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Microstructure of bacterial poly(β-hydroxybutyrate-co-β-hydroxyvalerate) by FAB mass-spectrometry

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SUMMARY

Bacterially synthesized Poly (β-hydroxybutyrate-co-β-hydroxyvalerate), P(HB-co-HV), with different compositions were analyzed by NMR spectroscopy and HPLC-mass spectrometry. Partial methanolysis or partial ammonolysis of P(HB-co-HV) has been performed, the oligomers obtained were separated by high-performance liquid chromatography (HPLC) and identified by fast atom bombardment mass spectrometry, (FAB -MS). The normalized intensities of oligomer peaks provided an estimate of the copolymer composition and of the sequence distribution of monomeric units. The interpretation of the data indicated that the sequence distributions of all samples were statistically random (Bernoullian model), and permited the detection in one sample, of traces of pure poly(β-hydroxybutyrate), (PHB).

INTRODUCTION

Composition and sequence arrangements of monomer units in Poly(β-hydroxybutyrate-co-β-hydroxyvalerate) copolymers influence crystallinity, mechanical, and physical properties (1, 2).

^{13}C-NMR spectroscopy is frequently used for the study of sequence distribution of these copolymers by analysis of diads and triads (2-5), but it cannot precisely discriminate beyond the triad level. Results obtained by ^{13}C NMR spectra and differential scanning calorimetry, (DSC), support the conclusion that some commercially available P(HB-co-HV) are mixtures of random copolymers with different HV mole fractions (4,5). Accordingly, the characterization of more complex sequence arrangements due to a mixture of copolymers requires a more sensitive technique than ^{13}C-NMR. Recently, new methods for the structural analysis of copolymers based on fast atom bombardment mass spectrometry (FAB-MS) analysis of the partial methanolysis (6,7) or pyrolysis (8) products were described. The greater sensitivity of these new methods is based on fitting the calculated statistical abundances with the MS experimental values corresponding to higher oligomers than the diads and triads observed in ^{13}C- NMR spectra.

In this study, P(HB-co-HB) samples were degraded by partial methanolysis or partial ammonolysis, and the mixtures of oligomers were fractionated by HPLC. FAB analyses were performed on oligomer fractions, as well as on the unfractionated mixture of oligomers. FAB-MS data were used to identify the

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oligomers formed, to estimate the copolymer composition, and to determine the sequence distribution of monomeric units.

Ammonolysis with piperidine is a new method used for the degradation of P(HB-co-HV) in order to identify and quantify high level sequences in copolymer and to compare them with those obtained by methanolysis. Until now, this reaction was used only for qualitative purposes on aromatic copolycarbonates (9), poly(ethylene terephthalate) and the copolyester containing α-truxilic and terephthalic units (10).

EXPERIMENTAL

Materials. -Bacterial P(HB-co-HV) samples were produced by fermentation from *Alcaligenes eutrophus* grown in culture media containing glucose and propionic acid, at ICI, Agricultural Division, Billingham, U.K.(11,12). The compositions HB/HV of Samples 1-3 determined by ^{1}H NMR (1, 3) were respectively 83 /17, 80/20, and 73/27.

NMR -^{1}H and ^{13}C-- NMR spectra were recorded on a Varian XL-300 MHz spectrometer operating at 300 MHz (for ^{1}H) and 75.4 MHz (for ^{13}C).

Partial Methanolysis -The procedures used for partial methanolysis and HPLC fractionation of copolyesters oligomers are slight modifications of those reported in the literature (6,13) .100 mg of each sample were dissolved in 20 ml of chloroform and 3 ml of a freshly prepared 1 N solution of HCl in anhydrous methanol were added. The appropriate amount of HCl was generated directly in the reaction medium from acetylchloride and dry methanol. The mixture (prepared in duplicate) was left at room temperature for 48 hours, after which the solvent was evaporated. One residue was directly analysed by FAB-MS and the other was dissolved in 2 ml acetonitrile, fractionated by HPLC and each fraction was collected and analysed by FAB-MS.

Partial Ammonolysis -Ammonolysis was performed under experimental conditions similar to those used for polycarbonates (9).

HPLC Fractionation -A Waters 600 Multisolvent Delivery System with a manual injector UK6 and a Waters 990 Photoiodide Array Detector was used. 50µl of degraded sample in acetonitrile were injected in a stainless steel Bondapak C_{18} (19 mm x 150 mm) column and the fractionation was carried out with an elution gradient starting with 100 % water and ending with 100 % acetonitrile. Using program no.6, the gradient took 45 min. to reach the final state for a solvent flow of 4 ml/min. UV detection was made at 205 nm.

FAB Mass Spectrometry -FAB analyses were performed on a double focusing high resolution VG ZAB 2F HS mass spectrometer equipped with a standard FAB source. The bombarding gas was Xenon with a energy of 8 kV and a beam current of 1 mA. Spectra were obtained by using glycerol as a matrix.

RESULTS AND DISCUSSION

The sequence distribution of the monomeric units in copolyesters was investigated by analysis of 75 MHz ^{13}C-NMR spectra and the diad sequence distribution was interpreted in terms of Bernoullian statistics.The experimental sequence distribution of Samples 1-3 obtained from 75 MHz ^{13}C NMR spectra and that calculated assuming a Bernoullian model (4-6), are shown in Table 1. F_{XY} represents the mole fraction of XY sequence.

Table 1 contains also the values of an additional parameter D, which indicates whether a sample is a random copolymer or not, and is defined as (4,5):

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

Usually, when D has a value of 1 or near 1, the sample is a simple random copolymer. D values smaller than 1 indicate an alternating copolymer, and D values larger than 1.5 indicate a block copolymer or a mixture of HB and HV rich random copolymers. The differences between experimental and calculated values

Table 1. Experimental and Calculated Sequence Distribution of P(HB-co-HV) Samples from NMR Data.

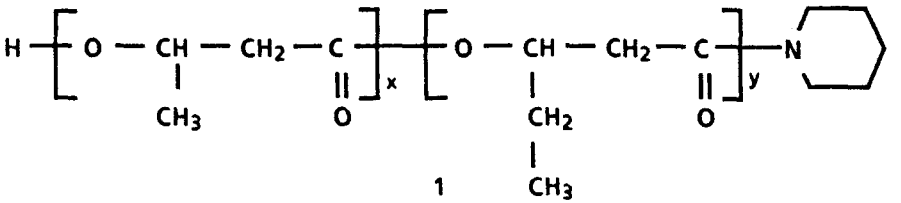
Sample	Model	F_V	F_B	F_{VV}	F_{BV+VB}	F_{BB}	D	S.D.
1	exp.	0.17	0.83	0.04	0.29	0.67	1.27	0.03
	Bernoull.			0.03	o.28	0.69		
2	exp.	0.20	0.80	0.04	0.33	0.63	0.93	0.02
	Bernoull.			0.04	0.32	0.64		
3	exp.	0.27	0.73	0.11	0.37	0.52	1.67	0.07
	Bernoull.			0.07	0.39	0.53		

were expressed in terms of error by means of the Hamilton normalized standard deviation (S.D.) (14).

$$S.D. = \sqrt{\frac{\Sigma_i (F_{exp.,i} - F_{calc.,i})^2}{\Sigma_i F^2_{exp.,i}}} \qquad (2)$$

D values shown in Table 1 suggest that Samples 1 and 2 are simple random copolymers, while Sample 3 may be a mixture of two random copolymers. On the other hand, the sequence distribution of the Samples 1 and 2 are well described by Bernoullian model, but the fit is not good enough for Sample 3, supporting the same conclusion as previously. Because the reliability of conclusions obtained by considering only diad sequences is questionable, the sequence distribution of chain segments containing up to 12 repeating units was characterized by using HPLC and FAB-MS techniques.

Figure 1 reproduces the HPLC fractionation of the methanolysis products of Sample 3. Table 2 reports the peak assignments obtained from FAB spectra of each fraction collected. As it can be seen, the majority of peaks are complex, containing mixtures of oligomers. Similarly, Figure 2 and Table 3 show the corresponding data for partial ammonolysis products of Sample 4. FAB data in Table 3 indicate that all oligomers obtained by degradation are amides 1 with alcohol/piperidine as end groups. The presence of some secondary products with two piperidine or piperidine/acid end groups which were generated by ammonolysis of poly(ethylene terephthalate), was not detected.



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The quantitative analysis of the oligomer mixtures obtained by both methods was achieved assuming that the relative abundances of the $(MH)^+$ ions present in the FAB spectrum are proportional to the statistical probability of the corresponding oligomer B_xV_y having the molecular weight M. Assuming Bernoullian statistics, for a simple random copolymer the statistical probability (abundance) of one oligomer B_xV_y is given by:

$$P_{x,y} = \frac{(x+y)!}{x!y!} F_B{}^x F_V{}^y \qquad (3)$$

The best fit between the experimental (FAB-MS) and calculated statistical abundances, using formula (3) indicates the most probable copolymer composition. Comparison between experimental and calculated statistical values used the normalized intensities of the peaks corresponding to dimers, trimers, tetramers, etc. in the unfractionated mixture obtained by degradation.

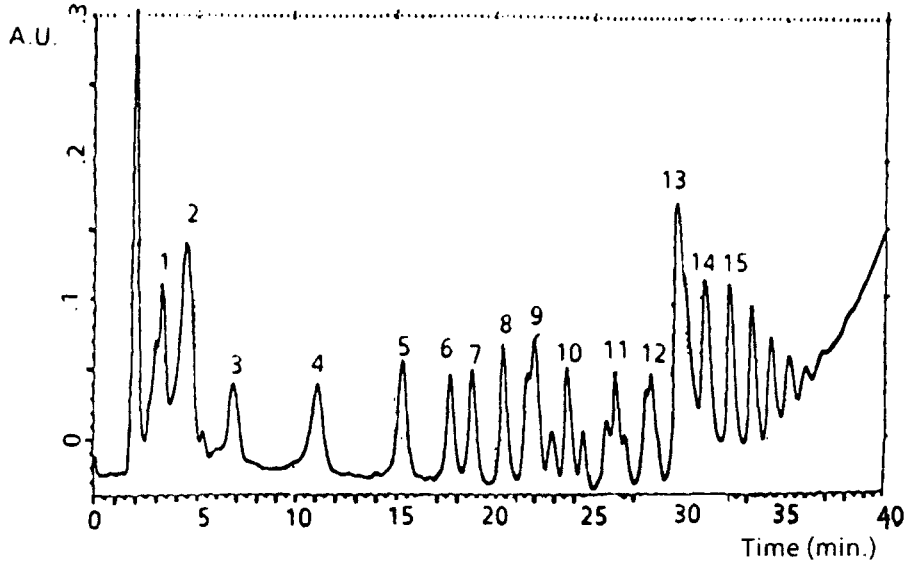

FIGURE 1: HPLC SEPARATION OF THE PARTIAL METHANOLYSIS PRODUCTS FROM SAMPLE 3

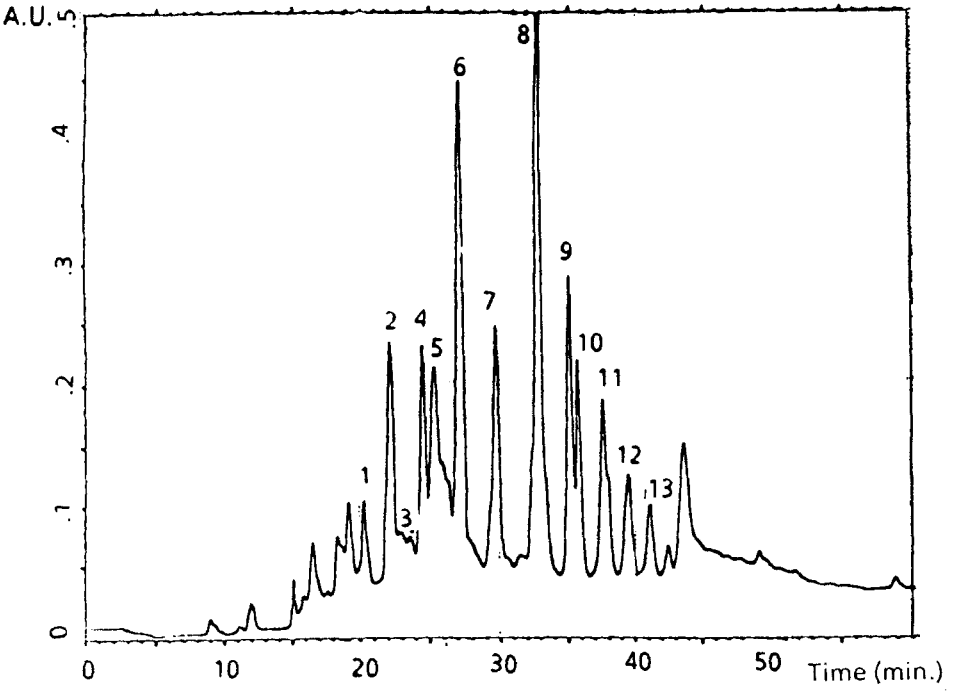

FIGURE 2: HPLC SEPARATION OF THE PARTIAL AMMONOLYSIS PRODUCTS FROM SAMPLE 3

Table 2. Identification of the Methanolysis Products B_xV_y = H[-OCH (CH_3)-CH_2-CO-]$_x$--[O-CH (CH_2 CH_3)-CH_2-CO-]$_y$ OCH_3 from Sample 3 by HPLC and FAB-MS.

peak	B_xV_y	MH +	peak	B_xV_y	MH +
1	B_2*	205	12	B_3V_3	591
2	BV	219		B_5V_2	663
3	B_3	291		B_7V	735
4	B_2V	305		B_9	807
5	B_4	377	13	B_4V_3	677
6	B_3V	391		B_6V_2	749
	B_5	463		B_8V	821
7	B_2V_2	405		B_{10}	893
	B_4V	477	14	B_3V_4	691
8	B_6	549		B_5V_3	763
9	B_3V_2	491		B_7V_2	835
	B_5V	563		B_{11}	979
10	B_7	635	15	B_4V_4	777
11	B_2V_3	505		B_6V_3	849
	B_4V_2	577		B_{12}	1065
	B_6V	649			
	B_8	721			

* the peak B_2 did not appear in FAB mass spectra, it was identified by the thermospray technique.

Table 4 reports the normalized experimental and calculated abundances of partial methanolysis products for P(HB-co-HV) samples. Analogous to the interpretation of NMR results, the differences between experimental and calculated values were expressed in terms of the Hamilton normalized standard deviation (S.D.) equation. There is a good agreement between calculated values (from Bernoullian statistics), with the experimental ones for Samples 1 and 2. These results confirm the HB/HV composition of Samples 1 and 2 and also the hypothesis on which the calculations are based, i.e. that the copolymer possesses a random distribution of monomeric units.

S.D. values are higher for the data corresponding to Sample 3. In order to get better agreement, the statistical calculations for three different HB/HV compositions (78/22, 68/32 and 63/37) close to that obtained by NMR (73/27) were made and the results are presented in Table 5. Inspection of the data in this table reveals that the best fit corresponds to the HB/HV composition of 68/32. To reconcile the composition value of Sample 3 obtained by NMR with that indicated by FAB-MS analysis, a mixture of two Bernoullian random copolymers may be taken as model (4,7). According to this model, when a mixture of two Bernoullian copolymers of P(HB-co-HV) with HB mole fractions A and B, respectively, are mixed in a molar ratio of X:(1-X), the values of A, B, and X can be calculated from the experimental mole fractions $F_{B_xV_y}$ of all sequences by solving sets of equations corresponding to dimers, trimers, etc. In these equations the values $F_{B_xV_y}$ should correspond to the normalized intensity of the MS peaks of B_xV_y oligomers, the normalization factor being the sum of intensities within each set of equations. A program using a minimization routine has been written to find the composition (A,B,X values) which best fits the experimental normalized intensities. In Table 4 the calculated normalized abundances for oligomers of Sample 3 (according to this model), as well as the best fit of A, B, and X are listed. The sequence distributions are consistent with a mixture of two polymers: 4% of PHB and 96% random copolyester P(HB-co-33% HV). The existence of the mixture of polymers is also supported by the D value larger than 1.5, calculated previously (cf. Table1).

Table 3. Identification of the Ammonolysis Products from Sample 3 by HPLC and FAB-MS Analyses.

peak	B_xV_y	$(MH)^+$	peak	B_xV_y	$(MH)^+$
1	B_2	258	11	B_5V	616
2	BV	272		B_2V_3	558
3	V_2	286	12	B_7V	788
4	B_3	344		B_6V_2	802
5	B_2V	358		B_5V_2	716
6	B_4	430		B_4V_3	730
7	BV_2	372		B_3V_3	644
8	B5	516		B_3V_2	544
	B_3V	444	13	B_8V_2	974
	B_2V_2	458		B_7V_2	888
9	B_4V	530		B_6V_3	902
10	B_3V_2	544		B_5V_4	916
	BV_3	472			

Table 5. Experimental and Calculated Normalized Abundances of the Partial Methanolysis Products from Sample 3 (FAB-MS).

Oligomers	Experimental	Calculated			
		63/37	68/32	73/27	78/22
B_3	32	25	31	39	48
B_2V	44	44	44	45	41
BV_2	24	31	25	16	11
S.D.		0.15	0.02	0.17	0.35
B_4	24	16	21	27	37
B_3V	37	37	40	40	42
B_2V_2	27	33	28	25	17
BV_3	12	14	11	6	4
S.D.		0.19	0.08	0.14	0.32
B_5	16	9	14	20	29
B_4V	30	29	34	38	41
B_3V_2	30	34	32	28	23
B_2V_3	17	19	15	12	7
BV_4	6	9	5	2	0
S.D.		0.18	0.11	0.23	0.45
B_6	14	6	12	15	23
B_5V	27	22	30	33	39
B_4V_2	34	32	35	31	27
B_3V_3	25	40	22	21	11
S.D.		0.34	0.09	0.15	0.42

A similar comparison between the experimental and calculated sequence distribution was made for oligomers obtained by partial ammonolysis, but the values of standard deviations between experimental and calculated normalized abundances were higher. This suggests that the aminolytic chain scission of the ester groups is not random. The scission by piperidine of the ester groups belonging to an HV unit could be less favored compared to an HB unit, due to the steric hindrance of the longer HV chain.

Table 4. Experimental and Calculated Normalised abundances of partial methanolysis products for P(HB-co-HV) Samples.

Copolymer #	B/V[a]		B_2V		V_3	B_3V		BV_3	B_4V		B_2V_3	B_6	B_4V_2		B_7	B_5V_2		B_3V_4	B_2V_5
		B_3		BV_2		B_4	B_2V_2		B_5	B_3V_2	BV_4		B_5V	B_3V_3		B_6V	B_4V_3		
1	83/17	Expl[b]	53	34	10 3	48	32	18 2	39	38	19 4	34	37	23 6					
		Calc[c]	57	35	7 1	57	35	7 1	40	40	17 3	33	41	21 5					
		S.D.[d]		0.08			0.15			0.05			0.08						
2	80/20	Expl[b]	51	34	12 3	38	38	17 7	34	37	22 7	25	38	26 10	21	34	26	16 3	
		Calc[c]	51	38	10 1	41	41	15 3	33	41	21 5	27	40	25 8	21	37	28	11 3	
		S.D.[d]		0.07			0.10			0.08			0.06				0.12		
3	73/27	Expl[b]	32	44	24	24	37	27 12	16	30	30 17 6	14	27	34 25	9	19	28	25 14	4
		Calc[c]	39	45	16	27	41	25 7	20	38	28 12 2	15	33	31 21	11	29	32	19 7	2
		S.D.[d]		0.17			0.14			0.23			0.15				0.31		
		Calc[e]	32	43	21 4	23	38	28 9	17	32	31 15 4	17	27	34 22	10	20	29	24 12	5
		S.D.[d]		0.01			0.07			0.08			0.09				0.06		

a) composition determined by NMR

b) relative intensities of the peaks in FAB mass spectrum

c) relative intensities calculated by eq.3 (Bernoullian)

d) standard deviation calculated by eq.2

e) relative intensities calculated by equations corresponding to a mixture of two Bernoullian copolymeres [4,7]

A/B/X = 1/0.67/0.04

CONCLUSIONS

Microstructure of P(HB-co-HV) samples can be characterized with high accuracy by partial methanolysis and HPLC-MS techniques. Detection of a small quantity of PHB mixed with the major constituent P(HB-33% HV) in Sample 3 demonstrates the superiority of this technique with respect to NMR and DSC. Because PHB and P(HB-co-HV) cocrystallise, one cannot depend on DSC to detect a small % of PHB. The presence of PHB in bacterial copolyesters of this kind is the result of processing conditions and bacterial physiology phenomena. Studies of biosynthesis conditions leading to the production of bacterial polyhydroxyalkanoates (PHA) have revealed that the substrate composition of the medium changes during bacterial growth because of buildup and subsequent utilisation of metabolic intermediates (15). Consequently, one would expect a change in the monomer composition of PHA as a function of the age of the culture. In the specific conditions of biosynthesis of P(HB-co-HV)copolyesters, small quantities of PHB are formed in the early growth stageFurther, it is known that *Alcaligenes eutrophus* can degrade PHB by secretion of an esterase, and use the polymer as a carbon source (16). This explains the contamination with traces of PHB of copolymer obtained as major product.

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