polymer communications

The amorphous state of bacterial poly[(*R*)-3-hydroxyalkanoate] *in vivo*

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Poly[(*R*)-3-hydroxyalkanoate]s (PHAs) are bacterial storage polyesters, which are accumulated by a range of micro-organisms as intracellular granules. In contrast to the isolated PHA, the nascent polymer in the granules does not crystallize but remains amorphous at ambient temperature. A lot of studies have been devoted to this phenomenon and all claimed the *in vivo* presence of a highly effective plasticizer. In contrast, this paper demonstrates that the native granules do not contain a plasticizer and that the amorphous state of *in vivo* PHA can be explained simply by straightforward crystallization kinetics.

(Keywords: poly[(*R*)-3-hydroxyalkanoate]; poly(hydroxybutyrate); PHB; bacterial polyesters; crystallization kinetics; native granules)

Introduction

Poly[(*R*)-3-hydroxyalkanoate]s (PHAs) are microbial storage polyesters, accumulated by a variety of bacteria as a reserve of carbon and energy[1,2]. The polymer appears as distinct intracellular granules, which are typically 0.2–0.7 μm in diameter[3] and possess a membrane-like coating composed of lipid and protein, representing some 0.5 and 2%, respectively, of the granule weight[4]. The membrane lipids and phospholipids are assumed to stabilize the interface between the hydrophobic contents of the granule and its aqueous environment, whilst the activities of the PHA polymerase and depolymerase are associated with the membrane proteins.

Due to their natural origin, PHAs have an exceptional stereochemical regularity, which enables the polymer to crystallize[5,6]. For example, bacterial poly[(*R*)-3-hydroxybutyrate] (PHB), which is abundant in nature, possesses a crystallinity typically around 60% and a crystalline melting temperature (T_m) at ~175°C when crystallized from the melt[7].

Initially, the native PHB granules were believed to be crystalline like the isolated polymer[8]. However, the ability of bacteria to readily dissimilate their granule reserves raised the question of how the depolymerase enzyme could so efficiently gain access to a crystalline substrate. Furthermore, this susceptibility of the granules to depolymerase action appeared to be lost in several treatments[4,9]. Surprisingly, recent findings[10–14] demonstrated that PHB *in vivo* is in fact in the amorphous state and that crystallization is induced by specific treatments[13,15], which typically form part of the isolation procedure. These treatments include sonification, centrifugation, drying, several enzyme treatments, and exposure to acetone, alkalines or extreme ionic strengths.

As early as 1968, it was stated[4] that the granules must contain some factor of which removal or damage results in loss of depolymerase susceptibility. Several studies have been devoted to estimate this factor, which according to the recent findings should prevent crystallization of the nascent polymer. Since intensive drying induces crystallization, water was claimed[12,15] to be involved, although it is possibly not the only factor since granule crystallization can also be initiated in an aqueous environment. Accordingly, Mas *et al.*[16] estimated the density of *in vivo* PHB to be 1.16 g cm^{-3}, whereas isolated PHB possesses a density of ~1.24 g cm^{-3}, and they attributed this discrepancy to a water content of 40% in the granules. Their conclusion was incorrect however, since the *in vivo* density in fact matches that of amorphous PHB[17]. Alternatively, the factor was suggested to be a lipid[13], which is inactivated on exposure to acetone, alkalines or lipase. Thin-layer chromatography of the acetone extracts disclosed the presence of two lipid components, but the amounts present were minor.

Both factors proposed were referred to as highly effective plasticizers[13,15,18]. The main reason for this was the observation[3,18–20] that PHB granules show ductile deformation upon fracturing in liquid nitrogen. This suggests that the PHB glass transition temperature (T_g), which was established[17] at ~5°C, is reduced *in vivo* by at least 200 K. However, a plasticizer does not prevent crystallization unless it is present in a large enough quantity to dissolve the polymer. In contrast, ^{13}C n.m.r. analysis[12] showed that the mobility of PHB *in vivo* matches that of a polymer melt, which is considerably lower than the mobility in solution.

This paper illustrates that the native granules do not contain a highly effective plasticizer and that the amorphous state of *in vivo* PHA should be explained by straightforward crystallization kinetics.

Experimental

Bacteria of the strain *Alcaligenes eutrophus* H16, containing ~70% wt/dry wt PHB, were provided by ICI Bioproducts & Fine Chemicals (Billingham, UK). Additionally, PHB polymer was supplied as a commercial Biopol® sample ($M_w = 539$ kg mol^{-1}, $M_w/M_n = 3.5$, $T_m = 174$°C), which was isolated from the biomass using the commercial method[21].

Another type of PHA was provided by B. Witholt of Groningen Biotechnology Centre. This PHA was

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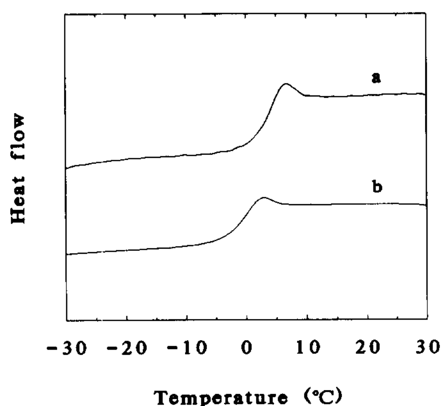

Figure 1 Thermograms of the glass transition of amorphous PHB (a) in lyophilized cells of the strain *A. eutrophus* grown on glucose and (b) isolated from the bacterial cells

accumulated in *Pseudomonas oleovorans* (ATCC 29347) using n-octane as the sole carbon source. Biosynthesis and isolation of the polymer were established according to the procedure described previously by Preusting *et al.*[6], yielding a transparent film ($M_w = 190\,\mathrm{kg\,mol^{-1}}$, $M_w/M_n = 1.9$, $T_m = 56°\mathrm{C}$).

Polymer containing cells of both strains were harvested by centrifugation and freeze dried. Centrifugation was performed below 5000 *g* and freeze drying was not entirely completed in order to prevent premature crystallization of the native granules. The wet bacteria pellet, the lyophilized cells, and the isolated polymer were subjected to calorimetric analysis. Prior to this, the isolated polymer was quenched from the melt to obtain the amorphous state. Thermograms were recorded using a Perkin-Elmer DSC-7 differential scanning calorimeter in the temperature range from -70 to 200°C at a heating rate of $10\,\mathrm{K\,min^{-1}}$. Indium was used for temperature and heat of fusion calibration.

Results and discussion

Using calorimetric analysis, a comparison was made between PHA *in vivo* and the isolated polymer. The amorphous state of the *in vivo* polymer was confirmed by the absence of a melting endotherm in the thermograms of the bacteria. The isolated polymer was made amorphous by quenching from the melt. Assuming the presence of a plasticizing component in the granules, the T_g of the *in vivo* polymer is expected to be considerably lower than that of the isolated polymer. *Figure 1* clearly demonstrates that this is not the case for PHB. However, it should be noted that only the native granules in the dried cells showed a distinct T_g, whereas its determination in wet bacteria was hindered by the vicinity of the ice melting temperature. Still, similar results were obtained for PHA, synthesized by *P. oleovorans* grown on n-octane, which could as well be studied in an aqueous environment (*Figure 2*). The results clearly indicate that native PHA does not contain a highly effective plasticizer.

The absence of a plasticizer is not inconsistent with the amorphous state of the native polymer, since this state can be attributed to crystallization kinetics. Crystallization is known to occur via nucleation and subsequent crystal growth. Nucleation sites are usually provided by impurities. In the absence of these so-called heterogeneous nuclei, crystallization can only be induced by homogeneous nucleation, which is a stochastic event with a relatively low frequency. It is well known[22–24] that crystallization can be retarded to a large extent by subdividing a system into isolated regions, while avoiding heterogeneous nucleation. This way, a nucleus will only affect the region in which it resides and not the whole mass of crystallizable material. In an early study[25], this was demonstrated for polyethylene droplets suspended in viscous oil, and the same principle is used in practice to supercool water[26]. The *in vivo* PHA granules form a natural analogon.

PHA granules are free of extraneous particles and the granule membrane forms a barrier against other cell constituents, so avoiding heterogeneous nucleation. Consistently, in a study concerning the crystallization kinetics of bacterial PHB[27], it was recognized that PHB has an exceptionally low nucleation density. At 20°C, the homogeneous nucleation rate was measured to be as low as 0.1 event $\mathrm{s^{-1}\,mm^{-3}}$. Notably, this value should be regarded as an upper limit, since some heterogeneous nucleation might have occurred at the sample surface or at impurities introduced during the sample preparation. Possessing a typical volume of the order of $10^{-11}\,\mathrm{mm^3}$, the nucleation rate of a PHB granule amounts to a maximum of 10^{-12} events $\mathrm{s^{-1}}$. The probability P that a nucleus appears and the granule crystallizes before time t can be described by an exponential function:

$$P = 1 - \mathrm{e}^{-t/\tau}$$

where the average time until a nucleus appears τ is $\sim 10^{12}$ s.

Of course, if observing an 'infinite' number of granules, as in ^{13}C n.m.r. and X-ray techniques, this same equation gives the crystallized fraction as a function of time. Strikingly, this implies that, assuming a realistic detection limit of $\sim 3\%$ crystallized granules, experimental indications of crystallization *in vivo* can only be obtained after 10^3 years.

All treatments which induce crystallization at much shorter notice, somehow damage the protecting membrane around the granule. Exposure to acetone or alkaline extracts the membrane lipids. Complete removal of water by drying techniques will change the nature of the hydrophobic–hydrophilic interface and affect the membrane conformation. The same accounts for extreme ionic strengths. Intensive centrifugation and sonification damage the membrane by deformation of the granules.

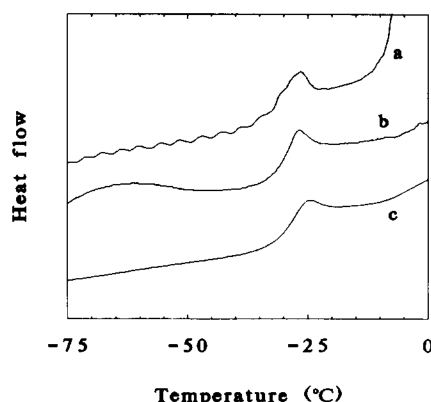

Figure 2 Thermograms of the glass transition of amorphous PHA (a) in living cells of the strain *P. oleovorans* grown on n-octane, (b) in lyophilized cells and (c) isolated from the bacterial cells

Finally, enzymes like lipase, trypsin and subtilisin digest certain membrane constituents. In all cases, the resulting defects in the membrane allow direct contact between the amorphous polymer content of the granule and its environment. This allows exogenous particles to act as (heterogeneous) nucleation sites for crystallization, which highly enhances the crystallization process. Accordingly, a recent study[18] demonstrated that native granules develop crystallization at their surface upon isolation. Of course, the rate of crystallization is related to the damage level of the membrane. The milder the treatment, the lower the rate of crystallization, which was confirmed experimentally[13,15] for centrifugation, alkaline treatment and acetone treatment.

Conclusions

This paper illustrated that, in contrast to earlier claims, native PHA contains no plasticizing component to prevent crystallization of the polymer. The amorphous state of the native polymer should be attributed to the crystallization kinetics of the submicrometre granules, which are dominated by homogeneous nucleation.

Still, the absence of a highly effective plasticizer seems to be inconsistent with the observed ductile behaviour of the granules at temperatures far below their T_g. However, the same behaviour has been observed[28] for submicrometre spheres of polystyrene at temperatures as far as 300 K below their T_g, and this phenomenon should be explained in terms of dimensional effects[29].

Possible future experiments to ascertain the stated theory of granule crystallization are the *in vitro* simulation of the described phenomena using a PHA latex, and the crystallization of a granule in a bacterial cell by introducing a nucleus using micromanipulators.

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