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Production and characterization of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxybutyrate) synthesized by *Ralstonia eutropha* in fed-batch cultures

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Abstract

Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxybutyrate) [P(3HB-*co*-3HV-*co*-4HB)] terpolymers were produced in fed-batch cultures of *Ralstonia eutropha* (reclassified from *Alcaligenes eutrophus*) using glucose, propionic acid and either 4-hydroxybutyric acid or γ-butyrolactone as carbon sources. The thermal and mechanical properties of terpolymers containing different proportions of the constituent monomers were characterized. Most of the terpolymers possessed low melting temperatures and the majority possessed crystallization half-times between 4 and 16 min, compared with 0.5 min for poly(3-hydroxybutyrate) [P(3HB)]. For certain polymer compositions, containing approximately 10 mol% of both 3-hydroxyvalerate (3HV) and 4-hydroxybutyrate (4HB), the mechanical properties were superior to those of P(3HB-*co*-0–30% 3HV) range of polymers and did not display any signs of deterioration over a one month test period. A compression molded polymer film containing 78 mol% 3HB, 11 mol% 3HV and 11 mol% 4HB was found to possess an elongation to break of 700% after one month, compared with 740% after molding, but ultimately declined to 35% after 13 months. The aging process was found to depend upon the primary crystallization rate. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Polyhydroxyalkanoates; Terpolymers; Mechanical properties

1. Introduction

Many bacteria accumulate polyhydroxyalkanoates (PHA), of which poly(3-hydroxybutyrate) [P(3HB)] is the best known example. The composition of PHA is determined both by the organism and the carbon sources available during polymer accumulation. Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] is another well-studied PHA, which is stronger and more flexible than P(3HB) [1]. P(3HB-*co*-3HV) can be produced in fed-batch culture from glucose and propionic acid using the bacterium *Ralstonia eutropha* (reclassified from *Alcaligenes eutrophus* [2]). The composition of P(3HB-*co*-3HV) can be varied in the range 0–30 mol% 3HV by altering the amounts of glucose and propionic acid supplied during polymer accumulation because 3-hydroxyvalerate (3HV) monomers are produced from propionic acid [3]. As the 3HV mole fraction is increased the polymer becomes more flexible.

When stored at room temperature after melt-processing, both P(3HB) and P(3HB-*co*-3HV) undergo an aging process [4,5], characterized by serious embrittlement of these plastics. For example, the elongation to break of P(3HB) decreased from approximately 50% (immediately after molding) to less than 10% after 2 weeks, and attained a final value of approximately 5% after storage at room temperature for 100 days [5]. The aging process was 90% complete within one month and was accompanied by an exponential decline in elongation to break [5]. Dynamic mechanical analysis (DMA) studies [6] revealed a decrease in the storage modulus (E') of the polymer at the T_g, over the same period of time, and a large reduction in dynamics loss peak was also observed.

A broadening of the T_g was observed for P(3HB) over a period of 150 days. This was attributed to constraints imposed on the amorphous phase by progressive (secondary) crystallization [7]. Dilatometry measurements showed that storage of P(3HB) is accompanied by a significant

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increase in density, again indicating that crystallization is continuing. This comparatively small loss of the amorphous phase leads to a marked change in properties, and relates to the reduction in capacity of the amorphous phase to dissipate energy by viscous flow. The proximity of the T_g of P(3HB) to room temperature limits the rate of secondary crystallization due to hindered diffusion of the polymer chains [6]. Lowering the T_g by the addition of a plasticizer may therefore have an effect on the rate of the secondary crystallization of P(3HB-*co*-3HV).

The effect of secondary crystallization on the amorphous phase was easily demonstrated by comparison of the solubilities of as-molded P(3HB) with an aged sample. As-molded P(3HB) was observed to dissolve readily in chloroform, while the aged sample had to be refluxed with chloroform, indicating a reduction in accessibility to the amorphous phase [5]. It was concluded [5] that aging is an intrinsic property of the polymer and not related to extraneous compounds or physical aging, as previously suggested [4].

PHA of high flexibility is produced by *Pseudomonas oleovorans*. These polymers (PHA_m) are heteropolymers of C_6–C_{12} 3-hydroxyalkanoates and display typical elongation to break of 250–350% [8]. No yield stress was observed for a sample of PHA_m produced from sodium octanoate by *P. oleovorans*, as PHA_m polymers are elastomers [9]. The same sample had a low ΔH_m (17.5 J g^{-1}) and an elongation to break of 380%. It has been noted that a slow rate of crystallization and a low heat of fusion is characteristic of PHA_m, in comparison with P(3HB) [8]. PHA_m produced from octanoate by *P. oleovorans* [10] was found to reach maximum crystallinity (33%) after storage at room temperature for 1 week [8]. Based on this observation, PHA_m appear to reach maximum crystallinity relatively quickly and hence no deterioration in mechanical properties would be expected after this time, but no aging study has yet been reported for PHA_m. The properties of PHA vary from tough brittle thermoplastics to elastomers depending on the nature and proportions of their constituent monomers, but it is not known whether aging is a general characteristic.

In an attempt to prevent or retard the detrimental aging of P(3HB-*co*-3HV), the effect of the incorporation of a third monomer, 4-hydroxybutyrate (4HB), into PHA produced by *R. eutropha* was investigated. It has been demonstrated that *R. eutropha* is able to synthesize 4HB monomer from structurally related carbon sources such as 4-hydroxybutyric acid [11], γ-butyrolactone [12] or 1,4-butanediol [12]. Introduction of 4HB into the polymer chain should limit the extent of crystallization, as the 4HB monomers are unable to cocrystallize with 3HB [13]. A previous study showed that the mole fraction of 4HB in P(3HB-*co*-4HB) had a significant effect on mechanical properties [14]. Tests on solvent cast films revealed an increase in elongation to break, from 5 to 1320%, as the mole fraction of 4HB increased in P(3HB-*co*-4HB) from 0 to 82 mol% [14].

The aim of the present work was to produce PHA terpolymers in large quantities by using phosphate-limited fed-batch culture and to characterize their thermal and mechanical behavior. Further tests were carried out to assess the effect of storage on mechanical properties.

2. Experimental

2.1. Production of P(3HB-co-3HV-co-4HB)

R. eutropha NCIMB 40529 was cultivated as previously described [15]. For the production of PHA terpolymers the feed was composed of the following: glucose, propionic acid and either γ-butyrolactone or sodium 4-hydroxybutyric acid. The feed was prepared by dissolving glucose in water and then sterilizing by autoclaving (121°C, 15 min). After cooling, propionic acid and either γ-butyrolactone or 4-hydroxybutyric acid was added. The total amount of carbon available in the feed was 500 g in each experiment. The volume of the feed was adjusted to 1.5 l by the addition of sterile water. The feed was pumped into the fermenter (37.5 ml h^{-1}) over a 40 h period, unless otherwise stated, after the exhaustion of glucose. At the end of the feeding period the bacteria were recovered by centrifugation (8000 g, 15 min, 4°C), washed twice with water, and lyophilized. Determination of residual glucose and phosphate, and purification of polymer were carried out as previously described [15].

The PHA content of bacteria was determined by GC of methylhydroxyacids produced by methanolysis [16] of lyophilized cells as previously described [15]. P(3HB), P(3HB-*co*-98% 3HV) (produced by *Rhodococcus ruber* from valeric acid) and 4-hydroxybutyric acid (Sigma) were used as standards for estimation of polymer content.

2.2. Analysis of PHA

NMR analysis was performed on terpolymers (40–70 mg) in $CDCl_3$ (1 ml). The polymers were dissolved by shaking, and mild heating was applied if required. Chromium acetyl acetonate (3 mg) was added to the solution to act as a relaxation agent. ^1H and ^{13}C NMR spectra were acquired with Varian Unity 400 spectrometers operating at 400 MHz nominal ^1H frequency. The probe temperature was 30°C. The data were acquired and processed on Sun computers using Varian-based Monsanto-developed software (MNMR). Intensities were determined using a deconvolution program also developed by Monsanto.

Weight average molecular mass (M_w) of purified polymers was determined by GPC as previously described [15].

Differential scanning calorimetry (DSC) measurements were performed on a Perkin–Elmer DSC7 instrument and calibration was performed with indium and zinc. Samples (5–10 mg) were heated (20°C min^{-1}) from 20 to 200°C and then cooled to 20°C at a rate of 20°C min^{-1}. To determine the glass transition temperature (T_g), samples were cooled from +200 to −70°C at a rate of 50°C min^{-1} and then

Table 1
Production of PHA terpolymers

Feed composition[a]			Biomass content ($g\,l^{-1}$)	PHA (% w/w)	PHA composition[b] (mol%)			$10^{-5}\,M_w$ ($g\,mol^{-1}$)	M_w/M_n	[η] ($dl\,g^{-1}$)
Glc	Pr	γBl			3HB	3HV	4HB			
85	5	10	136	62.3	93	2	5	4.73	5.80	4.42
70	20	10[c]	66	63.6	91	4	5	5.41	3.96	2.82
60	20	20	67	52.6	89	6	5	5.54	4.90	4.74
60	20	20	122	51.0	88	4	8	4.10	4.42	nd[d]
65	35	5[c]	64	50.0	87	7	6	6.27	4.03	3.87
60	37	3	93	54.0	85	14	1	7.44	3.66	3.81
50	40	10	106	45.6	78	11	11	5.39	6.46	3.77
50	35	15	104	57.7	78	9	13	2.57	4.12	2.16
60	30	10	84	62.0	74	20	6	3.21	5.42	1.96

[a] Feed composition refers to the percentage of the total carbon available from each substrate: Glc, glucose; Pr, propionic acid; γBl, γ-butyrolactone.
[b] From carbonyl region of ^{13}C NMR spectrum.
[c] Sodium-4-hydroxybutyrate used instead of γ-butyrolactone.
[d] nd = Not determined.

heated to 200°C at a rate of 20°C min^{-1}. Crystallization rates were determined by DSC. Samples (5–10 mg) were heated (20°C min^{-1}) from 20 to 200°C, held for 1 min and then quench–cooled (100°C min^{-1}) to temperatures in the range of 60–90°C. The crystallization half-time ($T_{1/2}$) was taken as the fastest rate of crystallization.

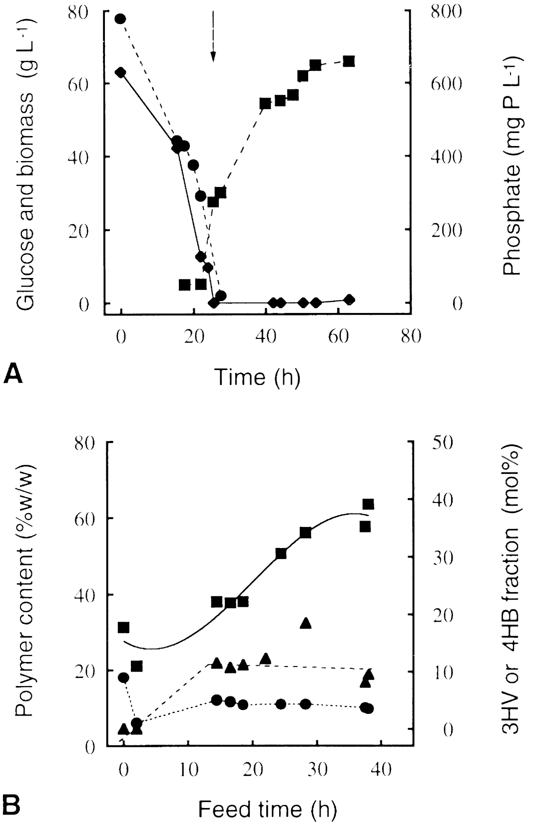

Fig. 1. Typical progress of fed-batch culture showing: (A) glucose (●); phosphate (◆) and biomass (■) concentrations throughout the growth and polymer accumulation phase; and (B) polymer content of bacteria (■), and mole fraction of 3HV (●) and 4HB (▲). Arrow denotes initiation of feeding.

Compression molded films (thickness between 0.17 and 0.20 mm) were made by molding the polymer powder (100 g) at T_m + 10°C under a pressure of 4.4 MPa. After molding, the films were annealed at 75°C for 15 min in a vacuum oven with nitrogen bleed to facilitate crystallization. Samples were aged at constant temperature (22°C) and humidity (50%). Tensile test pieces (2.54 mm width, 10.16 mm gauge length) were cut from polymer films using steel ASTM regulation punches. Mechanical testing was carried out using a Monsanto tensometer, using a cross-head speed of 10 mm min^{-1}. Samples were analyzed according to ASTM D638 [17].

3. Results and discussion

3.1. Production of PHA terpolymers in fed-batch culture

Fed-batch culture experiments were undertaken in which a 4HB-precursor substrate was included in a mixed substrate feed with glucose and propionic acid. The amount of each carbon source supplied determined the composition of the PHA produced (Table 1). The composition and weight-average molecular mass (M_w) of each polymer are shown in Table 1. The M_w of the terpolymers was within the range previously observed for PHA produced by *R. eutropha* [18]. The growth phase and subsequent polymer accumulation phase for the production of P(91/4/5) (representing a polymer containing 91 mol% 3HB, 4 mol% 3HV and 5 mol% 4HB) can be seen in Fig. 1. The feeding of the PHA substrates was initiated at the time of glucose exhaustion, which occurred at about the same time as phosphate exhaustion. Throughout the feeding period, 3HB, 3HV and 4HB monomers were synthesized and the composition of the accumulated polymer remained approximately constant.

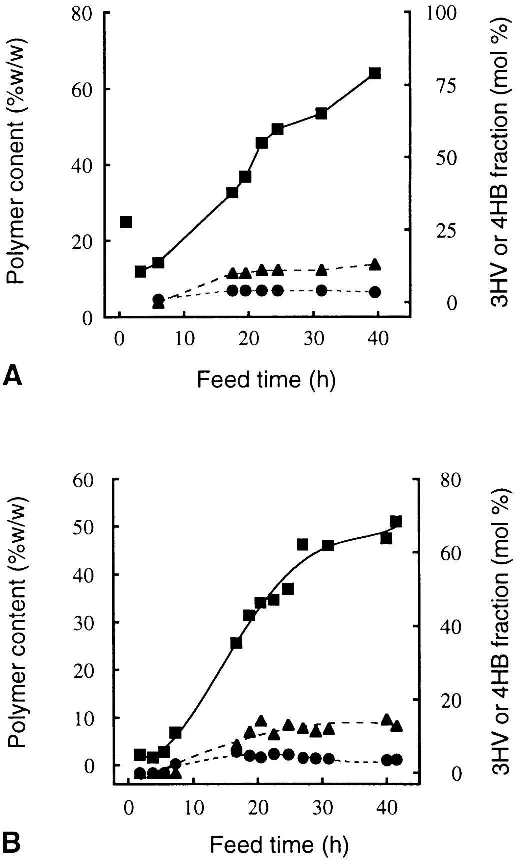

Fig. 2. Polymer accumulation phase during the production of: (A) P(88/5/7); and (B) P(88/4/8). Shown are the polymer content (■), and mole fraction of 3HV (●) and 4HB (▲).

This was also found to be the case during the production of P(88/5/7), P(88/4/8) (Fig. 2) and the other terpolymers produced in this study.

The range of polymer composition produced by *R. eutropha* in fed-batch culture appears to be quite narrow, with a maximum of about 30 mol% 3HV plus 4HB (Table 1). The industrial process used to manufacture P(3HB-*co*-3HV) did produce polymers containing up to 30 mol% 3HV and used a *R. eutropha* strain with an increased 3HV yield from the propionic acid [3]. Increased mole fractions of 3HV in terpolymers were achieved using valeric acid; however, due to its toxicity and reduction of the polymer yield in fed-batch culture (data not shown) it was not used as a substrate for terpolymer production in this study. To produce PHA containing over 15 mol% 3HV or 4HB, up to 40% of the total carbon in the substrate feed has to be derived from either γ-butyrolactone or propionic acid, respectively. Using larger amounts of 3HV or 4HB precursor resulted in the accumulation of these substrates in the culture to a concentration, which inhibited further polymer synthesis (data not shown).

^{13}C NMR analysis of the terpolymers revealed that the number-average sequence length [NASL] [19] of the three monomers was in agreement with what would be expected for a random (Bernoullian) distribution along the polymer chain (Table 2). The polymer would be expected to be random because the monomer composition remained constant throughout the accumulation phase, and P(3HB-*co*-3HV) produced from mixed carbon sources have proved to be random copolymers [20]. A typical NMR spectrum of a PHA terpolymer, with peak assignments is shown in Fig. 3.

3.2. Thermal properties of PHA terpolymers

The incorporation of both 3HV and 4HB monomer into P(3HB) to produce terpolymers had a profound influence on the thermal properties of the polymer. In most cases the T_m was in the range 106–147°C (Fig. 4), which is markedly lower than that observed for P(3HB-*co*-3HV) or P(3HB-*co*-4HB) containing the same mole fraction of 3HB [18]. Most of the terpolymers were slow to crystallize, relative to P(3HB), possessing crystallization half-times ($T_{1/2}$) of approximately 4–5 min. The decrease in crystallization rate can be explained by the disruption of crystallization by the relatively bulky 4HB monomers. The 4HB monomer cannot cocrystallize within the P(3HB) lattice because it possesses a longer backbone than that of 3HB [13]. It has also been suggested that steric hindrance exists between the

Table 2
^{13}C NMR analysis of PHA terpolymers

Composition			Number average sequence length observed (calculated for Bernoullian)		
3HB (mol%)	3HV (mol%)	4HB (mol%)	3HB	3HV	4HB
93	2	5	20.30 (15.15)	1.00 (1.01)	1.00 (1.05)
91	4	5	9.10 (11.36)	1.00 (1.04)	1.00 (1.05)
89	6	5	9.41 (9.90)	1.00 (1.06)	1.00 (1.04)
88	4	8	8.15 (8.33)	1.00 (1.05)	1.00 (1.09)
87	7	6	7.28 (8.26)	1.03 (1.05)	1.00 (1.08)
85	14	1	5.84 (5.59)	1.23 (1.20)	1.00 (1.01)
78	11	11	5.46 (4.55)	1.27 (1.13)	1.10 (1.12)
78	9	13	4.41 (4.69)	1.16 (1.08)	1.09 (1.16)
74	20	6	3.83 (3.86)	1.41 (1.25)	1.40 (1.06)

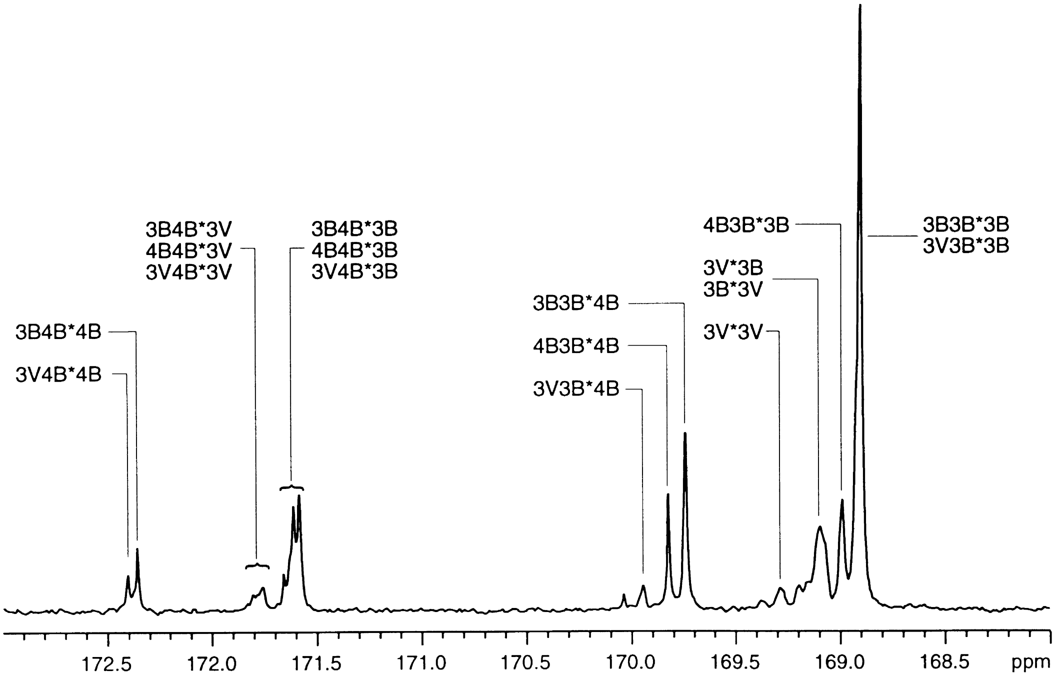

Fig. 3. Expansion of the carbonyl region of a ^{13}C NMR spectrum of P(63/12/25) showing diad and triad assignments.

methyl (or ethyl) side-chain of the 3HB (or 3HV) monomer and the adjacent carbonyl oxygen atoms of the 4HB monomer, requiring deformation of the P(4HB) lattice to accommodate 3HB monomers [21]. The 3HV monomers can, to a certain extent, be incorporated within the P(3HB) lattice, where they act as defects in the lattice causing an increase in the unit cell dimensions and hence a decrease in the melting temperature [21]. It is therefore proposed that the amorphous phase of the polymer contains the majority of 4HB and a proportion of 3HB monomers, thus reducing the overall crystallinity of terpolymers compared with that of P(3HB-*co*-3HV).

The presence of both 3HV and 4HB monomers within the PHA terpolymer has therefore a concerted effect on the thermal properties. The heats of fusion (ΔH_m) for terpolymers were found to be low relative to P(3HB), with most in the range of 12–50 J g^{-1} (Fig. 4). The low ΔH_m of certain terpolymers is indicative of a largely amorphous polymer. One PHA terpolymer, P(74/20/6) possessed a melting temperature close to that of P(3HB). This polymer contained the lowest proportion of 3HB monomers and very low ΔH_m. The crystalline regions of this polymer are small and are probably composed primarily of 3HB monomers, with the larger amorphous phase containing a soluble mixture of all three monomers. It appears as though only a limited amount of crystallization can take place within polymers containing at least 10 mol% 3HV and 10 mol% 4HB monomers.

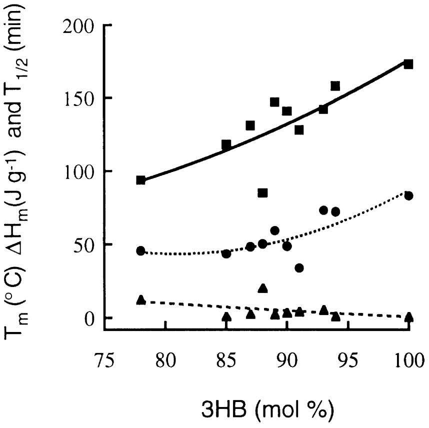

Fig. 4. T_m (■), ΔH_m (●) and crystallization half-time (▲) of P(3HB-*co*-3HV-*co*-4HB) as a function of 3HB content.

In a previous study on the crystallization rate of PHA it was reported that a sample of P(79/21/0) continued to crystallize for several weeks after casting from solution [22]. An investigation of segmental dynamics in P(3HB-*co*-4HB) revealed that the crystalline regions of the polymer were comprised solely of 3HB monomers [23]. This is reflected in a slight decrease in melting temperature up to approximately 40 mol% 4HB [21]. The degree of crystallinity of P(3HB-*co*-4HB) is, however, greatly reduced from 55% for P(3HB) to just 23% for P(58/0/42) [21]. In the same study the crystallization rate of P(82/0/18) was found to be slightly slower than that observed for P(3HB), but significantly faster than that recorded for P(79/21/0). This is due to the exclusion of 4HB from the crystalline phase in P(82/0/18), and the slow cocrystallization of P(79/21/0).

Table 3
Effects of storage on the mechanical properties of PHA terpolymers

Terpolymer	Age (days)	Strength[a] (MPa)	Elongation[b] (%)	*E*[c] (MPa)
P(91/4/5)	0	11	45	272
	7	31	12	567
	30	33	13	391
P(89/6/5)	0	19	87	313
	7	13	28	260
	30	21	7	1095
	180	21	12	930
P(88/4/8)	0	27	534	232
	7	17	300	485
	26	14	230	473
	92	11	120	764
P(85/14/1)	0	26	344	380
	7	19	208	501
	30	nd[d]	10	1068
P(78/11/11)	0	17	746	78
	7	25	755	207
	30	23	699	201
	120	14	556	158
	395	4	35	386
P(78/9/13)	0	15	753	146
	7	8	588	89
	30	10	260	327
	180	6	68	396
P(74/20/6)	0	33	492	285
	7	19	477	131
	30	16	299	519
	180	15	243	503

[a] Tensile strength at break (mean standard deviation 26%).

[b] Elongation to break (mean standard deviation 33%).

[c] Young's modulus (mean standard deviation 12%).

[d] Nd = not determined, 3–5 sample bars were used for each determination.

3.3. An aging study of PHA terpolymers

It has previously been reported that P(3HB-*co*-4HB) are generally more flexible than P(3HB-*co*-3HV) [24]. Tensile test data obtained from solvent cast films showed that the elongation to break increased as the 4HB-mole fraction increased [24].

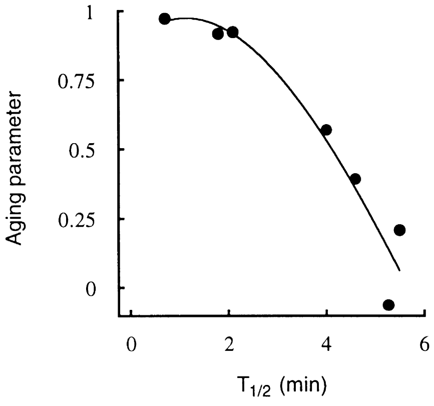

Fig. 5. The relationship between crystallization half time and aging parameter (●) in PHA terpolymers.

Terpolymers with either a 3HV or 4HB mole fraction below 10 mol% were found to age in a similar manner to 3HB/3HV copolymers; a reduction in elongation to break and a corresponding increase in Young's modulus were observed (Table 3). The tensile strength of these polymers decreased with time. The incorporation of around 10 mol% 3HV and 10 mol% 4HB appears to prevent short-term aging and no significant change in the elongation to break of such terpolymers was observed over one month (Table 3). The tensile strength increased slightly over a one-month period, as did the Young's modulus. These terpolymers were found to possess a very low heat of fusion (typically 12–15 J g^{-1}) when compared with P(94/6/0) (72.2 J g^{-1}). This may indicate a reduction in crystallinity or the molar heat of fusion. If the polymers are of low crystallinity, this could explain their improved mechanical properties. Obviously a larger proportion of amorphous phase would lead to an increased capacity to dissipate energy, and increase the flexibility of the polymer. P(74/20/76), P(89/6/5) and P(78/9/13) displayed signs of aging. This is most notable in the large increase in Young's modulus observed over a one-month test period (Table 3). A reduction in elongation to break was also seen, although the decline was not as pronounced as for other copolymers and terpolymers. P(89/6/5) initially possessed a low elongation to break, which decreased further upon storage and was accompanied by a large increase in the Young's modulus. This polymer was found to have quite a high ΔH_m (59.2 J g^{-1}) and crystallized faster than other terpolymers. Tensile test data generated after 13 months storage showed that P(78/11/11) possessed an elongation to break of 35%. These data convincingly demonstrate that these terpolymers do age, albeit over a longer period of time than P(3HB) or P(3HB-*co*-3HV).

3.4. Relationship between aging and crystallization rate in PHA terpolymers

Of the three parameters tested, the elongation to break showed the greatest change during storage of the PHA terpolymers. To facilitate comparison of aging, an aging parameter, calculated from the elongation to break at 0 and 28 days, was defined (Eq. (1)).

$$\text{Aging parameter} = \frac{(\text{elongation to break}_0 - \text{elongation to break}_{28})}{\text{elongation to break}_0} \quad (1)$$

where subscripts 0 and 28 indicate storage time in days.

A plot of the aging parameter of PHA terpolymers against the crystallization half time can be seen in Fig. 5. It is clear that an increase in crystallization half time leads to a reduction in the effect of short-term storage on mechanical properties. To achieve the longer crystallization half-times the polymers must contain around 10 mol% 3HV and 10 mol% 4HB as is the case with P(78/11/11) and P(80/11/9). Terpolymers with crystallization half times greater than 5 min displayed much slower deterioration in mechanical properties due to a decreased rate of secondary crystallization upon storage. The reduction in the crystalline phase caused by the incorporation of approximately 10 mol% 3HV and 10 mol% 4HB may hinder secondary crystallization due to the steric considerations discussed previously.

4. Conclusions

The presence of <10 mol% of 4HB units within a terpolymer of 3HB, 3HV and 4HB monomers has a negligible effect on the aging characteristics of the PHA, although a retardation of the process was observed. On a macro scale the effects of aging are manifested in the deterioration of the mechanical properties of the polymer with time and are linked to the process of crystallization. PHA terpolymers which aged quickly were those containing a relatively low proportion of 4HB monomers (<10 mol%) and crystallization half-times below 4 min. In contrast, P(3HB-*co*-3HV-*co*-4HB) containing 10 mol% 3HV and 10 mol% 4HB displayed no signs of short-term aging. The mechanical properties remained relatively unchanged during a one month period, during which 90% of the aging process is completed in P(3HB-*co*-3HV). The PHA terpolymers, which aged slowly also, displayed very low heats of fusion and long crystallization half-times of over 5 min, indicative of a polymer of low crystallinity. These polymers possess a sufficiently long crystallization half time to preclude significant secondary crystallization within the one-month test period. The properties of these terpolymers offer some improvement in flexibility over P(3HB) and P(3HB-*co*-3HV) but display signs of long-term aging.

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