Viscoelastic and thermal properties of bacterial poly(D-(-)-β-hydroxybutyrate)

M. Scandola*, M. Pizzoli and G. Ceccorulli
Centro di Studio per la Fisica delle Macromolecole CNR and Dipartimento di Chimica 'G. Ciamieian', Università di Bologna, Via Selmi 2, 40126 Bologna, Italy

A. Cesaro*, S. Paoletti and L. Navarini
Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, Università di Trieste, Piazzale Europa 1, 34127 Trieste, Italy
(Received 3 March 1988; revised 12 April 1988)

Three poly(D-(-)-β-hydroxybutyrate) (PHB) samples from Rhizobium spp. have been characterized in order to evaluate the effects of different extraction procedures of the polymer. Only the molecular weight is found to change, being \(6 \times 10^4\) for the sample extracted with HCl (1 M) and of the order of \(10^4\) for the samples extracted with acetone. Thermogravimetric results on PHB with different molecular weights, indicate that the temperature at which weight loss becomes significant is lower than 230°C only for the lowest molecular weight examined (6 \(\times 10^4\)). The calorimetric properties strongly depend on thermal history. The d.s.c. curves of "room stored" samples show only a melting endotherm at 177°C, whose area increases with annealing. Quenching from the melt shows evidence an intense glass transition (\(\Delta C_p = 0.43 \text{ J/g deg}\)) in the vicinity of 0°C, followed by a 'cold crystallization' peak preceding melting. The viscoelastic spectrum shows three relaxations: a water-related low temperature relaxation (-80°C), the glass transition (apparent activation energy \(\Delta H_m = 356 \text{ KJ/mol}\)) and a broad relaxation in the temperature range between \(T_g\) and \(T_m\) due to motions in the crystalline phase.

Keywords: Poly(D-(-)-β-hydroxybutyrate); biopolymer; viscoelasticity; thermal properties

Introduction

Many micro-organisms accumulate poly(D-(-)-β-hydroxybutyrate) (PHB), an optically active aliphatic homopolyester, and use it as a source of energy and carbon\(^1\). This polyester can be produced in large amounts in some micro-organisms; *Alcaligenes eutrophus*, for example, grown in particular conditions, is able to accumulate PHB in the late storage phase up to 78% of the total dry mass\(^2\,3\).

*Azotobacter* and *Alcaligenes* are currently used in the fermentation process to produce PHB with commercialization purposes\(^4\). In spite of the increasing industrial interest, literature data on solid state mechanical properties are scarce. In addition to studies on PHB solution properties, aimed at defining the polymer conformation in solution\(^5\,\,6\), investigations on solid state properties have been mainly focused on powder and fibre X-ray diffraction analysis\(^7\,\,9,11\).

This paper presents an investigation on the viscoelastic and thermal properties of PHB. Extraction procedures yielding products with different molecular weight have also been given attention, in order to determine the influence of this parameter on the properties under study.

Among the different micro-organisms producing PHB, *Rhizobium* has been chosen because of its non-pathogenicity and importance in the agricultural field.

Experimental

Fermentation and polymer recovery

The screening of the strains of *Rhizobium* spp. conformed to the following criteria: (1) productivity of PHB under given conditions; (2) low production of extracellular polysaccharides (EPS) which cause the viscosity to increase and make the centrifugation of the cells from the culture broth very difficult.

PHB was produced from the two following strains: *R. meliloti* SU-255 (samples A and B) and *R. leguminosarum* (sample C).

The growth conditions for *R. meliloti* SU-255, in a definite medium (at pH 7.5), were: glucose 20 g/l; glutamic acid 1.0 g/l; MgSO\(_4\) 0.2 g/l; CaCl\(_2\) 0.04 g/l; traces of elements: FeCl\(_3\) 0.01 g/l; ZnSO\(_4\cdot7\text{H}_2\text{O}\) 0.01 g/l; MnCl\(_2\) 0.01 g/l; NaMoO\(_4\cdot2\text{H}_2\text{O}\) 0.01 g/l; Vitamins: biotin 1.0 mg/l; thiamine 1.0 mg/l; methionine 0.01 mg/l; MgSO\(_4\cdot7\text{H}_2\text{O}\) 0.01 g/l; NaCl 1.0 mg/l; NaNO\(_2\) 0.01 mg/l; NaCl 1.0 mg/l; NaCl 1.0 mg/l; NaCl 1.0 mg/l.

The growth was carried out in a 300 ml Erlenmeyer flask with 100 ml of medium on a rotatory shaker at 100 rev/min at 25°C (sample A) or in a 10 litre vessel at 25°C with forced aeration (sample B), respectively.
Growing conditions of \textit{R. leguminosarum} (sample C) are described elsewhere\textsuperscript{12}.

During PHB production, the PHB content was determined in all cases with the gas-chromatographic method described by Braunegg \textit{et al.}\textsuperscript{13}. The final yield of PHB was 47\% of cell dry weight for samples B and C and 40\% for sample A, respectively.

Two different methods were used for PHB extraction:

(a) \textit{sample A}: the culture broth was centrifuged at 1000 \text{rev/min} for 10 min at \(25^\circ\text{C}\). Cell pellets obtained from 1 litre of cell culture were suspended in 200 ml \text{HCl} 1 M. The suspension was heated for 1 h at \(100^\circ\text{C}\) in a water bath, centrifuged and washed with water. Pellets were suspended in 100 ml of \text{CHCl}_3 and shaken overnight. Cell debris was removed by centrifugation (30000 \text{rev/min}) and the solution was then evaporated to give PHB in a film-like form.

(b) \textit{samples B and C}: the 'mild' extraction procedure using acetone is described elsewhere\textsuperscript{12}.

Two commercial samples of PHB from Sigma Co. (P 8150) and Aldrich GmbH (28,250-2) were used. Another sample was received indirectly from ICI Ltd.

Characterization methods

Infrared spectra between 4000 and 400 cm\(^{-1}\) were recorded with a Perkin-Elmer G983 spectrophotometer. Samples were mixed with KBr and pressed into discs.

\textsuperscript{1}H n.m.r. spectra of the CDC\(_1\)\(_3\) solutions were recorded on a Varian EM3360A n.m.r. spectrometer using TMS as internal standard.

Viscosity measurements were carried out in chloroform, by using a Schott-Gerate AVS/G equipment with an Ubbelohde viscometer (\(\theta = 0.46\) mm) in a thermostatic bath at 30\(\pm\)0.01°C.

Calorimetric (d.s.c.) measurements were carried out by means of a DuPont 9900 Thermal Analysis System (heating rates 10 or 20 \text{deg/min}) in the temperature range \(-100\) to 200°C. The temperature scale was calibrated with high purity standards: benzene and indium.

Thermogravimetric (TG) measurements were made with a Mettler Thermal Analyser TA 2000 C. Investigations were carried out under air flow (flow rate = 50 ml/min) using Al\(_2\)O\(_3\) as a reference, with a scan rate of 10 \text{deg/min}.

Dynamic-mechanical (DMA) measurements were performed with a Dynamic Mechanical Analyser (Polymer Laboratories, Ltd) operated in the dual cantilever bending mode, in a range of frequencies (\(\nu\)) from 0.33 to 30 \text{Hz}. PHB samples in the form of small injection moulded bars (30 \(\times\) 7.5 \(\times\) 1.5 mm) were investigated in the temperature range \(-150\) to 160°C (heating rates 0.5 and 3 \text{deg/min}).

Results and discussion

Molecular characterization

The viscosity-average molecular weight of the PHB samples was determined from the viscosity-measurements, using the Mark–Houwink equation \([\eta] = KM^n\) with \(a\) and \(K\) values taken from the literature\textsuperscript{9}; the \([\eta]\) and molecular weight values for PHB samples denoted A, B and C (see Experimental) are reported in Table 1. The viscosity-average molecular weight for other samples resulted to be \(2 \times 10^8, 4.5 \times 10^8\) and \(6.8 \times 10^8\) respectively.

Infrared spectra for all PHB samples are qualitatively identical, and they correspond to the spectra for PHB obtained from \textit{Azotobacteriaceae}\textsuperscript{14}, \textit{Halobacterium mediterranei}\textsuperscript{11}, \textit{Bacillus megatetarum}\textsuperscript{1}. Also \textsuperscript{1}H n.m.r. spectra are in agreement for all PHB samples and correspond to the spectra reported in the literature for PHB obtained from \textit{Bacillus megatetarum}\textsuperscript{10}, and \textit{Bacillus cereus}\textsuperscript{17}. These findings confirm the suggestion by Doi \textit{et al.}\textsuperscript{18} that the structural identity of PHB is independent of the bacterial origin of the polymer.

The only difference among the samples examined is the molecular weight, which is rather high for samples B and C as compared to that of sample A. This difference is likely to be related to the procedure followed during polymer extraction. The mild extraction method identified in the experimental section as 'method (b)' yields molecular weights of the order of \(10^6\), higher than commonly reported for both laboratory and industrial PHB preparations.

Thermal and dynamic-mechanical properties

Figure 1 shows two subsequent calorimetric scans of sample B (carried out from \(-100\) to 220°C at a heating rate of 20 \text{deg/min}). After the first thermal scan the sample was rapidly quenched to \(-100^\circ\text{C}\) in the d.s.c. Whereas the only significant feature in the first scan is the endothermic melting peak of PHB, the second scan on the quenched sample is more complex: an endothermic baseline shift corresponding to the glass transition in the vicinity of 0°C is followed by an exothermal 'cold crystallization' peak and by a melting endotherm analogous to that observed in the first scan. Provided that the polymer is subjected to the same thermal history

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure1.png}
\caption{Calorimetric curves for PHB (sample B): broken line: first scan; full line: second scan (after quenching at \(-100^\circ\text{C}\)). Heating rate: 20 \text{deg/min}}
\end{figure}

<table>
<thead>
<tr>
<th>Sample</th>
<th>Source</th>
<th>([\eta]) \text{ (dl/g)}</th>
<th>(M_w \times 10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>\textit{Rhizobium} meliloti SU-255</td>
<td>0.63</td>
<td>60</td>
</tr>
<tr>
<td>B</td>
<td>\textit{Rhizobium} meliloti SU-255</td>
<td>7.50</td>
<td>1400</td>
</tr>
<tr>
<td>C</td>
<td>\textit{Rhizobium leguminosarum}</td>
<td>8.39</td>
<td>1600</td>
</tr>
</tbody>
</table>

* In \text{CHCl}_3 at 30°C
the heat of fusion ($\Delta H$) and melting temperature ($T_m$) are 851 J/g and 177°C respectively (for a scan rate of 10 deg/min). Crystallization and melting of PHB have been extensively studied by Barham et al.\textsuperscript{20,21} who estimated, on the basis of calorimetric, density and X-rays results, that the heat of fusion of 100\% crystalline PHB is 146 J/g \textsuperscript{20}. From this value the amount of crystallinity of our PHB samples may be calculated; for all samples stored at room temperature the crystalline fraction is rather constant (55–60\%) and increases, as expected, on annealing at temperatures close to $T_g$, as it is also shown by the powder X-ray diffraction data\textsuperscript{12}.

It is well known that high temperature treatments such as annealing or melt processing may influence the physical properties of PHB, due to thermal degradation. Grassie et al.\textsuperscript{22} suggested that random chain scission is the main degradation mechanism in PHB, resulting in a decrease of molecular weight. Evolution of volatile products is observed only when extensive chain breakdown yields oligomeric products, the process becoming significant at temperatures well above 200°C during TG scans at 10 deg/min, irrespective of the nature of the atmosphere (N\textsubscript{2}, air, vacuum)\textsuperscript{22}. The thermal stability of PHB with molecular weight ranging from $6 \times 10^4$ to $1.4 \times 10^6$ has been investigated here by subjecting the polymers to a discontinuous heating cycle, i.e. to a scan interrupted by subsequent 1 h-isothermal treatments at 150, 170 and 190°C. Figure 2 shows the thermogravimetric (TG) results obtained under air flow as well as the temperature versus time profile of the experiment. The TG curves clearly indicate that the temperature at which weight loss becomes significant is higher than 230°C for all samples but sample A. Moreover, the 'initial' temperature of evolution of volatile products shows an appreciable, albeit small, dependence on molecular weight in the range $2 \times 10^5$–$1.4 \times 10^6$. Comparison with literature results obtained with dynamic TG scans\textsuperscript{22} suggests that the three isothermal heat treatments preceding the last temperature ramp (Figure 2) do not appreciably influence the temperature range of complete degradation.

A quite useful characterization of the physical properties of polymeric solids comes from the determination of their viscoelastic spectrum, as it may be obtained from dynamic-mechanical measurements. Dynamic-mechanical results available in the literature...
Properties of poly(α(−)-β-hydroxybutyrate): M. Scandola et al.

Figure 4 Multifrequency dynamic-mechanical measurements in the β transition region: A 0.33 Hz; B 1.0 Hz; C 3.0 Hz; D 10.0 Hz; E 30.0 Hz. Heating rate: 0.5 deg/min. Inset: Arrhenius plot refer only to the Tg range and the relaxation spectra of PHB recently reported have been obtained by means of dielectric and piezoelectric measurements in the range −150 to 150°C. The dynamic-mechanical spectrum of PHB has been presently determined over a wide temperature range it is shown in Figure 3. The temperature dependence of the loss tangent (tan δ) and of the storage modulus (E) refer to sample B stored at room temperature for several weeks after moulding. Analogous results have been obtained for lower molecular weight samples (2 x 10^5 and 4.5 x 10^5, respectively), subjected to the same thermal history. The dynamic-mechanical loss tangent spectrum shows three relaxation peaks (α, β, γ in order of decreasing temperature) in agreement with the piezoelectric results of Ando et al..

The low temperature relaxation is related to the presence of moisture absorbed by the polymer during room storage: in fact, its intensity decreases either by heating above 100°C or by drying under vacuum over P2O5. Furthermore, the γ absorption cannot be detected in a PHB sample tested under dry nitrogen purge after heat treatment to 155°C, but it reappears in the dynamic-mechanical spectrum after storage of the sample in a room with normal humidity conditions. Accordingly, TG results in the range 30–120°C indicate a reversible weight loss of about 1% for 'room stored' samples.

Low temperature relaxations related to the presence of water have been found in a number of polymers containing polar groups. The attribution of this relaxation peak to the mere presence of water (some kind of 'glass transition' of water) or to more complex motions of water associated with some part of the polymer chain is still controversial. In the case of PHB, it is the authors' opinion that the latter attribution is more likely to be correct, in view of the recent results on the influence of water on an analogous secondary relaxation shown by cellulose acetate. Therefore, while Ando et al. assigned the piezoelectric relaxation of PHB to 'local molecular motion of main chains in the amorphous region', we suggest that it might well be the motion of water molecules interacting with the macromolecules through polar interactions to originate the dynamic-mechanical dispersion.

On the basis of its temperature location (see d.s.c. results) and of the associated modulus drop, the β relaxation, shown in Figure 3, is easily attributed to the glass transition of the amorphous fraction of PHB, as it was already recognized by previous studies. The small size of the tan δ peak and of the corresponding decrease of the modulus are clear indications of the partially crystalline character of the polymer. Indeed, in the range of the β relaxation the modulus shows a peculiar behaviour, i.e. a decrease clearly composed of two contiguous steps previously not observed. This feature is found whenever 'room stored' PHB samples are examined. Due to the proximity of the β dispersion (Tg) to room temperature, the PHB samples undergo physical ageing during storage. In the case of partially crystalline polymers this phenomenon also occurs at temperature slightly above Tg producing changes in both shape and intensity of absorption peak and associated modulus drop. The effect of physical ageing on the β dispersion region of PHB is presently under investigation.

In Figure 4 the effect of frequency on the temperature location of the β absorption of PHB is reported. The insert shows the linear dependence of log v versus 1/Tmax, where Tmax is the peak temperature. The linearity implies that, in the range of frequencies investigated, the Arrhenius equation \( v = v_0 \exp(-\Delta H_a/RT) \) satisfactorily applies to the main glass-to-rubber transition of PHB allowing the calculation of the apparent activation energy, \( \Delta H_a = 356 \text{ kJ/mol} \).

The last transition in the spectrum of Figure 3 is the very broad γ relaxation, which is located between the glass transition and the melting of PHB. The proximity of melting is indicated by the very steep drop of the modulus starting at about 150°C. Concerning the possible molecular origin of the γ relaxation, it has been suggested that it originates in the crystalline regions. In fact, the authors report a shift of the transition temperature to higher values with increasing annealing temperature. Thickening of lamellae of PHB upon annealing has been also reported. The suggestion of the involvement of the crystalline phase as the origin of the dynamic mechanical γ relaxation is presently supported by DMA results.

Figure 5 Calorimetric curves for PHB (sample B): A 'room stored'; B annealed at 150°C for 18 h. Heating rate: 10 deg/min

carried out on a sample re-run after heat treatment at 150°C; the intensity of the α peak is strongly reduced. The same evidence is drawn from the d.s.c. calorimetric curves reported in Figure 5 on sample B both 'room stored' and annealed at 150°C. The former curve shows a gradual endothermal deviation of the baseline between 120 and 160°C that results in a sort of long tail preceding melting; the curve of the annealed sample indicates that in addition to an overall increase of crystallinity, the heat treatment also eliminates the pre-melting tail. Taking into account the different heating rates of the d.s.c. and DMA measurements (10 versus 3 deg/min) the two observed phenomena (α relaxation and endothermal baseline deviation) might well be related and both evidence some kind of reorganization of the crystalline phase, such as a partial melting and re-crystallization.

Conclusions

Three relaxations have been found in the viscoelastic spectrum of PHB at temperatures lower than melting and have been assigned as follows: the secondary γ relaxation at about -80°C is related to the presence of absorbed water, the main β relaxation (ca. 20°C) is the glass-to-rubber transition and the broad α relaxation, centred at about 130°C, is due to motions in the crystalline phase. The glass transition has also been evidenced by d.s.c, in about 130°C, is due to motions in the crystalline phase.

The properties under study are not affected by differences in molecular weight (6 x 10⁴-1.4 x 10⁶) with the exception of thermal stability, which is quite low for the sample of lowest molecular mass (significant evolution of volatile products at 170°C). For molecular weights comprised between 2 x 10⁶ and 1.4 x 10⁶ weight loss becomes appreciable only at T > 230°C.

Acknowledgements

The authors are grateful to Dr R. Tombolini for growing the bacteria strains and to Professor L. P. T. M. Zevenhuizen for the hospitality. This work has been carried out with the financial support of the Italian Ministry of Public Education (MPI).

References

5 Desarte, J. and Weill, G. Macromolecules 1974, 7, 450
8 Akita, S., Einaga, Y., Miyaki, Y. and Fujita, H. Macromolecules 1976, 9, 774
10 Cornibert, J. and Marchessault, R. H. J. Mol. Biol. 1972, 71, 735
17 Agostini, D. E., Lando, J. B. and Reid Shelton, J. Pol. Sci. 1971, 9, 2775
19 Wunderlich, B. J. Phys. Chem. 1960, 64, 1052
21 Barham, P. J. J. Mater. Sci. 1984, 19, 3826
23 Owen, A. J. Colloid & Polymer Sci. 1985, 263, 799
24b Fukada, E. and Ando, Y. Int. J. Biol. Macromol. 1986, 8, 361
26 Cecconulli, G., Fizzioli, M. and Scandola, M. Polymer Communication 1986, 27, 228