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Use of Poly(ethylene glycol) to Control the End Group Structure and Molecular Weight of Poly(3-hydroxybutyrate) Formed by *Alcaligenes latus* DSM 1122

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Abstract

Poly(ethylene glycol), PEG, which is known to associate with lipid membranes and proteins, was used to control the molecular weight and end-group structure of poly(3-hydroxybutyrate), P3HB, produced by *Alcaligenes latus* DSM 1122. A series of PEGs with varying molecular weights (PEG-106 to PEG-10,000) was added to the *A. latus* incubation medium. In general, PEGs of relatively lower molecular weight and high concentrations of PEG in the medium had deleterious effects on cell growth, polymer yield and P3HB productivity. PEGs with molecular weights at or below 1000 g/mol resulted in the formation of P3HB with reduced molecular weights. The extent of molecular weight reduction was dependent on the concentration and molecular weight of the PEG added to the incubation medium. Larger molecular weight reductions at identical medium concentrations were achieved by using PEG of relatively lower molecular weight. PEG-106 was most effective in regulating P3HB molecular weight in that only a 1% (w/v) medium concentration was required to reduce the number average molecular weight (M_n) by 89% (from 267,000 to 30,000 g/mol). Study of the purified polymers showed that for PEG molecular weights up to 1,000 g/mol, P3HB chains were esterified at their carboxyl terminus with PEG chain segments. Thus, products having "tailored" molecular weights and end-group structure were formed. This was explained by that for P3HB formation by *A. latus*, PEG of sufficiently low molecular weight served as a chain terminating agent. In other words, the *in-vivo* esterification of P3HB chain ends with PEG increases the rate of chain termination relative to propagation and, thereby, results in reduced P3HB molecular weight. © 1997 Elsevier Science Ltd.

Keywords: *Alcaligenes latus*, poly(3-hydroxybutyrate), poly(ethylene glycol).

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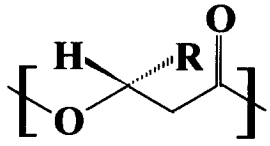
Introduction

Polyhydroxyalkanoates (PHAs) are a family of polyesters that can be biosynthesized from numerous prokaryotic sources. The repeat units that make up these poly(β-esters) are all in the [R]-configuration which results in isotactic polyesters.[1,2]



β-linked PHA Structure, R=side chain substituent

A number of reviews has been published that describe the range of PHA structures formed by various bacteria, biochemical pathways involved in polymer formation, genetic control of the process and various applications of PHAs.[3-8] Variation of PHA repeat unit structure has provided a useful mechanism by which the physico-mechanical properties of PHAs can be "tailored" for various applications. Based on a recent review by Steinbuchel and Valentin[9] it was reported that 91 different repeat unit structures have thus far been found or specifically incorporated into PHAs.

Poly(3-hydroxybutyrate), P3HB, was the first member of the PHA family to be discovered and characterized.[9] PHAs are found in the cytoplasm of cells in the form of inclusion bodies or granules. The outer membrane structure of granules which are believed to have an atypical membrane consisting of primarily protein and a lipid monolayer has been described in detail elsewhere.[10-12] The detailed mechanism by which the PHA synthase converts monomer to polymer is still unknown.[13] A two-step polymerization reaction has been proposed involving an acyl-S-enzyme intermediate.[14] It is thought that 2 active thiol groups may contribute to the polymerization reaction.[15] One of the thiol groups locates the incoming 3HB monomer while the second locates the polymer chain. Condensation occurs through a four-membered transition state, which vacates one of the thiol groups and allows incorporation of the next monomer. This model presumes that the synthase is also involved in chain transfer which, ultimately, controls PHA molecular weight.

A significant disadvantage of microbial polyester biosynthesis as opposed to traditional chemical catalyzed polymerization strategies is the lack of rational routes to regulate product molecular weight and the inability to "tailor" product end-group structure. By the manipulation of polymer molecular weight, optimal

properties for variable applications can be realized. End-group control can result in the preparation of telechelic polymers. Such products may contain one or more end-groups that can be used to react with other molecules creating intriguing possibilities for new conjugates between PHAs and other natural or synthetic polymers. Furthermore, regulation of molecular weight and end-group structure may ultimately result in a new family of structures with desirable interfacial properties. In this report, we describe a novel microbial synthetic strategy which, when further extended, may offer a route to many of the above structural control elements.

Numerous papers were published on the biological properties of poly(ethylene glycol), PEG.[16-18] In a recent paper, we summarized some of the interesting properties of PEGs.[19] Based on reports of PEG-phospholipid membrane interactions[20] and the current description of the PHA granule as having a membrane-like outer structure,[10-12] work was initiated in our laboratory to investigate whether the addition of PEGs to fermentation media would affect PHA biosynthetic systems. In one study, PEG (M_n = 200 g/mol, PEG-200) was added (up to 4% w/v) to incubations of *Alcaligenes eutrophus* (ATCC 17699) using 4-hydroxybutyric acid as the carbon source for PHA formation.[19] It was found that PEG-200 resulted in the following: i) changes in the mol% of 3HB, 3-hydroxyvalerate (3HV) and 4-hydroxybutyrate (4HB) repeat units, ii) formation of complex product mixtures which could be separated into high 4HB and high 3HB containing fractions and iii) a method to prepare PHA-PEG diblock copolymers where the carboxylate terminus of PHA chains were covalently linked by an ester bond to PEG chain segments. This was the first report describing the *in-vivo* formation of a natural-synthetic diblock copolymer. In another study where fructose was the carbon source for *Alcaligenes eutrophus* (ATCC 17699) cultivations, PEG was found to be useful in controlling product molecular weight.[21,22] For example, the addition of 10% (w/v) PEG-106 (ethylene glycol dimer) to the *A. eutrophus* incubation medium on fructose resulted in a decrease in P3HB number average molecular weight (M_n) by 91% to 19,400 g/mol.[22] Interactions leading to molecular weight reduction were enhanced when relatively lower molecular weight PEGs were used. Furthermore, molecular weight reduction required the presence of at least one PEG chain end functionality.[22] Moreover, molecular weight reduction was not accompanied by P3HB-PEG diblock formation. It was hypothesized that PEG interacted with the PHA polymerase and that this interaction resulted in an increase in the rate of chain termination by water relative to propagation reactions.[22]

To extend our understanding of "PEG modulated fermentations", the effects of PEGs on the production of P3HB by *Alcaligenes latus* DSM 1122 was studied. PEGs with number average molecular weights (M_n) of between 106 g/mol (PEG-106) and 10,000 g/mol (PEG-10,000) were added to the *A. latus* fermentation medium and the effects of each PEG analog on cell yield, polymer yield, P3HB productivity, P3HB molecular weight, and

polymer composition was determined. The formation of P3HB-PEG diblock copolymers using various molecular weight PEGs was investigated. The PEG segment length of P3HB-PEG diblock copolymers was compared to that of PEGs added to the medium. Insight was gained into the extent of diblock formation by comparison of experimental M_n measurements with theoretical values based on the model that all P3HB chains were terminated by PEG. Comparisions between P3HB formed when PEGs were added to incubation media of *A. latus* DSM 1122 and *A. eutrophus* (ATCC 17699) lead to important differences in the mechanism by which PEG regulates molecular weight.

Materials and Methods

Bacterial Strain Information and Preservation. *Alcaligenes latus* DSM 1122 was kindly supplied by Dr. Urs Hanggi (Biomer Biodegradable Polymers, Krailling, Germany) and was used in this study. For long term cell preservation the organism was cryogenically stored in liquid nitrogen. The strain was first grown in liquid culture (medium composition described below) under aerobic conditions in 500 mL Erlenmeyer flasks (100 mL culture volumes) at 30°C to the late log phase of growth (about 19 h) in a shaker incubator at 250 rpm. The culture was then diluted 1:1 with sterile 20% glycerol and aseptically transferred to 1.5 mL cryogenic vials. The vial contents were frozen in a dry ice/ethanol bath and then transferred to liquid nitrogen for long term storage. The cells contained in the vials were used as inocula for all the fermentation reactions described below.

Fermentations. Single-stage fermentations were carried out at 200 mL volumes in 500 mL Erlenmeyer flasks. The growth medium was initially prepared in 6 liter stock solutions (2) using nanopure distilled water. Special care was taken to make each stock solution compositionally identical. The medium contained the following (in grams/liter): Na_2HPO_4, 3.4; KH_2PO_4, 1.5; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.01; ferric ammonium citrate, 0.06; glucose, 10.0 (added separately, see below); and 1 mL of a micro-element solution. The micro-element solution contained the following (in grams/liter of 0.1 N HCl): H_3BO_3, 0.3; $CoCl_2 \cdot 6H_2O$, 0.2; $ZnSO_4$, 0.1; $MnCl_2 \cdot 4H_2O$, 0.03; Na_2MoO_4, 0.03; $NiCl_2 \cdot 6H_2O$, 0.02; $CuSO_4 \cdot 5H_2O$, 0.01. After the pH of the medium was adjusted to 7.0, 200 mL aliquots were transferred from a stock solution to 500 mL Erlenmeyer flasks. This procedure minimized flask to flask variations in the medium composition. PEG was then added to the growth medium of each flask at concentrations ranging from 0 to 10% (w/v). Each flask was then sterilized by autoclaving at 121°C for 20 minutes. Glucose was prepared as a 20% (w/v) stock solution and

autoclaved separately to avoid carmelization. It was then aseptically added to make the final glucose concentration in each flask 1.0%. Control flasks (no PEG added to the medium) were run for each PEG tested. Standard deviations were reported for values of residual cell yield (R-CY), polymer yield, and cellular productivity by using results from the 8 replicate control flasks. The PEGs having M_n values of 106 (ethylene glycol dimer), 200, 300, 600, 1000, 2000, 4600, and 10000 g/mol (PEG-106, PEG-200, PEG-300, PEG-600, PEG-1000, PEG-2000, PEG-4600, and PEG-10000, respectively) were all obtained from Aldrich Chemical Company, used as received and the molecular weights given were as specified by the manufacturer. The PEG molecular weights were confirmed by proton (^{1}H) nuclear magnetic resonance (NMR) spectroscopy by end group analysis (comparison of intensities for terminal CH_2-OH and internal -[-O-CH_2-CH_2-]- proton resonances at 3.62 and 3.66 ppm, respectively) and were found to be 194, 300, 584, and 1008 g/mol for PEG-200, PEG-300, PEG-600, and PEG-1000, respectively. Cultures were inoculated (0.1%, v/v) using the contents of rapidly thawed cryovials (see above) containing *A. latus*. Polymer production was then carried out in the above medium at 30°C for 48 hours with shaking at 250 rpm. The cells were then separated by centrifugation (6000 × g, 20 minutes), washed once with water, lyophilized and weighed. The total cell dry weight (CDW) minus the weight of accumulated microbial P3HB (see below for isolation procedure) was used to determine the non-PHA residual cell yield (R-CY) in g/L.

Polymer isolation. The intracellular P3HB was isolated from lyophilized cells by extraction with an excess of chloroform (60 mL per 1 g of biomass, 25°C, 48 h). The products were purified exactly as was described elsewhere.[22] Samples analyzed by ^{1}H NMR spectroscopy were purified by an additional (two instead of one) precipitation/washing cycle. The yield of P3HB in g/L was determined as follows: {cell dry weight (g/L)}X{% P3HB of the cell dry weight}X{0.01}

Analytical methods. The molecular weights of P3HB products were determined by gel permeation chromatography (GPC) using a Waters HPLC system with 500-, 10^3-, 10^4- and 10^5-A° Ultrastyragel columns placed in series. The detectors used included a Waters 410 refractive index (RI) and a Viscotek T60 viscometer. Chloroform was used as the eluent at a flow rate of 1.0 mL/min, sample concentrations were typically 4 mg/mL and the injection volume was 100 μL. Molecular weight values reported in Figure 1 were determined by RI detection using a calibration curve based on polystyrene standards (Aldrich) of low polydispersities with no further corrections. Experimental molecular weights given in Table 2 were determined by using dual RI/viscosity

detection. TriSEC software was used to generate a universal calibration curve which corrected for effects of hydrodynamic volume. The dn/dc of P3HB in chloroform (632.8 nm, 25 °C) determined by using a Wyatt OPTILAB 903 interferometric refractometer was 0.0490 ml/g.

A UNITY-500 NMR spectrometer was used for 1 and 2-D ^{1}H-NMR experiments described below. ^{1}H-NMR spectra were recorded at 500 MHz. Chemical shifts in parts per million (ppm) were reported downfield from 0.00 ppm using tetramethylsilane (TMS) as an internal reference. The experimental parameters for 1-D spectra were exactly as was previously reported[22] except that the number of transients was varied between 45 and 583 to compensate for differing signal intensities of PEG proton signals. The signal-to-noise ratio for PEG signals was a function of the PEG M_n chosen for the fermentation. ^{1}H-NMR peak areas were determined by spectrometer integration. For the COSY experiment, the experimental parameters were identical to those previously used.[22]

Results and Discussion

PEG effects on P3HB yield and P3HB productivity. *A. latus DSM 1122* produces P3HB from glucose.[23] In this study, PEG was added before inoculation to the medium which contained glucose as the carbon source. The %-PEG (w/v) in the medium and the PEG M_n was varied. Specifically, PEG-106, PEG-200, PEG-300, PEG-600, PEG-1,000, PEG-2,000, PEG-4,600, and PEG-10,000 were used (see Materials and Methods Section). Generally, the molecular weight and the concentration of PEGs in the medium determined the biomass accumulation as well as the P3HB yield. For control experiments where PEG was not in the medium, the biomass formed which was reported as the non-P3HB residual cell yield (R-CY) was 2.0 ± 0.1 g/L. The P3HB formed was 53% of the total cell dry weight or 2.3 ± 0.3 g/L. Regardless of the PEG molecular weight, PEG medium concentrations of ≤1% had no significant effect on either the R-CY or the P3HB yield (Table 1). At PEG medium concentrations of 2% or greater, PEGs of lower molecular weight caused increased negative effects on both cell growth and polymer yield. For example, *A. latus* grew and produced P3HB in the medium containing 10% PEG-10,000. In contrast, when 4% of either PEG-106 or PEG-200 was added to the medium, cell growth was not observed. One explanation for this effect of PEG molecular weight is that lower PEG molecular weight at equivalent PEG concentrations (w/v) results in a relatively larger osmotic stress on cells. While 10% PEG-10,000 was tolerated by *A. latus*, this high medium concentration did result in large decreases in both R-CY and polymer yield.

Table 1. Effects of PEG concentration and molecular weight on *Alcaligenes latus* cell growth and P3HB accumulation

PEG[a] mol. wt. (g/mol)	%-PEG added (w/v)	Non P3HB residual cell yield[b] (R-CY) (g/L)	Polymer yield (g/L)	Cellular Productivity[c] (mg polymer/ mg R-CY)
Control	0	2.0 ± 0.1	2.3 ± 0.3	1.2 ± 0.1
10,000	1	2.1	2.5	1.3
	2	1.9	2.5	1.3
	5	2.0	2.0	1.0
	10	0.5	0.1	0.2
4,600	1	2.0	2.3	1.2
	2	1.9	2.2	1.2
	5	1.7	1.8	1.1
	10	0.6	0.2	0.3
2,000	1	2.1	2.5	1.2
	2	2.1	2.5	1.2
	5	1.2	0.9	0.8
	10	0	0	N/A
1,000	1	1.8	2.3	1.3
	2	1.8	2.0	1.1
	3	1.6	1.8	1.1
	4	1.2	0.8	0.7
	5	0	0	N/A
600	1	1.9	2.2	1.2
	2	1.8	2.4	1.3
	3	1.6	2.1	1.3
	4	1.0	0.7	0.7
	5	0	0	N/A
200	1	1.8	2.4	1.3
	2	1.6	1.8	1.1
	3	1.1	0.4	0.4
	4	0	0	N/A
106	1	1.7	2.1	1.2
	2	1.6	1.4	0.9
	3	0.8	0.3	0.4
	4	0	0	N/A

[a]All PEGs were added to the cultivation media at the onset of the fermentations; [b]The non-P3HB residual cell yield (R-CY) is the total cell dry weight minus the weight of accumulated microbial polyester [R-CY = total biomass (g/L) - polymer yield (g/L)]; [c]Cellular productivity = polymer yield/R-CY.

The effects of PEG molecular weight and concentration on P3HB productivity (mg P3HB{mg R-CY}$^{-1}$h^{-1}) were determined (see Table 1). In the control medium the P3HB productivity was 2.5 ± 0.2 (x 10^{-2}). With the exception of PEG-106, addition of ≤2% PEG did not substantially alter P3HB productivity. When 3% PEG was added to the medium, only PEG-106 and PEG-200 showed decreased productivity relative to the control (from 2.5 x 10^{-2} to 0.8 x 10^{-2}). Furthermore, there was little effect on P3HB productivity for 5% PEG-4600 and PEG-10,000. Thus, for the lower molecular weight PEGs (106 and 200 g/mol), P3HB productivity was negatively affected at relatively lower medium concentrations when compared to the higher molecular weight PEGs. Results in Table 1 where the addition of PEG to the medium caused decreased P3HB productivity shows that corresponding decreases in P3HB yield was due not only to poorer cell growth, but also to decreased cellular production efficiency.

Therefore, the addition of the relatively lower molecular weight PEGs to the *A. latus* growth medium caused relatively larger decreases in cell yield, polymer yield and P3HB productivity. In contrast, for similar studies where PEGs of variable molecular weight were added to the polymer production medium of *A. eutrophus*,[22] it was not possible to find regular trends between PEG molecular weight and its effects on cell growth and P3HB yield.

P3HB molecular weight. Measurements of P3HB molecular weights for Figure 1 were determined by GPC relative to polystyrene without further correction. Figure 1 shows the plot of the molecular weight change ($M_n/M_{n(o)}$, see Figure 1 legend) verses the % PEG in the medium. For control incubations, P3HB molecular weight ($M_{n(o)}$) was 267,000 ± 31,000 g/mol). When the PEG added to the medium had an M_n above 1000 g/mol (PEG-2,000, PEG-4,600, and PEG-10,000), there was no change in P3HB M_n relative to $M_{n(o)}$ for PEG concentrations up to 10%. By adding PEGs to the medium with M_n values ranging from 106 to 1,000 g/mol a controlled reduction in the P3HB molecular weight was achieved. The extent of the molecular weight reduction was dependent on the PEG molecular weight and the PEG medium concentration. Larger molecular weight reductions at identical PEG medium concentrations resulted by using PEGs of relatively lower molecular weight. Thus, the largest decrease in P3HB M_n occurred by using PEG-106. The addition of only 1% PEG-106 to the medium resulted in a decrease in the product M_n by 89% to 30,000 g/mol. Further increase in the medium PEG-106 concentration from 1 to 3% resulted in little to no further change in M_n. With 1% of either PEG-200, PEG-300, PEG-600, or PEG-1,000 in the medium, the corresponding molecular weight changes were 0.15, 0.21, 0.79 and 0.89, respectively. Thus, by using PEG medium concentrations of ≤1% which did not decrease either the

P3HB yield or the productivity (see above), large changes in P3HB M_n were achieved. Similar experiments carried out with *A. eutrophus* ATCC 17699 where PEGs were added to the P3HB production medium resulted in similar trends but differing magnitudes of molecular weight change.[22] For example, the P3HB molecular weight change for 1% PEG-106 was 0.10 in this work and 0.26 in the study using *A. eutrophus*.[22]

It should be noted that by adding to the *A. latus* medium increasing concentrations of PEGs having M_n values between 106 and 1,000 g/mol, the polydispersity of the products (M_w/M_n) increased slightly from 1.6 to ~2.1. In contrast, the addition to the medium of either PEG-2,000, PEG-4,600 or PEG-10,000 had little to no effect on M_w/M_n which ranged from 1.6 to 1.8.

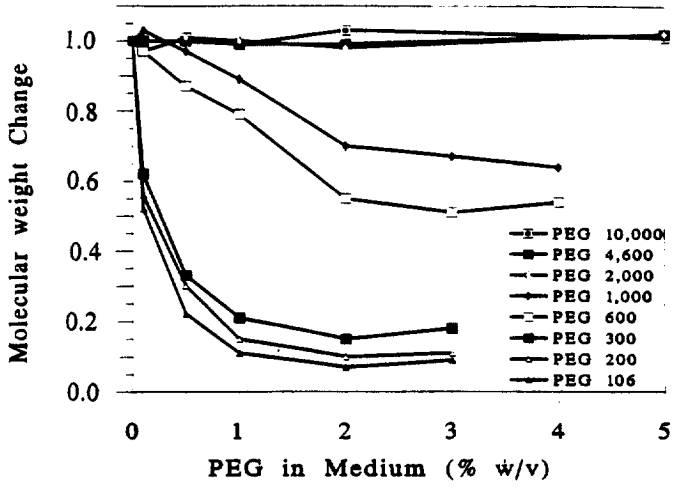


Figure 1. P3HB molecular weight change as a function of the medium PEG concentration (% w/v). The values of molecular weight change are given as $M_n/M_{n(o)}$ where M_n and $M_{n(o)}$ are the GPC number average molecular weights (relative to polystyrene without further correction) of products formed in the medium with and without PEG, respectively.

Formation of P3HB-PEG diblock copolymers. The polymers formed by *A. latus* were analyzed by ^{1}H NMR spectroscopy. The 1-D ^{1}H-NMR spectrum of the P3HB formed during the incubation where 2% PEG-200 was added to the medium was shown in Figure 2. Interestingly, weak ^{1}H-NMR signals between 3.60 and 3.76 ppm were observed that correspond to protons of ethylene glycol (EG) repeat units. P3HB formed in the absence of PEG-200 does not show ^{1}H NMR signals in this region. Furthermore, the relative intensity of signals in this region did not change if the material was purified by 1, 2 or 3 precipitation/washing cycles (see Materials and Methods). Moreover, PEG-200 added to P3HB (1:2 w/w) was easily removed by one precipitation/washing cycle. A 2-D ^{1}H-NMR (COSY) experiment performed on the same P3HB sample was used to assign EG ^{1}H-NMR signals (spectrum not shown). Assignments of signals corresponding to protons b, c and e (see Figure 2) at 4.24, 3.66 and 3.61 ppm, respectively, were made based on our previous study.[19] The 2-D ^{1}H-NMR COSY spectrum showed that the signal corresponding to protons b at 4.24 ppm has a crosspeak with the signal at 3.69 ppm. Furthermore, the signal assigned to protons e at 3.61 ppm showed a crosspeak with the signal at 3.73 ppm. Based on these ^{1}H-^{1}H coupling interactions, the signals at 3.69 and 3.73 were assigned to protons a and d, respectively. ^{1}H-NMR spectra were also recorded for products formed by adding 2% of either PEG-106, PEG-600 or PEG-1,000 to the medium and the expanded spectral regions corresponding to EG signals were shown in Figures 3a, 3b and 3c, respectively. Peak assignments of EG signals in Figure 3 were based on those shown in Figure 2. As expected, the expanded region in Figure 3a does not show signals due to protons c, since PEG-106 does not have internal EG repeat units. Assuming that the contribution of overlapping signals in the 3.64 to 3.76 region can be estimated by Bernoullian curve fitting, the area under peaks was measured by cutting and weighing. The signal intensity ratios for protons *b* to *d*, *b* to *e* and *d* to *e* were each 1.0:1.0 for the P3HB products formed by adding 2% of either PEG-106, PEG-200, PEG-600, or PEG-1,000 to the medium. Based on the above, the addition of relatively low molecular weight PEGs (PEG-106 to PEG-1,000) to the medium of *A. latus* incubations results in P3HB-PEG diblock copolymers where the PEG chain segments were covalently linked to the carboxylate terminus of P3HB chains. Interestingly, in related studies carried out with *A. eutophus*, P3HB formation in the presence of these identical PEGs did not result in diblock formation.[22] Thus, it appears that reactions between growing P3HB chains and PEGs to form PEG terminated microbial polyesters occur readily for *A. latus* but was not observed for *A. eutrophus*. However, *A. eutrophus* did form PHA-PEG diblock copolymers when microbial polyesters containing 4HB repeat units were accumulated.[19] This suggests that reactions between PEG and PHA chains may be dependent on the PHA chain terminal structure.

By comparison of the signal intensity ratios of protons c relative to *b*, *d* and *e* (Figures 2 and 3) it was estimated that n (internal EG units) were 0, 2.5, 8.4 and 12.0 for PEGs 106, 200, 600, and 1,000, respectively. These n values correspond to PEG chain segment molecular weights of 106, 198, 456, and 618 g/mol, respectively. These results show that chains from the oligomeric mixture PEG-200 which react with propagating P3HB have an M_n value which is nearly identical to that provided to the organism. In contrast, the chains from

PEG-600 and PEG-1000 which react with propagating P3HB have M_n values that were substantially less than what added to the medium. This preference for diblock formation by relatively lower molecular weight PEGs is consistent with that lower molecular weight PEGs interact to a greater extent with the PHA synthase resulting in relatively larger molecular weight reductions.[21,22] In fact, for P3HB formation by *A. latus* in medium to which PEG was added, it appears that the extent of molecular weight change is a function of the relative rates of chain propagation and chain termination where the latter occurs by formation of PEG esterified P3HB chain ends. It should be noted that ^{1}H-NMR spectra recorded of P3HBs formed by *A. latus* when 2% PEGs having M_n values of 2,000 g/mol or greater were added to the medium showed negligible PEG contents. This is consistent with that the addition to the medium of PEGs having M_n values of 2,000 g/mol or greater did not result in P3HB molecular weight reductions (see Figure 1). Therefore, it was concluded that, under the physiological conditions used herein for fermentations, these relatively higher molecular weight PEGs did not function as chain terminating agents.

For the following analysis it was assumed that all of the propagating P3HB chains were terminated by PEG and, therefore, are P3HB-PEG diblock copolymers. Using this model and with knowledge of PEG terminal chain segment lengths (see above), M_n values were calculated based on the relative intensity of P3HB and EG signals (see Table 2, legend *b*). M_n values of these products were also measured by GPC using dual refractive

TABLE 2. Comparison of experimental and calculated M_n values

PEG M_n (g/mol)	% PEG (w/v)	Experimental Product M_n[a] (g/mol)	Calculated P3HB-PEG M_n[b] (g/mol)
1000	2	161100	141000
600	2	66300	64000
300	2	25900	25000
200	2	17900	16000
106	2	11700	13000

a. Determined by GPC using dual refractive index/viscosity detection. The GPC data was processed by TriSec software which used a universal calibration curve to correct for effect of hydrodynamic volume.

b. PHB-PEG M_n assuming that all P3HB chains were PEG terminated was calculated by ^{1}H NMR using the following equation:

$$\frac{\frac{A_{CH_3} \times \frac{1}{3}}{\frac{A_{EG}}{\chi}} \times M + M_{n(PEG)}}$$

Where A_{CH_3} is the area under the ^1H NMR signals that correspond to the methyl group of P3HB repeat units, χ is the number of hydrogens in the PEG chain segment, A_{EG} is the area under the ^1H NMR signals that correspond to EG repeat units, M is the molecular weight of the 3HB repeat units and $M_{n(PEG)}$ is the number average molecular weight of the PEG chain segment.

index/viscosity detection. The resulting GPC data was processed by TriSEC software which corrected for differences in hydrodynamic volume between P3HB and polystyrene standards (see Materials and Methods). Inspection of Table 2 shows that there was excellent agreement between calculated (by NMR) and experimental M_n values. Thus, these results suggest that when incubations of *A. latus* were carried out in the medium containing 2% of either PEG-106, PEG-200, PEG-300, PEG-600 or PEG-1,000, a high fraction of P3HB chains were terminated by PEG thus forming P3HB-PEG diblock copolymers.

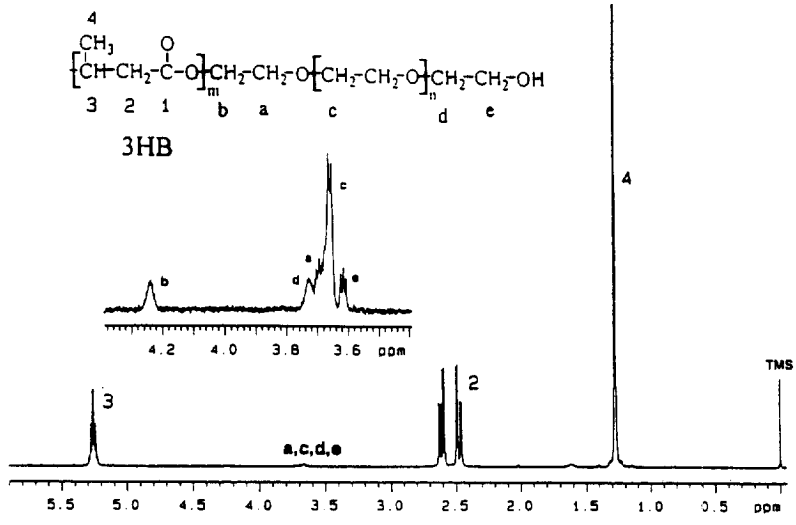


Figure 2. The 500 MHz ^1H NMR spectrum of the purified P3HB product formed by *A. latus* in the medium containing 2% (w/v) PEG-200. The expanded spectral region shows proton resonances which correspond with EG repeat unit signals.

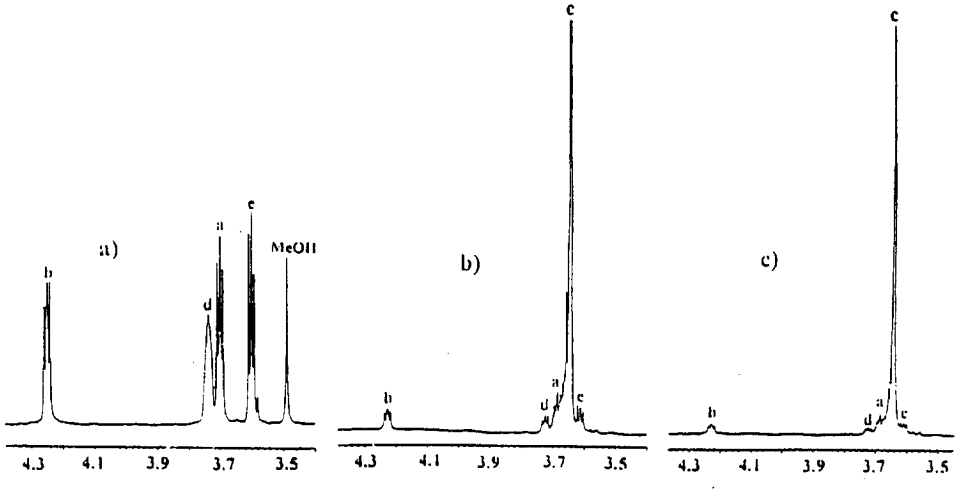


Figure 3. Expansions of the EG proton signal regions from 500 MHz ^{1}H NMR spectra recorded of purified P3HB products formed by *A. latus* in the medium containing 2% (w/v): a) PEG-106, b) PEG-600 and c) PEG-1000.

Summary of Results

PEG added to the *A. latus* incubation medium interacts with the cellular biosynthetic system responsible for P3HB synthesis. The PEGs with $M_n \leq 1000$ g/mol added to the medium functioned to control product molecular weight by acting as chain terminating agents. Furthermore, there was excellent agreement between experimental (by GPC, universal calibration curve) and theoretical M_n values calculated by ^{1}H-NMR. The theoretical M_n values were based on the model that all P3HB chains were terminated with PEG chain segments. Therefore, it was concluded that the fraction of chains formed that were terminated by PEG was indeed high. For P3HB synthesis by *A. eutrophus*, we hypothesized that PEG interacts with the *A. eutrophus* synthase in such a way to increase the rate of chain termination by water relative to propagation reactions.[22] In contrast, for P3HB synthesis by *A. latus*, molecular weight regulation results from reactions between the growing P3HB chain and PEGs to form P3HB-PEG diblock copolymers. It is interesting to note that *A. eutrophus* was capable of forming PHA-PEG diblocks when 4HB containing PHAs were formed.[19] Thus, these results suggest that there are significant differences in the structure of *A. eutrophus* and *A. latus* PHA synthase active sites which lead to variability in PEG binding and reactivity. Current work is being directed towards investigating interactions of PEG with intracellular granules and biosynthetic enzymes using cell free systems. These efforts will hopefully lead to important insights into "PEG-modulated fermentations" that will ultimately be used to direct future research.

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