

A Tunable Switch to Regulate the Synthesis of Low and High Molecular Weight Microbial Polyesters

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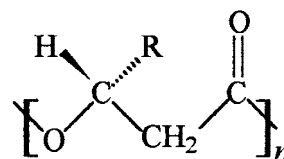
Abstract: The addition of poly(ethylene glycol) ($M_n = 200$ g/mol) (PEG-200) to the fermentation media of *Alcaligenes eutrophus* and *Alcaligenes latus* at various stages of growth resulted in the synthesis of poly(3-hydroxybutyrate) (PHB) with bimodal molecular weight distributions. The presence of 2% w/v-PEG-200 did not have deleterious effects on PHB volumetric yields and cell productivity. In general, the M_n values of the high (H) and low (L) fractions showed little variability as a function of the time at which PEG-200 was added to the cultures. By this approach, the H:L ratios (w/w) of the PHB synthesized by *A. eutrophus* and *A. latus* were varied from 9:91 to 76:24 and from 16:84 to 88:12, respectively. It is believed that the H fractions were formed prior to the addition of PEG-200 to the cultures. Also, once PEG-200 was made available to the cells, PEG-200 acted as a switch so that the reduced molecular weight fraction was formed. In addition, a necessary requirement for the above is that the frequency of transesterification reactions during polymer synthesis was small. The efficiency that PEG-200 reduced the molecular weight of the PHBs formed by both bacteria appears similar. Indirect evidence suggests that the PHB L fractions formed by *A. latus* subsequent to PEG-200 addition consist primarily of chains that have PEG terminal groups. This terminal chain structure was not observed for PHB formed by *A. eutrophus*. © 1999 John Wiley & Sons, Inc. *Biotechnol Bioeng* 62: 106–113, 1999.

Keywords: *Alcaligenes latus*; *Alcaligenes eutrophus*; poly(3-hydroxybutyrate); poly(ethylene glycol); bimodal molecular weight distributions

INTRODUCTION

Poly(hydroxyalkanoic acids), PHAs, are a family of optically active polyesters that are synthesized by numerous

bacterial species as intracellular carbon and energy reserve materials (Anderson and Dawes, 1990; Dawes and Senior, 1973; Doi, 1990). The general structure for the PHA subset of β -linked PHAs is shown below. When R is methyl, the polymer is poly(3-hydroxybutyric acid), PHB.



A number of reviews on PHAs that describe biochemical aspects of polymer formation, structural variability, and properties have been published (Anderson and Dawes, 1990; Brandl et al., 1990; Doi, 1990; Gross, 1994; Steinbuechel, 1991; Steinbuechel and Valentin, 1995). An important benefit of many microbial polyesters is that they have been found to be biodegradable upon disposal (Abe and Doi, 1996; Molitoris et al., 1996; Doi et al., 1990).

Although the biochemistry of PHA (mainly PHB) biosynthesis has been the subject of much recent work (Byrom, 1994; Kidwell et al., 1995; Steinbuechel et al., 1995; Wiczorek et al., 1995), the mechanisms of polymer growth and control of chain molecular weight are not understood. Hence, rational methods for PHA molecular weight control other than our investigations into the use of polyethylene glycol (PEG) in culture media (Shi et al., 1996b; Ashby et al., 1997) have not been reported. However, the variation of PHA molecular weights remains of considerable interest.

Examples of work carried out to study molecular weight variation are as follows. PHB molecular weight was affected by the method of polymer isolation from cells. Neutral solvent extraction results in higher degree of polymerization (n) values than alkaline hypochlorite or acid hydrolysis treatment (Doi, 1990). Also, the bacterial strain used is an important factor. For example, *Azotobacter vinelandii* (Akita et al., 1976) has been reported to produce PHB with

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molecular weights up to 3.39×10^6 g/mol, where the molecular weights of PHB from *A. eutrophus* are generally less than 10^6 g/mol. The polymerization degree also may vary depending on the time selected for harvesting cells, the limiting nutrient used, and by changing other culture physiological conditions (Anderson and Dawes, 1990; Suzuki et al., 1988). For example, by using a two-stage batch cultivation of *A. eutrophus* on fructose or butyric acid in nitrogen-free medium, the molecular weight of PHB was time, temperature, and pH dependent (Doi et al., 1989). The PHB M_n increased rapidly with time during the course of PHB accumulation to reach a maximum value ($(10 \pm 2) \times 10^5$) at a 6 h incubation time. Extended cultivation time beyond 6 h resulted in lower M_n PHB. PHB M_n also varied under identical cultivation conditions but with variation of the carbon source (fructose versus butyric acid) (Doi, 1990). In another study, the molecular weight of PHB produced in a fed-batch culture of *Protomonas extorquens* was affected by the culture temperature, pH, molar ratio of methanol to ammonia, and concentration of methanol in the medium (Suzuki et al., 1988). Also, ultrahigh molecular weight PHB ($M_n = (5-20) \times 10^6$) was produced under specific fermentation conditions by using a recombinant *Escherichia coli* XL-I Blue (pSYL105) (Lee et al., 1994) which harbored *A. eutrophus* PHB biosynthesis (*phbCAB*) genes (Kusaka et al., 1997).

Recently, our laboratory showed that the addition of PEGs with molecular weights ≤ 1000 g/mol to the fermentation medium decreased the molecular weights of the resulting polymers to varying degrees. The extent of molecular weight reduction depended on the PEG molecular weight and concentration in the incubation medium (Shi et al., 1996b; Ashby et al., 1997). This technology has resulted in the biosynthesis of PHBs with molecular weights (M_n) ranging from 1.1×10^4 to 1.2×10^6 g/mol. In one report, control of the PHB molecular weight occurred due to the termination of propagating PHB chains by PEG (Ashby et al., 1997). This resulted in the *in vivo* formation of PHB-PEG diblock copolymers (natural-synthetic hybrids) by *A. latus*. In a related publication using *A. eutrophus*, PEG also provided control of PHB molecular weight. However, in this later case, diblock copolymers were not formed (Shi et al., 1996b). It was hypothesized that PEG interacted with the PHA synthase of *A. eutrophus* in such a way that there was an increase in the rate of chain termination events by water relative to propagation reactions.

The *phbB* (acetoacetyl-CoA reductase) and *phbC* (PHA synthase) genes from *A. eutrophus* were transferred to and successfully expressed in the plant *Arabidopsis thaliana* using an *Agrobacterium tumefaciens* Ti vector system (Poirier et al., 1995). The resulting transgenic *A. thaliana* demonstrated the feasibility of plant mediated PHB biosynthesis. Analysis of the resulting plant-generated PHB showed that a substantial portion of the product had molecular weights of about 10^6 g/mol. Such molecular weights are similar to bacterial PHB synthesized by *A. eutrophus*. However, unlike bacterial PHB, the plant-generated polymer had a molecular weight distribution that was multimodal. Substantial product fractions had chains of molecular weights in the

range of 10^4-10^5 g/mol. Undoubtedly, by controlling the molecular weights of each fraction as well as the ratio of high to low molecular weight product, important changes in the physico-mechanical properties as well as the bioerosion rates will result.

In this paper, we report on a route to bacterial polyesters having complex but controlled molecular weight distributions. This was accomplished by adding PEG-200 at different times to the culture broths of *A. eutrophus* and *A. latus* fermentations. In other words, PEG-200 was added to the incubations at various stages of bacterial growth and polymer formation.

MATERIALS AND METHODS

Strain Information

A. eutrophus ATCC 17699 was purchased from the American Type Culture Collection (Rockville, MD) while *A. latus* DSM 1122 was kindly supplied by Dr. Urs Hänggi (Biomer Biodegradable Polymers, Krailling, Germany). For long-term cell preservation, each organism was grown in liquid culture to the late-log phase and cryogenically stored in liquid nitrogen. These frozen cells were used for inoculation of cultures in all experiments. Details of the method used have been described elsewhere (Shi et al., 1996a; Ashby et al., 1997).

Inoculation, Growth, and PHB Production in Liquid Culture

Shake flask cultures of each organism were carried out at 200 mL volumes in 500 mL Erlenmeyer flasks. All medium formulations were prepared using deionized water and reagent grade media components that were purchased (unless otherwise specified) either from Aldrich Chemical Company (Milwaukee, WI) or Fischer Scientific (Fair Lawn, NJ). With the exception of fructose that was sterilized by filtration, fermentation media were sterilized by autoclaving at 121°C for 20 min. All cultures were inoculated using the contents of rapidly thawed cryovials at a concentration of 0.1% (v/v). PHB production by *A. latus* was conducted under single-stage fermentation conditions. In contrast, for *A. eutrophus*, the fermentation involved a two-stage process. The specific culture conditions and the medium formulations for both *A. eutrophus* and *A. latus* fermentations are exactly as was described previously (Shi et al., 1996a; Ashby et al., 1997). In all experiments, the organisms were cultivated at 30°C with shaking at 250 rpm and a pH of 7.0. Polyethylene glycol having a M_n of 200 g/mol (PEG-200, molecular weight specified by the manufacturer) was obtained from Aldrich and was used as received. The PEG M_n was also determined in our laboratory by (^1H) nuclear magnetic resonance (NMR) spectroscopy. Comparison of intensities for terminal $\text{CH}_2\text{-OH}$ and internal $[\text{O-CH}_2\text{-CH}_2\text{-}]$ -proton resonances at 3.62 and 3.66 ppm, respectively,

showed that the NMR-determined M_n for PEG-200 was 194 g/mol.

For *A. latus* incubations, 2% (v/v) sterile PEG-200 was added to separate cultures at 16, 20, 24, 28, and 32 h post-inoculation. All *A. latus* fermentations were conducted for 48 h. The bacterial cells were harvested by centrifugation (5900g, 20 min), washed once with water, and lyophilized. PHB production by *A. eutrophus* was carried out under two-stage fermentation conditions. Growth of *A. eutrophus* was initiated in 200 mL volumes (in 500 mL Erlenmeyer flasks) of nutrient-rich media under aerobic conditions for 24 h (see reference Kunioka et al. (1989) for media composition and fermentation conditions). The cells were then harvested aseptically by centrifugation (5900g, 20 min). Typically, the cell dry weight (CDW) of first-stage *A. eutrophus* cultivations was approximately 3.5 g/L and the extent of polymer formation was less than 2.7% of the CDW (<0.1 g/L). The cells were then washed in sterile Na_2HPO_4 - NaH_2PO_4 buffer and aseptically transferred into 500 mL Erlenmeyer flasks containing 200 mL of nitrogen-free medium and 2.0% filter sterile fructose as the polymer producing substrate (see reference Shi et al. (1996a) for media composition and fermentation conditions). Two percent (v/v) PEG-200 was added to separate flasks at 4 h intervals from 4 to 36 h during the 48 h second or PHB production stage. At the end of the *A. eutrophus* fermentations, the cells were separated, washed, and lyophilized as above.

Polymer Isolation

The intracellular PHB produced by each organism was isolated by stirring a suspension of the lyophilized cells (about 0.5 g) with an excess of chloroform (100 mL) at room temperature for 48 h. The insoluble cellular material was removed by filtration, and the solvent was evaporated leaving the crude PHB product. This crude product was then redissolved in chloroform (~0.1 g/mL) and was precipitated by dropwise addition of the chloroform/polymer solution into cold methanol. The PHB was isolated by filtration and washed once with cold methanol. The % PHB of the cellular dry weight and the volumetric PHB yields for cultivations of *A. latus* and *A. eutrophus* without adding PEG to the media were 56% (2.5 g/L) and 52% (4.5 g/L), respectively. Additional details of the procedure used are described elsewhere (Shi et al., 1996a).

Molecular Weight Determinations

The number- and weight-average molecular weights (M_n and M_w , respectively) and the polydispersity (M_w/M_n) of each PHB sample were determined by gel permeation chromatography (GPC). Polystyrene standards (Aldrich, Milwaukee, WI) with low polydispersities were used to generate a calibration curve from which product molecular weights were determined with no further corrections. Additional details of the GPC method were described elsewhere (Shi et al., 1996a; Ashby et al., 1997). Peak decon-

volution and integration of bimodal distributions was carried out by using Peakfit peak analysis software (Jandel Scientific, San Rafael, CA) assuming Gaussian peak distributions. Molecular weight averages of each peak in bimodal distributions were calculated by importing the generated data from Peakfit software into a spreadsheet format and calculating the molecular weight (M_i) at retention times (T) along the peak by using the following equation:

$$\text{Log } M_i = 11 - 0.208T. \quad (1)$$

Eq. (1) was generated by Millenium software for the polystyrene standard calibration plot $\text{Log } M_i$ versus T . The molecular weight averages M_n and M_w were calculated using Eqs. (2) and (3), respectively,

$$M_n = \frac{\sum_i h_i}{\sum_i h_i/M_i}, \quad (2)$$

$$M_w = \frac{\sum_i h_i M_i}{\sum_i h_i}, \quad (3)$$

where h_i is the refractive index detector output (mV) at a given data point, and M_i is the calculated value from Eq. (1) above.

Structural Analysis by $^1\text{H-NMR}$

Proton (^1H) NMR spectra were recorded at 250 MHz on a Bruker AMX 250 spectrometer. Chemical shifts in parts per million (ppm) were reported downfield from 0.00 ppm using tetramethylsilane (TMS) as an internal reference. The experimental parameters were as follows: 0.5% (w/v) polymer in chloroform-*d*, temperature 298 K, 2.4 μs (14°) pulse width, 3 s acquisition time, and 5000 Hz spectral width. The repeat unit composition of products was determined by the relative intensity of signals due to 3-hydroxybutyrate (HB) and ethylene oxide (EG) repeat units, respectively (Shi et al., 1996a; Ashby et al., 1997).

RESULTS AND DISCUSSION

In this work, we investigated whether the addition of PEG at different times during polymer production would result in "tailored" molecular weight distributions. In other words, this study attempted to experimentally validate the following hypothesis: the microbial PHB polymerization could be switched from the formation of higher to lower molecular weight PHB upon the introduction of PEG into the culture medium.

Polymer Yields and Cellular Productivities

The effect on both PHB yield and cellular productivity of adding PEG-200 to the medium at variable incubation times was studied (see Table I). Most bacterial storage polymers, whether polyesters or polysaccharides, are produced during

Table I. Poly(3-hydroxybutyrate) production in the presence of PEG-200 by *A. latus* and *A. eutrophus*.

Product	Supplement time ^a (h)	Non-PHB residual cell yield ^b (R-CY)	Polymer yield (g/L)	Cellular productivity (mg/mg R-CY) ^c
<i>Alcaligenes latus</i> DSM 1122 ^d				
La-1	Control ^e	2.0 ± 0.2	2.5 ± 0.3	1.3 ± 0.2
La-2	16	1.6	2.8	1.8
La-3	20	1.8	2.8	1.6
La-4	24	1.9	2.5	1.3
La-5	28	2.0	2.4	1.2
La-6	32	2.2	2.4	1.1
<i>Alcaligenes eutrophus</i> ATCC 17699 ^d				
E-1	Control ^e	4.2 ± 0.2	4.5 ± 0.2	1.1 ± 0.1
E-2	4	3.3	4.5	1.4
E-3	8	3.5	4.8	1.4
E-4	12	3.4	5.0	1.5
E-5	16	3.9	4.7	1.2
E-6	20	4.2	4.2	1.0
E-7	24	3.9	4.4	1.1
E-8	28	4.2	4.4	1.1
E-9	32	4.2	4.4	1.1
E-10	36	4.3	4.7	1.1

^aPEG-200 supplements were added at the polymer production stage of the fermentations. (1st stage for *A. latus* and 2nd stage for *A. eutrophus*).

^bThe non-PHB residual cell yield (R-CY) is the total cell dry weight (CDW) minus the weight of accumulated microbial polyester [R-CY = total biomass (g/L) – polymer yield (g/L)].

^cCell productivity = polymer yield/R-CY.

^dCarbon source used was 1% glucose for *A. latus* and filter sterile 2% fructose for *A. eutrophus* (for additional details on the fermentations see Experimental section).

^eControls contained no PEG-200 for the duration of the fermentations.

the stationary growth phase. However, it has been documented that *A. latus* produces PHB concomitantly with cellular growth (Hanggi, 1990). Hence, 2% (v/v) sterile PEG-200 was aseptically added to the growth medium of *A. latus* between the late-log (16 h post-inoculation) and mid-stationary (32 h post-inoculation) growth phases. In control experiments carried out in medium without PEG addition, the biomass accumulation of *A. latus*, expressed as the non-PHB residual cell yield (R-CY, see Table I, legend b), was 2.0 ± 0.2 g/L. After inspection of R-CY, polymer yields, and cellular productivity values for the *A. latus* cultivations, it was concluded that PEG-200 addition to the medium at 16–32 h did not significantly affect these values (see Table I). These results suggest that for the above incubations, the osmotic stress to the organism caused by adding PEG to the medium did not result in a deleterious affect on PHB formation.

In contrast to *A. latus*, *A. eutrophus* cultivations were conducted by a two-stage fermentation process (see Materials and Methods section). The nutrient-rich first stage was designed to increase the cellular biomass without PHB accumulation while the second stage was conducted so that PHB production was enhanced (see Materials and Methods section and Shi et al. (1996b)). Hence, the addition of 2%

PEG-200 to the *A. eutrophus* cultivations was carried out during the second stage of *A. eutrophus* incubations at times between 4 and 36 h. Control experiments conducted in medium without PEG addition resulted in an R-CY of 4.2 ± 0.2 g/L. In contrast to the *A. latus* system, the R-CY of *A. eutrophus* upon termination of the fermentations increased from 3.3 to 4.3 g/L as PEG addition to the medium was carried out later in the cultivations (see Table I). Thus, a 2% addition of PEG-200 to the PHB production medium early in the fermentation resulted in poorer cell growth. However, polymer yields remained relatively constant at about 4.5 ± 0.2 g/L regardless of the time of PEG addition to the culture (see Table I). Hence, the result was higher cellular productivity when PEG-200 was added early in the fermentation. This indicates that while PEG-200 caused a decrease in the cellular biomass (probably due to osmotic restraints), it also seemed to activate the PHB biosynthetic system in *A. eutrophus*.

Generation of PHB Having Controlled Molecular Weight Distributions

When PEG-200 was added at different times to the PHB production cultures of each organism, the result was that the products formed had very different molecular weight distributions. These distributions were bimodal and consisted of high (H) and low (L) molecular weight fractions (see Fig. 1). In each case, the ratio of H to L (H:L w/w) was dictated by the time at which the PEG-200 was added into the fermentation medium (Fig. 2). The results showed that the addition of PEG-200 early in the fermentations (≤24 h for *A. latus*, ≤16 h for *A. eutrophus*) produced a relatively larger concentration of low molecular weight products (H:L <1). Using the fermentation conditions described herein, the H:L ranged from 0.19 to 7.33 and from 0.10 to 3.17 for PHBs synthesized by *A. latus* and *A. eutrophus*, respectively (Fig. 2). Previous work in our laboratory showed that PEGs with $M_n \leq 1000$ g/mol appeared to increase the rate of chain termination relative to propagation for the reactions catalyzed by the PHB synthase systems (Shi et al., 1996b; Ashby et al., 1997). Hence, the presence of PEG at earlier stages of polymer production allows the PEG to reduce the molecular weight of a larger product fraction. In other words, it appears that the fraction of product which is produced during the time that PEG is available to the PHA synthase is similar in value to the fraction of PHB that was formed with reduced molecular weight.

Control experiments for *A. latus* and *A. eutrophus* performed where PEG was not added to the incubations resulted in unimodal molecular weight distributions and M_w/M_n values of about 2.5. For the culture conditions used herein, comparison of the control experiments showed that the PHB synthesized by *A. eutrophus* had a molecular weight that was about 3 times larger than that from *A. latus*. PEG addition to the *A. latus* culture at 16 h post-inoculation resulted in a single molecular weight peak corresponding to the L fraction. This indicated that PHB production by the *A.*

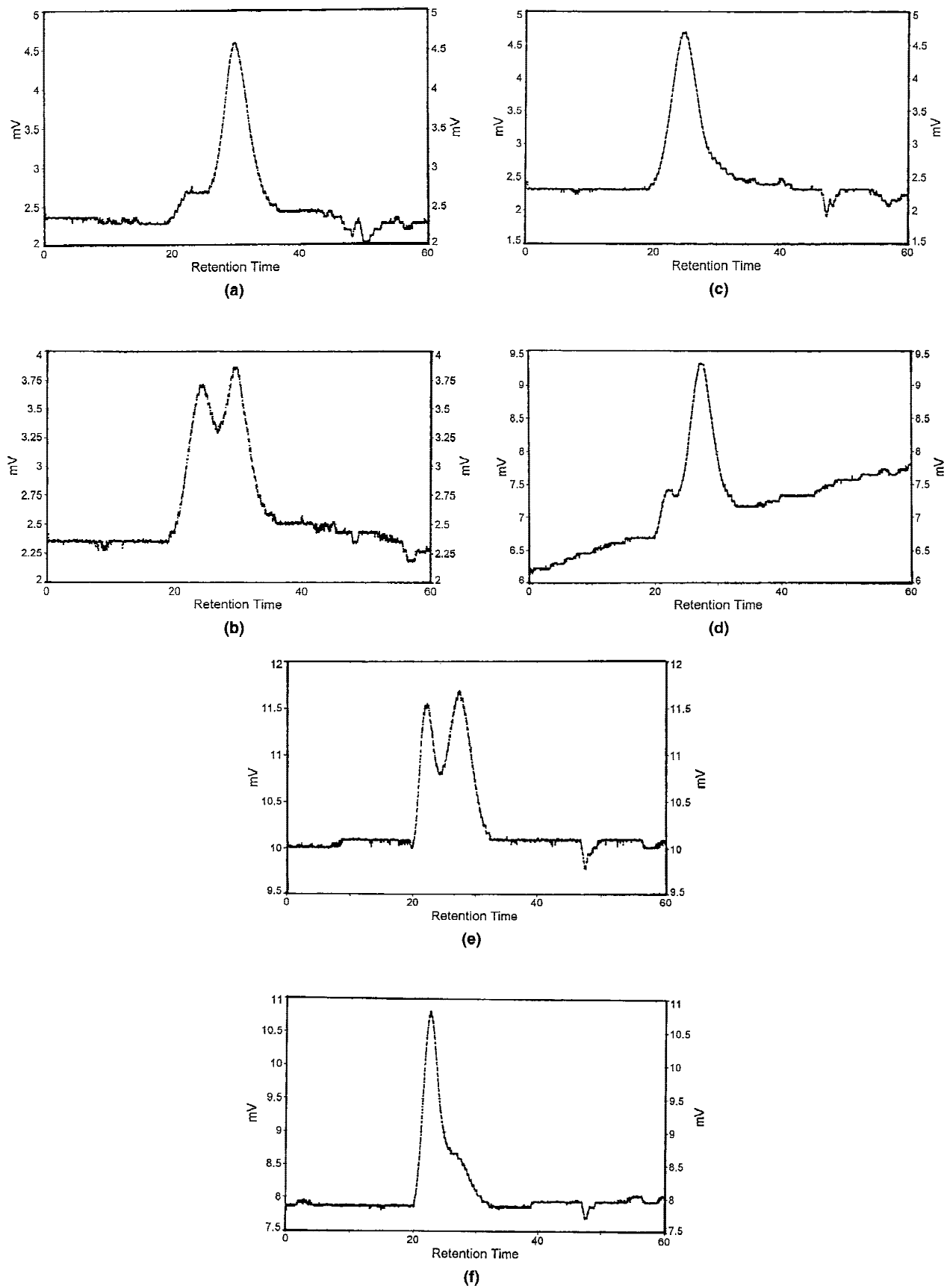


Figure 1. GPC traces of the products formed by *A. eutrophus* and *A. latus* based on the time of PEG-200 supplements: (A) *A. latus* product La-3, (B) *A. latus* product La-4, (C) *A. latus* product La-5, (D) *A. eutrophus* product E-3, (E) *A. eutrophus* product E-5, (F) *A. eutrophus* product E-10.

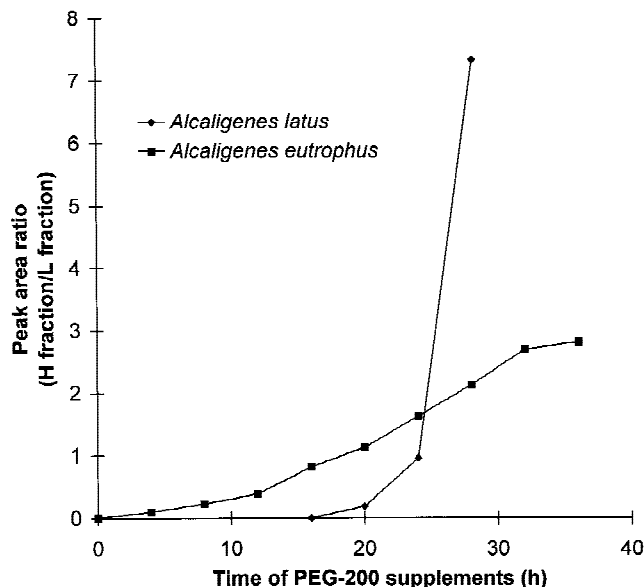


Figure 2. Peak area ratios (H/L) from the bimodal molecular weight distributions of PHB products formed by *A. eutrophus* and *A. latus*.

latus system was negligible prior to 16 h. Indeed, when *A. latus* cells were harvested after the 16 h culture time, the polymer yield was only 0.1 g/L. The molecular weights of the H and L components for bimodal products were determined by using peak fit software (see Materials and Methods). With the exception of sample La-3, M_n values for the H fractions of *A. latus* products remained almost unchanged

(~390,000 g/mol). The same was true for the H fractions of *A. eutrophus* products (M_n about 1.2 million g/mol). This supports the hypothesis above that the H fractions were formed prior to the addition of PEG-200 to cultures. Furthermore, the L fraction M_n values for PHBs formed by *A. latus* are almost invariable (~30,000 g/mol). Hence, it appears that once PEG-200 is available to the cells, PEG-200 acts as a switch so that the reduced molecular weight fraction is formed. On the basis of our previous work, the L fraction M_n can be controlled by variation in the PEG molecular weight and PEG medium concentration (Shi et al., 1996b; Ashby et al., 1997). Therefore, in principle, the molecular weight of the L fraction can be rationally varied by the selection of a specific PEG molecular weight and medium concentration. In the case of the L fractions produced by *A. eutrophus*, the results were more complex. Specifically, the L fraction molecular weights appear to increase as the time of PEG-200 addition to the medium increases (85,000 to 189,000 g/mol, see Table II). Also, M_w/M_n values tended to decrease as the time at which PEG-200 was added to *A. eutrophus* cultures increased. It is relevant to note that the peak-fit program assumes a Bernoulli distribution, which may not be an accurate model for all of the chromatograms and corresponding fractions. This may explain the observed deviations in molecular weight of *A. eutrophus* L fractions described above. In addition, when PEG is present in the medium prior to inoculation, the molecular weight distributions (M_w/M_n) of products did not exceed 2.5 (Shi et al., 1996b). This shows that the numerical

Table II. GPC molecular weight analysis of poly(3-hydroxybutyrate), PHB, produced in cultures to which PEG-200 was added at variable times.

Product	Suppl. time ^c (h)	H fraction ^a			L fraction ^b			Composition (mol%) [3HB(EG)]
		% Area	M_n ($\times 10^3$)	M_w/M_n	% Area	M_n ($\times 10^3$)	M_w/M_n	
<i>Alcaligenes latus</i> DSM 1122 ^d								
La-1	Control ^e	100	391	2.47	0	—	—	100 (0)
La-2	16	0	—	—	100	31	2.58	96.9 (3.1)
La-3	20	16	666	3.57	84	25	5.20	97.5 (2.5)
La-4	24	49	395	6.35	51	28	5.91	98.3 (1.7)
La-5	28	88	385	6.26	12	36	3.95	99.7 (0.3)
La-6	32	100	377	3.07	0	—	—	99.9 (0.1)
<i>Alcaligenes eutrophus</i> ATCC 17699 ^d								
E-1	Control ^e	100	1170	2.54	0	—	—	100 (0)
E-2	4	9	1180	2.77	91	85	4.54	100 (0)
E-3	8	19	1290	3.31	81	92	4.84	100 (0)
E-4	12	28	1290	3.26	72	104	4.37	100 (0)
E-5	16	45	1270	3.12	55	101	3.41	100 (0)
E-6	20	53	1210	3.33	47	99	3.30	100 (0)
E-7	24	62	1190	2.88	38	124	2.58	100 (0)
E-8	28	68	1180	2.77	32	133	2.38	100 (0)
E-9	32	73	1160	2.59	27	157	2.15	100 (0)
E-10	36	76	1150	2.41	24	189	1.99	100 (0)

^aH fraction corresponds to the higher molecular weight peak in the bimodal GPC distribution.

^bL fraction corresponds to the lower molecular weight peak in the bimodal GPC distribution.

^cSee Table I, legend a.

^dSee footnote d, Table I.

^eSee footnote e, Table I.

changes in M_n and M_w/M_n that were found for differing times of PEG-200 addition to the medium are not related to the presence of differing amounts of PHB in *A. eutrophus*. In other words, based on previous studies (Shi et al., 1996b), we have no reason to believe that the effectiveness of PEG-200 for PHB molecular weight reduction is related to the quantity of PHB that is already accumulated in cells. It is interesting to note that the L fractions from *A. latus* are of lower molecular weight than the L fractions of *A. eutrophus*. If one takes the L fractions from E-2 through E-6 as representative (they are $\geq 47\%$ (by wt) of the total product), then the L fractions from *A. eutrophus* are about 3 times greater than those from *A. latus*. Since the H fractions of *A. eutrophus* were also greater than those of *A. latus* by a factor of about 3, it appears that PEG-200 reduces the molecular weight of the PHBs from these bacteria with similar efficiency.

Previous work in our laboratory for PHB synthesis by *A. latus* showed that PEG acts as a chain-terminating agent. More specifically, a high fraction (apparently all) of the PHB chains were terminated so that an ester bond was formed between the terminal PHB carboxyl and a PEG hydroxyl group (PHB-PEG diblock copolymers) (Ashby et al., 1997). Hence, it was anticipated that PEG-200 acted similarly herein for the *A. latus* system so that the L fraction chains would have PEG terminal chain segments and the H fractions would not. Inspection of the ^1H NMR spectra for samples La-2, La-3, and La-4 showed the same chemical shifts for PHB-PEG terminal signals that were previously found (Ashby et al., 1997). This suggests that the low molecular weight fraction of the products from *A. latus* consist of chains that have PEG terminal groups. Inspection of Table II shows that, as anticipated, the mol% of EG in the *A. latus* formed products decreased regularly as the L fraction decreased (Table II). Furthermore, assuming that all chains of La-2 contain terminal PEG groups, Eq. (4) below was used to calculate whether the decrease in EG mol% for La-3 to La-5 is consistent with the corresponding decrease in L fraction wt%.

$$\{\text{La-X wt\%} \div 100\} \times 3.1 = \text{EG mol\%},$$

where X is 3, 4, or 5 (4)

Using Eq. (4), the calculated EG mol% values for the L fractions of La-3, La-4, and La-5 are 2.6, 1.6, and 0.4, respectively, which is in excellent agreement with the experimental values (2.5, 1.7, and 0.3, respectively, see Table II). Hence, this is indirect evidence that the L fractions of La-3, and La-4, and La-5 also consist of primarily chains that have PEG terminal groups. To obtain direct evidence for this claim, fractionation of sample L-4 was undertaken with the goal of separating and characterizing the pure L and H fractions of this product. Unfortunately, thus far, we have been unsuccessful in achieving a suitable fractionation by differential solubility methods.

We conducted previous studies on the regulation of PHB molecular weight by adding PEGs to *A. eutrophus* incubations. It was concluded that the formation of a covalent

linkage between PHB and PEG was not the operative mechanism for an observed PEG-induced reduction in PHB molecular weight (see above and Shi et al. 1996b). Thus, the fact that products E-2 through E-10 did not have EG repeat units based on ^1H -NMR analysis (see Materials and Methods and Table II) is consistent with that expected on the basis of our previous work.

SUMMARY OF RESULTS

The addition of PEG-200 at different times throughout the cultivations of *A. eutrophus* and *A. latus* resulted in PHBs with bimodal molecular weight distributions. These products consisted of high (H) and low (L) molecular weight fractions. By variation of the time that PEG was added to the incubations of these bacteria, it was possible to create a wide range of bimodal products with differing H:L ratios. Hence, the ability of PEGs to reduce PHB molecular weight can be turned on at will during a fermentation process to produce products with "tailored" molecular weight distributions. The fact that the H and L fraction molecular weights remained almost unchanged, regardless of when PEG was added to the medium, suggests that the frequency of transesterification reactions during microbial PHB synthesis was low. It is envisioned that improvements in the chain transfer or termination efficiencies of PEG-type analogs may eventually lead to methods which provide low molecular weight PHA plasticizers, amphiphilic surfactants, novel macromers and chains which bear physiologically active end groups. Indeed, future work in our group is being directed towards realizing these objectives.

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