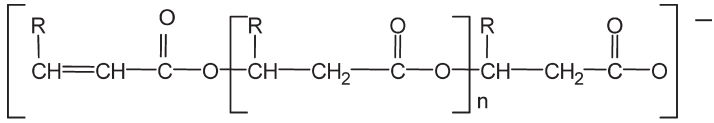
The PHAs oligomers were obtained *via* partial degradation of the original PHAs biopolyesters dissolved in chloroform. The reactions were performed in the presence of a 0.375 molL^{-1} aqueous solution of tert-butylammonium hydroxide or 5N aqueous solution of KOH containing 18-crown-6 complexing agent at 35 °C. The chloroform fractions containing low molar mass oligomers were protonated using the acid ion-exchange resin Dowex. The obtained oligomer samples were analyzed by SEC, ^1H NMR, and ESI-MS^n spectrometry

ESI-MS^n Experiments

Electrospray mass spectrometry analyses were performed using an LCQ ion trap mass spectrometer (Finnigan, San Jose, CA, USA). PHBV samples were dissolved in chloroform/methanol mixture (10/1; v/v). The solution was introduced to the ESI source by continuous infusion by means of the instrument syringe pump at a rate of 3 μLmin^{-1}. The LCQ 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 with an isolation width of 1 m/z and activated by collision with a 30–35% ejection RF–amplitude at standard He pressure. The experiments were performed in negative-ion mode.

Results and Discussion

In the MS spectra of copolymers, the relative intensities of the mass peaks



Scheme 1.

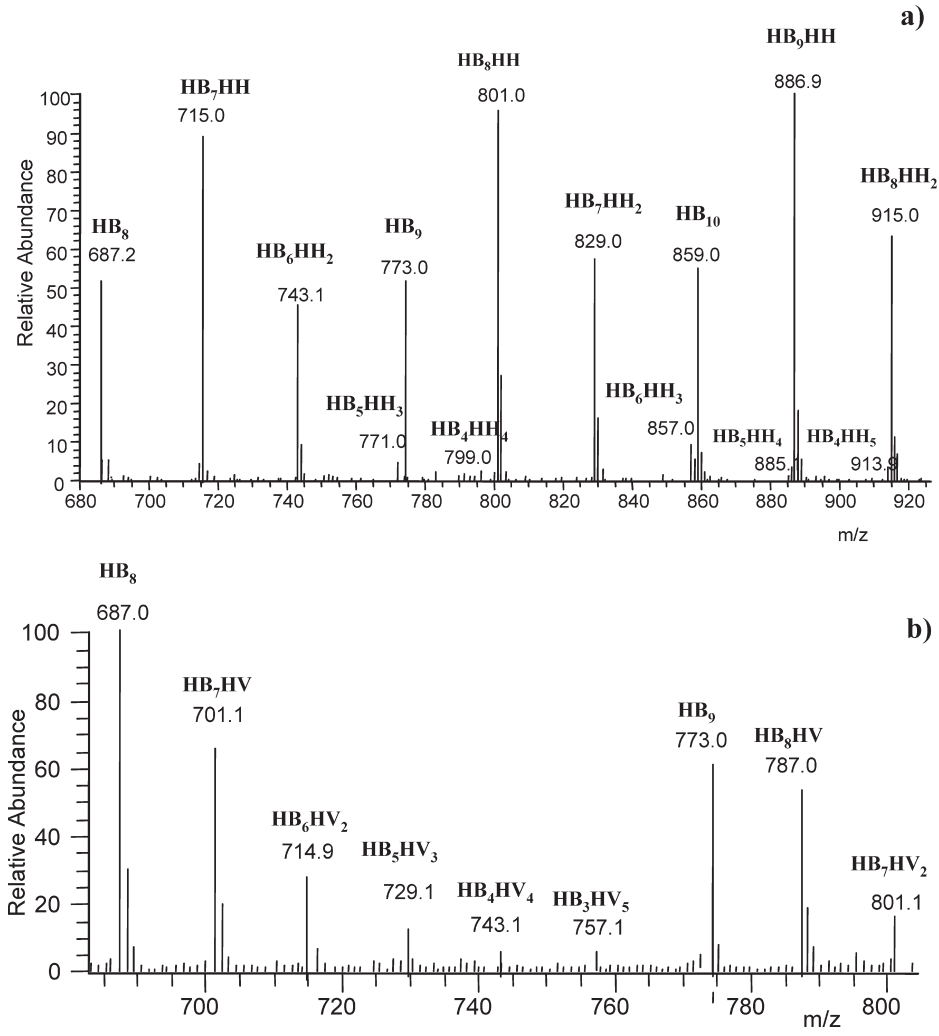


Figure 1.
Expanded ESI-MS spectrum (negative-ion mode) of PHAs oligomers: **a)** PHBH oligomers (13 mol% of HH units) in the mass range m/z 680–920 **b)** PHBV oligomers (13 mol% of HV units) in the mass range m/z 660–820.

appearing at the spectrum are dependent on composition and the type of distribution of comonomer units in the polymer chains. In order to decode the relative intensities of the peaks related to the individual copolymer molecules and to determine their composition and sequence distribution the theoretical mass spectra of copolymers are generated (based on the Bernoullian or first- or second-order Markovian chain statistic) and compared with those experimentally acquired with the aid of "soft" MS ionization techniques. This method has been successfully applied for the characterization of several copolymer systems including microbial and synthetic aliphatic copolyesters. [17–19]

The drawback of MS characterization of high molar mass (co)polymers is that with increasing mass the mass resolution decreases, while the number of potential isobaric structures increases. Further-

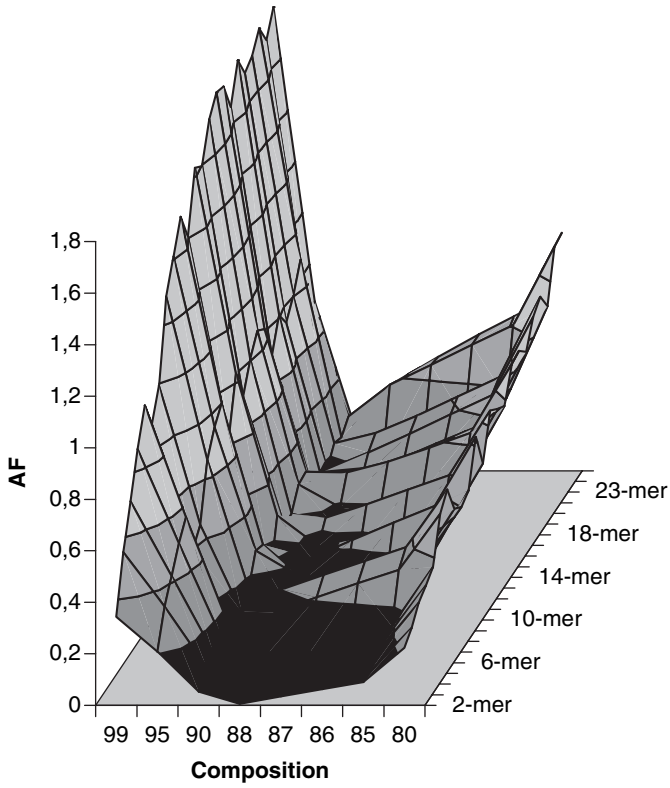


Figure 2.
AF factor as a function of PHBH biopolyester composition.

more, many MS instruments (such as those equipped with a quadrupole mass analyzer, a magnetic sector, a cyclotron cell, or an ion trap) are limited in the range of mass they can detect. In order to deal with these limitations of MS instruments, the original high molar mass copolymer is usually subjected to partial degradation to obtain lower molar mass analogues possessing the same composition and sequence distribution as the original one. Several methods of partial degradation have been evaluated for different copolymers including partial thermal degradation, hydrolysis, methanolysis, aminolysis, photolysis, ozonolysis, and methoxidation.[17] However, in the case of partial copolymer degradation the analysis needs an additional step in which the sequence of the starting copolymer is reconstructed from the data obtained on the partially degraded sample.

In the present work detailed structural characterization of two selected commercially available biopolyesters like as PHBV and PHBH was performed based on the ESI-MS analysis of the oligomers obtained thereof. The PHBH ($Mn_{GPC} = 900$) and PHBV ($Mn_{GPC} = 700$) oligomers were prepared by controlled, partial, alkaline depolymerization of PHBH and PHBV biopolyesters, catalyzed by KOH/18-crown-6 complex or tetrabutylammonium hydroxide, according to the procedure described in experimental section.

The ^{1}H NMR analysis revealed that oligomers obtained containing carboxylic and olefinic end groups and possess the same composition as that of the starting biopolyesters e.g. 13 mol% of HH units and

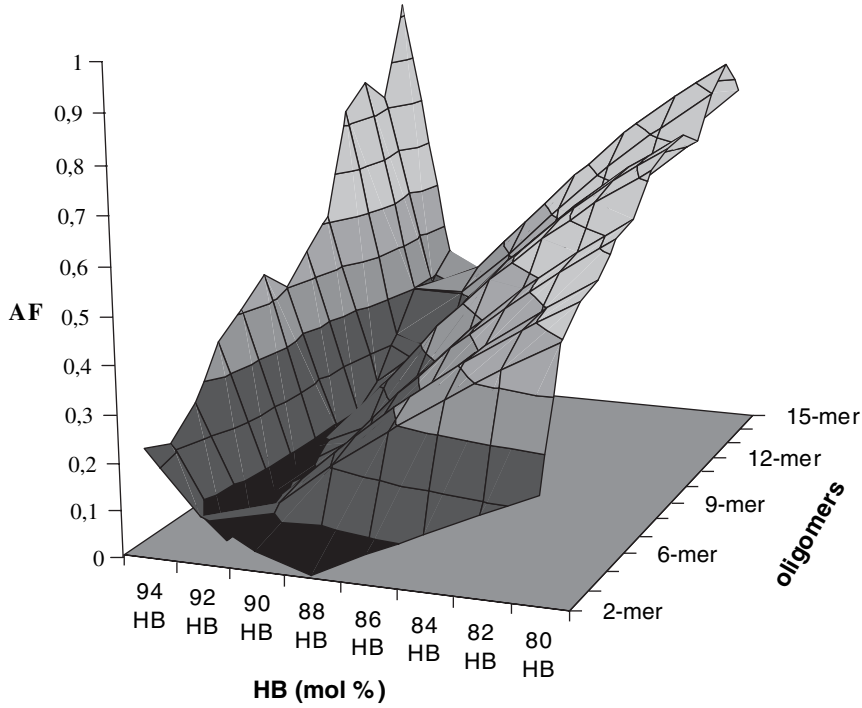


Figure 3.
AF factor as a function of PHBV biopolyester composition.

13 mol% of HV units in the case of PHBH and PHBV copolyesters, respectively.

The negative ESI-mass spectra of PHBH and PHBV oligomers show distribution of singly charged negative ions. The anions observed in the mass spectra correspond to the individual oligomer chains composed of HB and HH (PHBH) or HB and HV (PHBV) units and terminated by unsaturated and carboxylic end groups of the general structure shown in Scheme 1.

The ions in mass spectra were grouped into numerous clusters due to their different degree of oligomerization and composition. The mass spacing between molecular ions in each cluster corresponds to the difference in molar masses of individual HH and HB or HV and HB units and are equal 28 Da and 14 Da, in the case of PHBH and PHBV oligomers, respectively.

The ESI mass spectra of PHA oligomers allowed the assignment of each peak to a specific oligomer, and therefore the identification of the structure of the copolymer and the end groups of the individual chains. Figures 1a) and b) show as example the expanded region of negative ESI-mass spectra of the PHBH and PHBV oligomers together with the chemical assignment of the peaks. These mass range consisted of the signals corresponding to the oligomer chains of the 8-mer and 9-mer cluster.

The mass peaks intensities relative to the individual of PHBH and PHBV co-oligomers were used for determination composition and sequence distribution of these copolyester. The experimental intensities have been compared with the theoretical ones calculated for random copolymers of similar compositions according to the formula based on Bernoullian statistic.[17,18,20] The differences between observed and calculated distributions was a function of the P_{HB}/P_{HH} or P_{HB}/P_{HV} ratio (where P_{HB}, P_{HH} and P_{HV} are the molar fractions of HB, HH and HV in the copolymer), and were expressed by means of the Hamilton agreement factor (AF).

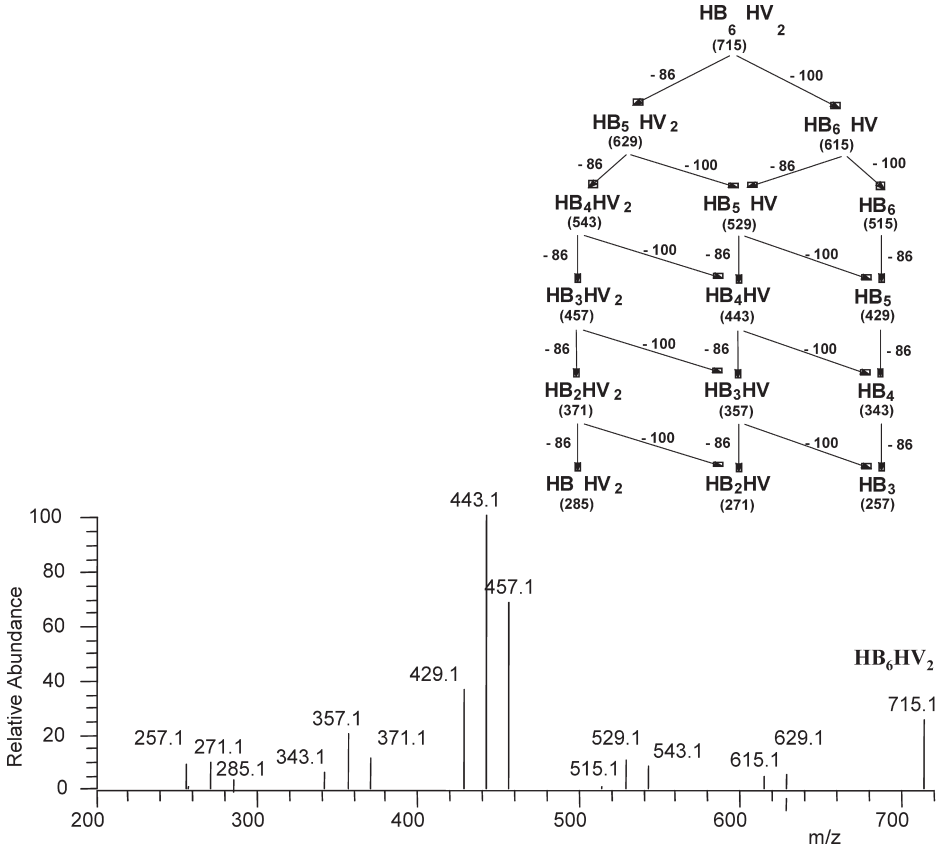


Figure 4.
The MS^2 spectrum of anion $[HB_6HV_2]^-$.

The best fit was taken to represent the correct value of this ratio.

The agreement factors (AF) calculated for each specific cluster of oligomers presented in ESI-mass spectra of PHBH and PHBV oligomers as a function of the oligomers composition are presented on Figures 2 and 3, respectively.

In Figure 2 distinct minima of AF factors calculated for each specific PHBH oligomers occur in the composition range of 85–89 mol% of HB units. This results is in good agreement which that NMR analysis of PHBH starting biopolyester.

The AF calculated for each specific oligomer clusters of PHBV oligomers (Fig. 3) shows a minimum in the composition range from 87–88 mol% HB units. These results indicated that the PHBV oligomers contain 12–13 mol% of HV units and in addition has randomly distributed comonomer units, which is in agreement with the sequence distribution determined by ^{13}C NMR spectroscopy on the dyad and triad level. [21]

The distribution of co-monomer units in the biopolyesters studied has been visualized by a tandem ESI mass spectrometry (ESI-MS/MS). In order to verify the random location of the HV units along individual chains of PHBV copolyester, MS/MS experiments were performed for molecular ion m/z 715 $[HB_6HV_2]^-$ selected from the 8-mer cluster.

A MS/MS spectrum of this molecular anion shows fragment ions grouped in clusters containing two fragment anions in the first step and three in the following steps. The fragmentation pathway shown in Figure 4 illustrates the results of the MS/

MS experiment of the molecular ion at m/z 715.

The fragment anions within the clusters having the same degree of oligomerization but a different content of HB and HV units (Figure 4) and were produced by the loss of crotonic acid (86 Da) or 2-pentenoic acid (100 Da), respectively (see fragmentation pathway in Figure 4). The proposed fragmentation pathway for a molecular anion m/z = 715 was consistent with the MS/MS mass spectrum obtained. The results from the MS^2 experiment thus confirmed that the PHBV biopoliester sample has a random distribution of HB and HV comonomer units in the copolyester chains.

Conclusion

The elaborated simple method of controlled depolymerization of natural PHAs leads to the formation of oligomers containing carboxylic and olefinic end groups with the same composition and sequence distribution as the starting biopolyesters.

The structural studies of two selected commercially available biopolyesters i.e. PHBV and PHBH were performed based on the ESI-MS analysis of their low molar mass oligomers. The molecular architecture of individual macromolecules, including the chemical structure of the end groups, was established based on mass-resolved signals of ESI-MS spectra of selected PHBH and PHBV oligocopolyesters studied. The composition and sequence distribution were determined based on measurement of the relative intensity of the individual oligocopolyesters peaks present at the ESI-mass spectra. The arrangement of comonomer structural units along the copolyester chains was verified by multistage MS experiments and investigation of the fragmentation pathway. Based on the present and previously published results the general utility of this method for the analysis of biopoliester macromolecules at the molecular level has been demonstrated.

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