Gas chromatographic, liquid chromatographic and gas chromatographic–mass spectrometric identification of degradation products in accelerated aged microbial polyhydroxyalkanoates

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(First received November 1st, 1993; revised manuscript received January 20th, 1994)

Abstract

The degradation products of alkaline hydrolysed poly(β-hydroxybutyrate) (PHB) and its copolymer with poly(β-hydroxyvalerate) were monitored using GC and LC. The one major degradation product detected was crotonic acid, a well known degradation product of 3-hydroxybutyrate. Using headspace GC–MS, volatile products were identified in thermo-oxidized native polyesters, e.g. 2-ethoxyethyl acetate and acetaldehyde in addition to crotonic acid. There is an inverse relationship between the amount of crotonic acid produced in thermo-oxidized samples of PHB and the average number-average molecular mass as shown by size-exclusion chromatography.

1. Introduction

Poly(β-hydroxybutyrate) (PHB) is produced by a wide variety of prokaryotic organisms such as bacteria and cyanobacteria, when their growth in a mineral medium is limited by the depletion of an essential nutrient such as nitrogen, oxygen, phosphorus, sulphur or magnesium. Many bacteria accumulate PHB, amounting to 30–80% of their cellular dry mass, because the polymer functions either as a carbon and/or energy reserve or as a sink for excess reducing equivalents. Depending on the mineral medium, copolyesters of PHB with e.g. poly(β-hydroxyvalerate) (PHV) can also be produced. PHB isolated from bacterial cells usually has a very high number-average molecular mass (10^5–10^6)

and is of high crystallinity. The native polyesters are optically active with an R absolute configuration in the chiral centre of 3-hydroxybutyric acid, but this is not the case for their synthetic analogues.

High-molecular-mass PHB and copolymers of PHV can, for example, be synthesized from racemic β-butyrolactone and β-valerolactone, respectively, with an oligomeric alumoxane catalyst, obtained by reaction of $AlEt_3$ with water [1]. In contrast to the bacterial copolyesters that have a random stereosequence, the synthetic, highly crystalline, polyesters are blocky and only partially stereoregular [2–4]. These differences in structure between synthetic and native polyesters have a great influence on their degradation properties.

The bacterially produced isotactic polyhydroxyalkanoates degrade very rapidly in differ-

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ent environments such as soil, sewage sludge and water. The synthetic polyester is however less susceptible to enzymatic degradation because the (S) stereoblocks halt the degradation. Low and medium crystalline samples show higher degradation rates since the greater amorphous chain mobility allows the depolymerase to penetrate more easily in to the surface and access the available (R) stereoblocks [5].

Hydrolytic degradation is an important process if the polyhydroxyalkanoates are to be used, for example, in medical applications. The simple hydrolytic degradation of microbial polyesters does not proceed as rapidly as the enzymatically induced hydrolytic degradation. It has been shown that the degradation rate of PHB and PHB–co-PHV samples in a phosphate buffer (pH 7.4) can be accelerated by increasing the temperature [1,6]. An induction period, that may correspond to the time required for water completely to penetrate the polymer matrix before the molecular mass loss starts, is observed at lower temperatures but becomes shorter with increasing temperature [4]. The effects of pH must be determined if the material is to be used *in vivo*, since relatively large localized pH changes occur that may alter the degradation profile [7]. For polyesters in general, the dependence of degradation on the pH is well known. In acidic and neutral solutions, hydrolysis proceeds by a protonation process followed by the addition of water and cleavage of the ester linkage. In alkaline media, hydroxyl ions are attached to the carbonyl carbons and the ester linkages are subsequently ruptured [8].

Although there is strong evidence that physiological degradation *in vivo* is substantially dependent upon a conventional hydrolytic process, the degradation behaviour of fabricated devices cannot be reasonably predicted simply on the basis of the properties of the unprocessed starting materials. This is because processing conditions used in polymer fabrication have a potentially marked effect on such properties as molecular mass and crystallinity of a material [9]. Polyhydroxyalkanoates are melt unstable which may cause problems during processing. The main decomposition of bacterial PHB and its copoly-

mers occurs at temperatures of about 250°C and results in the formation of thermal decomposition products of the HB and HV repeating units [10].

In the work described in this paper the influence of pH, temperature and time on the hydrolysis of PHB and PHB–PHV copolymer have been investigated and the degradation products subsequently monitored using gas chromatography (GC) and high-performance liquid chromatography (HPLC). In addition the thermal susceptibility of PHB and PHB–PHV has been analysed monitoring changes in molecular mass by size-exclusion chromatography (SEC) and the formation of low-molecular-mass compounds by headspace (HS) GC and HS-GC coupled to ion trap mass spectrometry (ITD-MS).

2. Experimental

Commercial Biopol MBL granules of PHB and PHB–co-PHV (7% PHV) were dissolved in chloroform at 20 g/l by refluxing. This solution was added to nine times its volume of methanol and the precipitated polymer was filtered and washed with methanol. Solution-cast films were made by dissolving this purified polymer in chloroform and films were cast on glass plates. The films were placed in solutions consisting of equal volumes of MeOH and 2 M NaOH at a temperature of −5°C. Films were removed after 1 h, 3 h and 6 days, and the solutions were neutralized with hydrochloric acid. A similar study has been carried out in phosphate buffers (pH 10) at 25 and 60°C for up to one year. The HPLC analyses were carried out using a Varian Vista 5500 liquid chromatograph connected to a Varian 4270 integrator. The samples were run isocratically with a mobile phase of 40% 8 mM orthophosphoric acid in water and 60% far-UV-grade acetonitrile, and the products were detected at 205 nm. Samples were dissolved in the same mixture prior to injection of 20 μl. The GC analyses were made using a Varian 3400 gas chromatograph with a flame ionization detector and a column type DB 1301. After injection of 1

μl at 90°C, the temperature was increased at a rate of 10°C/min up to 180°C. The samples were dissolved in diethyl ether prior to injection and 0.4 μl n-pentadecane was used as an internal standard in the mild alkaline hydrolysis study.

Commercial Biopol MBL granules, purified as described above were also used for thermal ageing. 200 mg of PHB and PHB–co-7% PHV were aged in sealed headspace vials at 100 and 180°C for up to 500 h. The subsequent HS-GC and HS-GC–MS analyses were carried out using a Perkin-Elmer 8500 flame ionization (GC) detector, a Perkin-Elmer headspace HS 101 unit and an ion trap (MS) detector. The column was a Chrompack wall-coated open tubular fused silica, 25 m × 0.32 mm; CP-SIL 19CB and a temperature programme from 40 to 180°C at 5°C/min used. The HS unit was thermostated for a period of 50 min at 100°C. The ITD operating conditions were: scan range 20–600 u; scan time 1.000 s; threshold 1 count; mass defects 0.03 u/100 u; multiplier voltage 1600 V and a transfer line temperature of 250°C.

The SEC analyses were performed using a Waters Associates M-6000A size-exclusion chromatograph equipped with a set of Styragel columns, thermostated 1 ml/min .flow, mobile phase chloroform. The molecular masses [number average (M_n) and mass-average (M_w)] were calculated using a calibration curve for polystyrene (PS).

3. Results

Figs. 1 and 2 show GC and HPLC results from the hydrolytic degradation of PHB and PHB–co-PHV in MeOH–NaOH for up to 3 h. The GC and HPLC analyses give similar results indicating that 3-hydroxybutyrate, 3-hydroxyvalerate and crotonic acid are formed as degradation products during the alkaline hydrolysis of PHB and PHB-co-PHV. Crotonic acid, that is also formed by the dehydration of monomeric 3-hydroxybutyrate, becomes the main product during prolonged degradation.

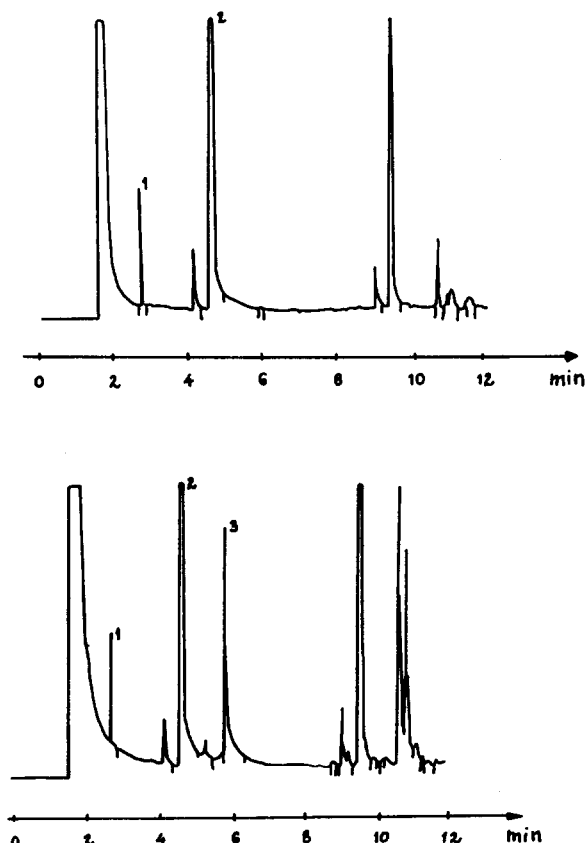Fig. 3 shows the conversion of β-hydroxybutyrate to crotonic acid. This reaction is very

Fig. 1. Gas chromatograms of PHB (top) and PHB–PHV (bottom) subjected to alkaline hydrolysis in MeOH–NaOH at −5°C for 1 h. Peaks: 1 = crotonic acid (2.9 min); 2 = β-hydroxybutyrate (4.6 min); 3 = β-hydroxyvalerate (5.8 min).

fast and it is therefore always difficult to detect the hydroxy acid.

With detection by UV absorption at 205 nm, for example crotonic acid absorbs over 400 times more strongly than the monomer itself, because of the conjugated C=C–C=O system present, and HPLC can therefore give a misleading indication of the major species present. Results from the mild alkaline hydrolysis in phosphate buffer show the same products but the degradation was much slower at the lower pH value. The increase in the degradation rate as the hydrolytic medium becomes more alkaline is possibly due in part to the increasing solubility of the polymer degradation products at higher pH as well as to the expected rise in hydrolysis rate

Fig. 2. Liquid chromatograms of PHB (left) and PHB–PHV (right) subjected to alkaline hydrolysis in MeOH–NaOH at −5°C for 3 h. Peaks: crotonic acid (1.2 min); β-hydroxybutyrate (1.6 min); β-hydroxyvalerate (2.6 min).

Fig. 3. Conversion of β-hydroxybutyrate to crotonic acid.

[11]. A rise in temperature from 25 to 60°C increased the degradation rate, as shown by the amount of crotonic acid formed at the different temperatures after one year in a phosphate buffer (pH 10.5) (Table 1). The crotonic acid was detected by GC and the numbers in Table 1 correspond to the amount crotonic acid detected

Table 1
Amount of crotonic acid in accelerated aged microbial polyhydroxyalkanoates as obtained by GC

Temperature (°C)	PHB sample (area counts)	PHB–PHV sample (area counts)
25	0.001	0.004
60	0.2	0.4

relative to the internal standard (n-pentadecane). It is suggested that changes at the surface occur concurrently with a bulk erosional process that results from the diffusion from the matrix of the products of the chain scission processes. Initially only very-low-molecular-mass fragments are able to diffuse out, but as the process proceeds the matrix becomes progressively more porous allowing an increasing loss of higher-molecular-mass degradation products [12]. Possible hydrolysis products of PHB include the monomer, oligomers and derivatives of these that have been further modified in side reactions. Oligomers 2–7 units long and with a methyl ester of the COOH-terminus could be isolated in low yield after mild alkaline hydrolysis of PHB. Oligomeric species that had been dehydrated at the OH-terminus to give a C=C double bond were also present [13].

HS-GC–MS analyses showed the presence of crotonic acid (HS-GC and MS), 2-ethoxyethyl acetate (MS) and acetaldehyde (HS-GC) in thermo-oxidized samples of PHB and PHB–co-7% PHV. The amount of 2-ethoxyethyl acetate decreased with increasing ageing time and it is thus presumably not a degradation product.

Fig. 4 shows the increase in the amount of crotonic acid derived from PHB and PHB–co-7% PHV aged at 100°C. Pure PHB yields an increasing amount of crotonic acid during ageing up to 350 h whereas no detectable amounts of

Fig. 4. Crotonic acid produced from thermo-oxidized PHB (▲, broken line) and PHB–co-7% PHV (△, solid line) at 100°C. y-Axis in surface units.

crotonic acid were produced by the copolymer during the first 50 h of ageing. The amount of crotonic acid produced during the whole period was lower from the copolymer than from the pure homopolymer. This may, to some extent, be due to the lower amount of PHB in the copolymer than in the homopolymer. It also indicates either that the copolymer is less susceptible to thermo-oxidative degradation than pure PHB or that the degradation of PHB–PHV proceeds through another mechanism.

The results of the SEC analysis of pure PHB are shown in Fig. 5. Both M_n and M_w decrease during the first 350 h of ageing at 100°C. After 500 h of ageing an increase in molecular mass is observed, indicating that some cross-linking has occurred. Cross-linking seems to occur earlier at higher ageing temperatures. After 15 min ageing at 180°C the sample is insoluble, presumably due to gel formation.

Fig. 6 presents the average M_n together with the amount of crotonic acid produced. The lowest value of M_n corresponds to a maximum crotonic acid produced in the samples. Fig. 6 also shows that the increase in M_n at 500 h corresponds to a decrease in the amount of crotonic acid produced.

It is suggested that the thermal decomposition of PHB and its copolymers is a result of random chain scission that occurs by the six-membered ring ester decomposition process, shown in Fig.
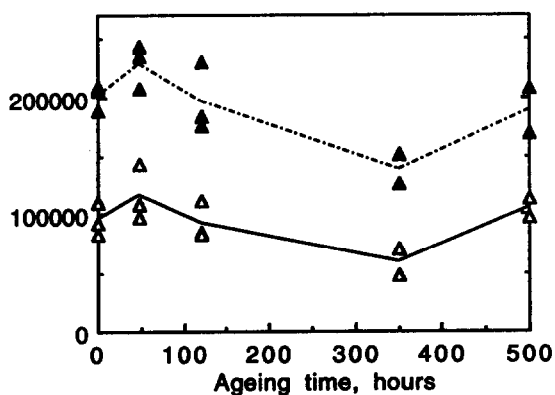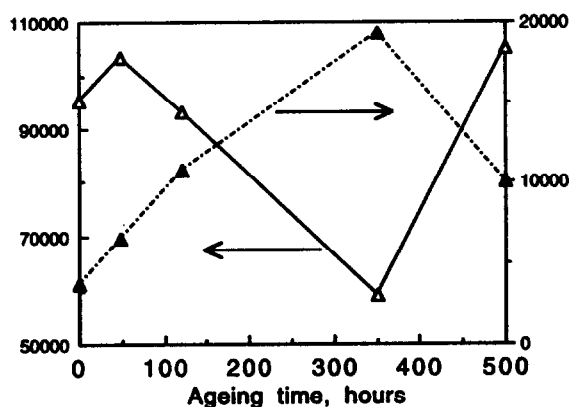

Fig. 6. M_n (△, solid line) and amount of crotonic acid (▲, broken line) produced as a function of ageing time at 100°C. y-Axes in surface units (to the right) and g/mol (to the left).

7, which forms carboxylic chain ends in the material [10,14].

The rapidly expanding production and use of plastic materials means that biodegradable plastics are urgently needed in view of the adverse environmental effects of non-biodegradable plastics. The development of plastics that do not pollute the global environment has become an extremely important objective, and biodegradable plastics such as the microbial polyesters might be the key to solving the waste disposal problem. Using the microbial polyesters in degradable materials thus demands a thorough knowledge of the degradation products formed and their interaction with the environment. Several studies have been made in order to investigate the low-molecular-mass products formed during degradation of inert and degradable polymers. For example in biodegraded casein, which is used as an additive in self-levelling concrete, it

Fig. 5. M_n (△, solid line) and M_w (▲, broken line) of PHB samples as a function of ageing time at 100°C. y-Axis in surface units.
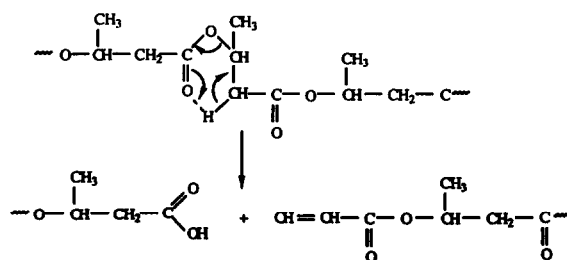

Fig. 7. Six-ring ester decomposition.

was possible to detect series of volatile organic acids and monoamines and [15] by using LC, polyamines were also found [16]. Using GC and GC–MS, degradation products such as hydrocarbons, carboxylic acids and ketones have been identified in thermo-oxidized low-density polyethylene (LDPE) and in degradable LDPE with corn starch and prooxidant [17,18].

Applications of biodegradable polyesters may include agricultural mulching films, packaging films, bottles and containers. These polyesters could also be useful in marine environments, such as fishing nets, where it has been estimated that one million marine animals are killed every year either by choking on floating plastic materials or by becoming entangled in non-degradable plastic debris [14]. The biocompatibility and slow resorption of microbial polyesters in biological environments (in vivo) makes it possible for PHB to be a safe medical material. PHB may for example be used as a slow drug-releasing material implanted inside the body. The final degradation product is D(−)-3-hydroxybutyrate, which is a physiological compound always present in the human body as an energy source [19]. The ready sorption of the polyhydroxyalkanoate latex by fibrous materials such as paper or non-woven fabric suggests applications as a binder, coating material or barrier. These applications in fibrous constructions become particularly attractive when the natural biodegradability of polyhydroxyalkanoates is considered [20].

4. Conclusions

Crotonic acid, β-hydroxybutyrate and β-hydroxyvalerate were detected and identified in accelerated aged microbial polyesters using GC, LC and GC–MS. The microbial polyesters are readily hydrolysed and biodegraded by microorganisms and enzymes. The hydrolysis reaction produces mostly the monomers. The crotonic acid produced is partly explained by the very rapid conversion of β-hydroxybutyrate to the corresponding acid. Using HS-GC–MS, volatile products are readily identified, avoiding extractions that can lead to loss of products.

5. Acknowledgement

6. References

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