Solution Properties and Molecular Size of Polyhydroxybutyrate from Recombinant *Escherichia coli*

by

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B.S., Mechanical Engineering (1993)

GMI Engineering & Management Institute

Submitted to the Department of Mechanical Engineering
in Partial Fulfillment of the Requirements for the Degree of
Master of Science

at the

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ABSTRACT

Polyhydroxybutyrate molecules produced by recombinant *Escherichia coli* were evaluated in terms of their size in solution to develop and test a hypothesis concerning the regulation of the molecular weight of PHB during *in vivo* polymer production.

Two recombinant strains of *E. coli* were used to produce material for this study: DH5α/pAeT41, containing the native operon from *Alcaligenes eutrophus* encoding the three enzymes necessary for the biosynthesis of PHB and DH5α/pSP2, a strain genetically engineered to optimize synthase production in *E. coli*. In the latter strain, the induction agent isopropyl-β-D-thiogalactopyranoside is used to induce synthase production.

Initial flask cultures of these two recombinant strains produced pure PHB showing greatly different intrinsic viscosities in chloroform. Strain pAeT41 produced PHB molecules nearly an order of magnitude larger in intrinsic viscosity than those produced by strain pSP2. This led to the hypothesis that high synthase concentration led to the production of shorter PHB chains. The same fermentation conditions in a larger scale 2 liter fermentation led to similar results with PHB chains from pSP2 being one half the length of those from pAeT41. In an attempt to regain the drastic difference in molecular weight previously discovered, the molar concentration of the induction agent (IPTG) added to the pSP2 culture was increased, and a lower PHB molecular weight resulted, presumably due to an increase in synthase production.

The molecular weight as a function of fermentation time was determined using ten liter fermentations. PHB produced by pAeT41 maintained a relatively constant molecular mass, while PHB from pSP2 distinctly decreased in molecular weight during the fermentation. This led to the hypothesis that initial PHB molecular weight was large due to very low enzyme production prior to IPTG induction, at which time high enzyme concentration caused the production of new shorter PHB chains. SEC and light-scattering showed that as enzyme production reached maximum levels, average PHB molecular weight dropped and the polydispersity index sharply increased due to an immediate increase in the number of short chains produced.

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This is my opportunity to thank those who have labored by my side for the past two and a half years and to give credit to those people without whom this project could not have happened. I have taken the liberty to shorten formal titles and locations as I saw fit.

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I must also thank all of the current and past members of the Biomaterials Science and Engineering Lab. Dr. Joon Ho Choi, Dr. Jin Ha Lee, Gillian Brown, and Sara Yu were all contributors to my work. Dr. Sung Koo Kim, a former student of this lab, first introduced me to fermentation technology.

Many thanks are also due Professor Anthony J. Sinskey of the Department of Biology at MIT for the use of his facilities and his valuable input into this project. The biotechnology which made this thesis possible was developed in his laboratory. Several members of the Sinskey Lab have assisted me over the course of my work. Most noteworthy is Dr. Kristi D. Snell, a post-doctoral associate in the Sinskey Lab. I credit Dr. Snell with the initiation of this entire project. Her knowledge, patience, interest, and unconditional support have been paramount, and I cannot thank her enough for her guidance. Also of note from the Sinskey Lab is Dr. Sang Jun Sim. Dr. Sim was instrumental in this project spending hour upon hour on such tedious work as large scale fermentations and enzyme activity determinations which found him in the lab 24 hours a day at times. Dr. Esperenza Troyano and Dr. Sam Pin Lee also had a hand in my education concerning chromatography and fermentation techniques, respectively.

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I. INTRODUCTION

Poly-β-hydroxybutyrate, more commonly referred to by its acronym PHB, has gained widespread attention over the past few decades due to its unique properties. PHB is a naturally produced polymer with thermoplastic properties. The attractiveness of a material of this nature is evident. In an age of increased environmental awareness, a plastic which can potentially be produced using renewable resources and which can be biologically degraded becomes very appealing.

This thesis is written nearly seventy years after the initial discovery of PHB. However, it has taken several advances in biotechnology to begin to understand how it is produced and how some of its properties may be manipulated. Some of the findings presented in this thesis are of both theoretical and practical significance. The prospect of controlling the chain length of a naturally occurring polymer is potentially useful in the large scale production of PHAs. Further, by understanding the control of PHB chain length, the mechanism by which the polymerization occurs can be better understood.

Chapter II will provide a history of PHB and the in the field. Explored here will be the structure and properties of the polymer, the solution properties of PHB including molecular weight analyses reported by other researchers, extraction techniques used to obtain the polymer from its host organism, the biosynthesis of PHB in *Alcaligenes eutrophus*, which is one of its native organisms, and some of the work done with PHB in

recombinant systems, particularly *Escherichia coli*. Following this literature survey, the procedures used to produce and analyze the PHB included in this study is. The results are presented chronologically. Through this sequence, a hypothesis regarding the molecular mass of PHB and how it is regulated in a recombinant system is clearly developed and proven. Following a summary of the conclusions reached in this study, a chapter on suggested future research can be found. Included in this section is the suggestion of a first-order model concerning the quantitative control of molecular weight using a synthetic polymerization analog which should be useful in understanding the role of certain enzymes in PHB production.

II. HISTORICAL BACKGROUND

Polyhydroxybutyrate (PHB) is the simplest of a family of bacterial polyesters, polyhydroxyalkanoates (PHAs), which are produced by a wide variety of microorganisms under nutrient deprivation[1-4] as an intracellular energy reserve.[1] Under appropriate conditions, PHAs have been shown to accumulate to up to 90% of the dry cell weight.[5] PHAs are thermoplastics which are biodegradable and biocompatible.[3] The idea of thermoplastic materials with a wide range of properties which are readily degradable by a wide variety of bacteria has become very attractive in an age of increased environmental concern.[6] Even more attractive is the fact that, with the advent of transgenic plant technology, this material could be produced using a renewable resource rather than a non-renewable one; i.e. atmospheric carbon dioxide becomes the carbon source instead of fossil fuels.

A bacterial PHA copolymer, Biopol, is currently being produced on a large scale by Zeneca (United Kingdom) using *Alcaligenes eutrophus* as the production organism. These materials have been widely discussed as potential biodegradable replacements for petroleum derived thermoplastics like polypropylene.[7-9] Degradability studies in soil, activated sludge, and seawater have shown PHB and some of its PHA copolymers to be degradable in all three environments.[10] A number of microorganisms are known to excrete

extracellular PHB depolymerase to degrade microbial polyesters for use as a nutrient source.[11]

STRUCTURE AND PROPERTIES

The repeating structure of PHB is shown in Figure 1 on page 13. The polymer was first described in the late 1920's by Lemoigne.[12] The structure and properties of was primarily of interest understanding microbial metabolisms into the early 1960's.[13] It is only relatively recently that its thermoplastic nature and biodegradability have been considered significant. The first data on PHB molecular weight and physical properties were reported in 1958 by Wilkinson and Williamson.[14]

Unlike most biological polymers such as polysaccharides and proteins, PHB is a thermoplastic material with a crystalline melting temperature of 180°C.[3,15] Due to its absolute stereoregularity, it is a highly crystalline material in bulk and crystallizes in a 2_1 right-hand helix.[16] The thermoplastic properties of PHB are most often compared to those of the petroleum-derived thermoplastic polypropylene due to the similar structure, melting temperature, glass transition temperature, and degree of crystallinity.[17] Although crystalline from the melt or from solvent, PHB has been shown to be completely amorphous in the host organism *A. eutrophus* by several groups.[3,18-22]

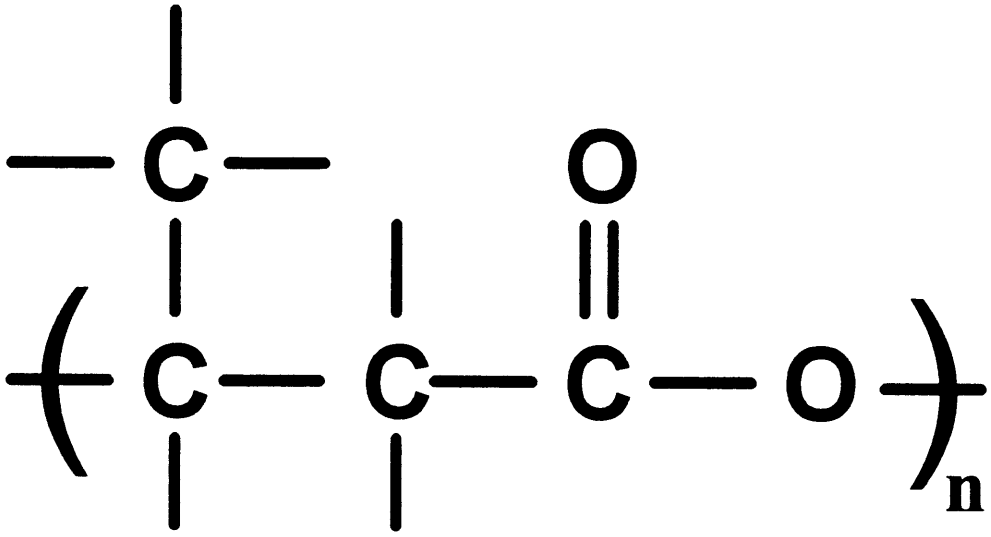


Figure 1. Repeating Unit of PHB

MOLECULAR WEIGHT AND SOLUTION PROPERTIES

In the literature, absolute molecular weight determinations have been very few for PHB and non-existent for other PHAs. Until the recent wide-spread use of gel permeation chromatography (GPC), molecular weight of PHB was not readily determinable. Currently, most studies on PHB use GPC to evaluate average molecular weights. Two groups have used absolute techniques, light scattering or osmometry, for PHB molecular weight determination.[23-26] These groups also reported the Mark-Houwink constants relating intrinsic viscosity to molecular weight for PHB in chloroform, 2,2,2-trifluoroethanol (TFE), and ethylene dichloride (EDC). TABLE I on page 15 summarizes the Mark-Houwink constants calculated by these groups for the relationship $[\eta] = KM_w^a$. Also presented in this table are Mark-Houwink constants calculated using a compilation of all data available from the authors mentioned. Some of the data used in these calculations was not used in the respective publications in Mark-Houwink calculations. For example, since Marchessault, *et. al.* and Cornibert, *et. al.* analyzed the same samples, molecular weight data from Cornibert, *et. al.* was used with some intrinsic viscosity data from Marchessault, *et. al.* in the chloroform composite calculation. Also, the 5 degree temperature difference in some TFE data was disregarded.

A few details are worth noting when deciding the constants to be used for either intrinsic viscosity conversion to molecular weight or for construction of a GPC universal calibration[27] curve. Marchessault, *et.al.* derived the molecular weights from sedimentation data, and the samples are unfractionated with polydispersities (M_w/M_n) ranging from 1.4 to

TABLE I. MARK-HOUWINK CONSTANTS FOR PHB, $[\eta] = KM_w^a$

Solvent	K	a	M_w (x 10^{-3})	Reference
Chloroform 30°C	0.77 x 10^{-4}	0.82	21 - 780	[23]Marchessault, *et.al.* 1970
Chloroform 30°C	1.18 x 10^{-4}	0.78	115 - 1640	[25]Akita, *et.al.* 1976
TFE 30°C	2.51 x 10^{-4}	0.74	21 - 1000	[24]Cornibert, *et.al.* 1970
TFE 25°C	1.25 x 10^{-4}	0.80	115 - 1640	[25]Akita, *et.al.* 1976
TFE 25°C	2.22 x 10^{-4}	0.76	20 - 9100	[26]Miyaki, *et.al.* 1977
EDC 30°C	0.92 x 10^{-4}	0.78	115 - 1640	[25]Akita, *et.al.* 1976
Chloroform	2.24 x 10^{-4}	0.73	21 - 1640	Composite
TFE	1.82 x 10^{-4}	0.77	20 - 9100	Composite

6.1, calculated using osmometry data for M_n. Ubbelohde type capillary viscometers were used for the determination of intrinsic viscosity.

Akita, *et.al.*[25] obtained the data using light scattering with fractionated samples having polydispersities between 1.4 and 2.3 for M_w 630,000 and less, also calculated using osmometry to obtain M_n. Osmometry is not accurate for measuring higher molecular weights since osmotic pressure is proportional to the inverse of M_n.[28], making osmotic pressures too low to measure with even the most sensitive instruments when high molecular weights are involved. Once again, Ubbelohde type capillary viscometers were used for the determination of intrinsic viscosity, except for molecular weights greater than 860,000, where a rotational viscometer was used to extrapolate viscosities to zero shear.

Cornibert, *et.al.*[24] used light scattering on the same samples of PHB used by Marchessault, *et.al.*[23] and used the intrinsic viscosities reported by Marchessault, *et.al.*[23] in TFE to calculate the Mark-Houwink parameters. Only four new data points were involved in this calculation.

The data of Miyaki, *et.al.*[26] covers the broadest range, however M_w's of 150,000 and below were actually determined with a stereo-isomer of PHB, copoly(D-L-β-methyl β-propiolactone) (PMPL). All samples with an M_w greater than 860,000 were subject to rotational viscometry to determine zero-shear intrinsic viscosities. Otherwise, Ubbelohde type capillary viscometers were used. No polydispersities were reported due to the M_n range being too high for osmometry and no suitable GPC solvent being yet available.

Akita, *et.al.*[25] and Miyaki, *et.al.*[26] isolated PHB from a strain of *Azobacter vinerandii*, and the other groups isolated PHB from other bacterial strains. All groups

showed the structure of their material to be the homopolymer PHB shown in Figure 1. Akita, *et.al.*[25] and Miyaki, *et.al.*[26] used NMR and IR spectra to confirm this, while Marchessault, *et.al.*[23] and Cornibert, *et.al.*[24] used samples which were shown to be PHB in a previous study.[55]

No Mark-Houwink constants have been calculated for any other PHAs. For this reason, GPC or intrinsic viscosity data from other PHAs cannot be converted to molecular weight.

The very first study of solution properties of PHB began in the early 1950's when intrinisic viscosities were correlated to extraction techniques.[29] The first reported value of PHB molecular weight was a very small 5000 Da, determined by isothermal distillation in chloroform.[14] More recently GPC results of weight-average molecular weights of PHB derived from *A. eutrophus* were reported to range from 600,000 Da to 2.4 million Da, depending on the carbon source with routine fermentations yielding polymer with a M_w just above 1 million.[3,30-33] These results are typically reported as M_n with a polydispersity index estimated from the shape of the GPC chromatogram. It is also well documented that PHB degrades in the cells upon depletion of the carbon source.[32,33]

The Mark-Houwink constants summarized above actually were the result of a separate controversy in the literature between the two groups involved. Marchessault, *et.al.*[23] suggested that the helical nature of PHB was somewhat retained in solution. Optical rotatory dispersion (ORD) measurements revealed what appeared to be a coil-helix transition at a given temperature or solvent composition. However, as is shown by the reported Mark-Houwink exponent, PHB appears to conform to a non-draining

random-coil configuration anticipated by most linear polymers in solution. [28] An interrupted helix model was described to account for the differences in hydrodynamic and optical data. Upon light scattering analysis of the same samples by Cornibert *et.al.*[24] in an attempt to obtain more information about the shape of the molecules, a new model based on a chain-folding hypothesis was offered. Akita, *et.al.*[25] set out to disprove these hypotheses with light scattering experiments of their own and repeated some of the ORD experiments performed by Marchessault, *et.al.*[23] They reported that PHB is a random coil in all solvents. More recently, Doi *et.al.*[34] used ^{13}C-NMR to conclude that the helical conformation of PHB is not retained in chloroform.

EXTRACTION TECHNIQUES

The extraction techniques used to purify PHB and other PHAs from the producing organism is worth mentioning. The molecular weight data reported in the literature has been obtained with a variety of extraction techniques. In fact, as mentioned previously, some of the first molecular weight data available on PHB is the result of extraction studies.[13,29] Also, it is the extraction step which is the primary obstacle to successful commercial exploitation of PHAs.[31]

There are essentially three methods which have been used to extract PHAs from their host cells: alkaline hypochlorite treatment, solvent extraction, and enzyme treatment.

The first method was developed by the first scientists to study PHB.[14] In this method, sodium hypochlorite is used to degrade most of the cellular macromolecules surrounding the PHA granule.[34] Unfortunately, it has been shown to severely degrade the PHA as well.[13,35,36] This technique has remained popular, however, due to its simplicity and effectiveness.

Solvent extraction is considered the mildest of the methods of extraction. In this method, an organic solvent such as chloroform, methylene chloride, dichloroethane, or propylene carbonate is used to extract the polymer from the freeze-dried cells. The cell wall material can then be removed by filtration or centrifugation and the polymer precipitated from solution using a suitable non-solvent such as hexane, diethyl ether, methanol or ethanol.[3] The precipitate is filtered off or centrifuged for collection and dried. Obviously, this procedure involves many more steps and is more costly. With high molecular weight PHB, separation of the cell debris from the resulting solution is not

trivial. However, it has been shown that this technique is much less degrading to the polymer than hypochlorite treatment.[13,30,31]

Enzymatic digestion of the cell material was developed by ICI (now Zeneca)[37] using an enzyme which degrades the cell membrane, but not the PHA.[38]

In an attempt to remove the disadvantages of solvent extraction alone and hypochlorite treatment alone, the methods were recently combined.[31] In this technique, a dispersion of sodium hypochlorite is used with chloroform to simultaneously degrade the cell mass and extract the polymer. The technique was successful and optimized to maximize the purity and recovery of the polymer and to minimize degradation.

BIOSYNTHESIS OF PHB

The biosynthetic pathway leading to PHB in *A. eutrophus* is fairly well understood.[39-42] Three enzymes play a role, as described in Figure 2, page 22. The thiolase enzyme reversibly condenses two units of acetyl-CoA into acetoacetyl-CoA. This in turn is reduced by the NADPH-dependent reductase enzyme into 3-D-hydroxybutyryl-CoA (HBCoA), the monomer for PHB. This is then polymerized by PHA synthase into P(3HB), or PHB.

Some effort has been exerted trying to understand the final step of PHB production, since it is the polymerization step. The three-step enzymatic process is generally looked at as two separate steps itself: 1) the production of substrate by the thiolase and reductase enzymes and 2) the polymerization of this substrate by the synthase enzyme (sometimes referred to as polymerase). Ballard *et.al.*[33] proposed a synthase mechanism and Kawaguchi and Doi[32] proposed a quantitative model demonstrating the regulation of the size of PHB molecules based on the assumption of constant synthase concentration, rapid initiation, and the presence of a chain transfer reaction. The assumption of constant synthase concentration was derived from activity measurements performed on *A. eutrophus* by Haywood, *et.al.*[42] The assumption of a chain transfer step was based on a yield/molecular weight ratio that show the number of polymer chains to be increasing during PHB production. If synthase concentration is indeed constant, the only way new chains could be produced is by a transfer step. They went on to calculate the number of synthase molecules present per cell using their model. Demonstrating the great similarity between Flory's most probable distribution, which is based on a chain-

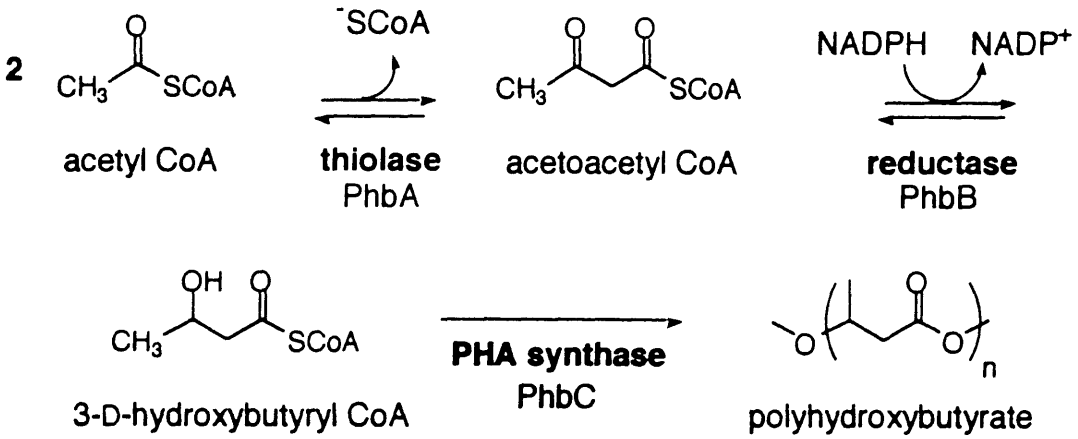


Figure 2. Biosynthesis of PHB

propagation and chain-termination model, and the molecular weight distribution of PHB from *A. eutrophus*, Doi[3] suggests that a chain-termination step occurs in PHA biosynthesis.

PHB PRODUCTION IN RECOMBINANT *E. COLI* AND *IN VITRO*

The DNA sequences of the genes encoding the PHB producing enzymes *in A. eutrophus* have been identified and analyzed.[44-46] This has led to their successful expression in *Escherichia coli* by several groups.[47-51] Little effort has been aimed at molecular weight determinations, however. Hahn, *et.al.*[30] has reported molecular weights of PHB from recombinant *E. coli* while studying extraction techniques and showing evidence of *in vivo* crystallinity of PHB granules. The number average molecular weight has been reported as 1.5 million Da by GPC, with a polydispersity of approximately 2.0, indicating an M_w of 3 million Da. This is about two times the average size of PHB molecules generally produced in *A. eutrophus*. Effort in this area seems to be focused on increasing yields and rate of PHB production, not on attempts at understanding the biosynthesis of PHB.

Utilizing the fact that PHA synthase from *A. eutrophus* has been successfully overproduced and purified,[52] *in vitro* polymerizations of HBCoA have been performed.[53] The reported molecular weight of the PHB from these macroscopic granules was over 10 million Da, determined by GPC, although the authors report some uncertainty about the accuracy of these measurements. In regard to the mechanism of the polymerization, it was concluded that the final molecular weight of the PHB was determined very early in the polymerization and was a function of PHA synthase concentration. No correlation between reaction time or substrate concentration was found. It was also concluded that no chain transfer reaction takes place in the *in vitro* reaction, contrary to Kawaguchi and

Doi's[32] hypothesis *in A. eutrophus*. Due to the inaccuracy of GPC measurements of high molecular weights, no model was proposed for molecular weight regulation.

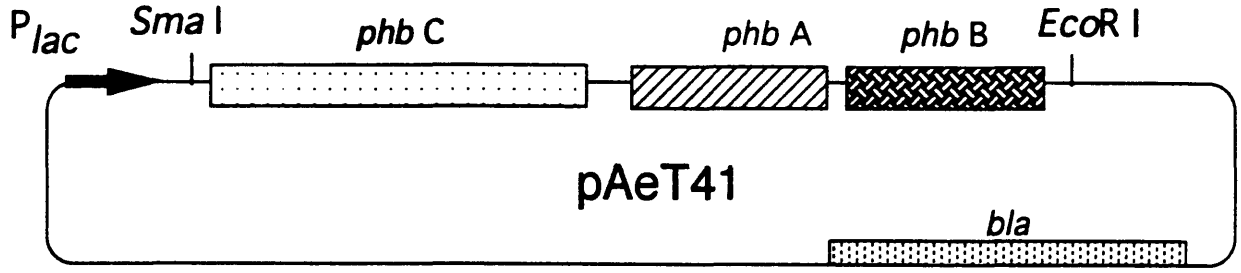
III. MATERIALS AND METHODS

PHB PRODUCTION

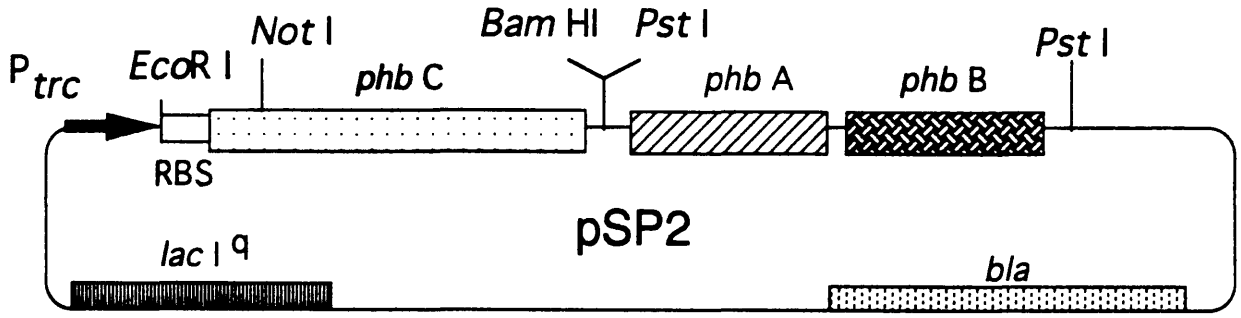
Strains

The PHB samples used in this study were produced using two recombinant strains of *Escherichia coli* provided by Professor Anthony J. Sinskey's Lab in the Department of Biology at MIT. Both strains contain plasmid-borne operon from *Alcaligenes eutrophus* encoding the three enzymes required for the biosynthesis of PHB. The plasmid maps are shown in Figure 3 on the following page.

Figure 3a shows the plasmid in the strain *E. coli* DH5α/pAeT41, which contains the native operon from *A. eutrophus* for PHB production.[45] Figure 3b shows the plasmid in the strain *E. coli* DH5α/pSP2, which contains an optimized ribosome binding site in front of the synthase gene to increase the efficiency of the production of this enzyme in *E. coli*, as shown by Gerngross, *et.al.*[52] The enzyme production is regulated by the addition of an induction agent, isopropyl-β-D-thiogalactopyranoside (IPTG). This regulation is included in the construction of the plasmid to inhibit enzyme production in the early stages of cell growth, as an excess of enzyme can be toxic to the cell and inhibit growth.



a)



b)

Figure 3. Construction of the plasmids designed to produce PHB in *E. coli*: a) pAeT41 and b) pSP2

Fermentation and Purification

Initial PHB samples were produced in shake flask cultures as follows. Both *E. coli* strains were grown into starter cultures overnight in LB media.[54] Inoculum (1%) was introduced to a 500 ml culture in a 1000 ml Erlenmeyer flask consisting of LB media and 20 g/L glucose as the carbon source, along with 50 mg/L ampicillin as the antibiotic. Cultures were incubated at 30°C. Optical density at 600 nm was monitored during each fermentation. When the optical density of the pSP2 culture reached 0.6, 0.4 mM IPTG was added to induce enzyme production. Fermentations were halted at 72 hours when the growth curves appeared to be reaching a plateau.

Subsequent fermentations were scaled up to either 1.8L or 10L by Dr. Sang Jun Sim of Professor Sinskey's Lab in the Department of Biology at MIT.

Upon the termination of each fermentation, the cells were harvested by centrifugation at 10,000g, washed with deionized water, and reharvested by centrifugation. Cells were then resuspended in a minimum amount of water and stored at -20°C overnight. Samples were then dried under vacuum and refluxed in chloroform at a concentration of 5 grams dry cell mass per 1 liter of chloroform for four to five hours. Cell mass was removed by filtration through a sintered glass funnel. The polymer was precipitated by the addition of one volume of n-hexane. The precipitate was then harvested using a sintered glass funnel and dried under vacuum.

Proton NMR spectra in deuterated chloroform, performed and examined at MIT by Drs. Monika Schoewölff and Kristi Snell, respectively, revealed the presence of only the pure homopolymer PHB. No further purification was attempted.

MOLECULAR WEIGHT DETERMINATION

Intrinsic Viscosity

All intrinsic viscosity determinations were performed using chloroform or 2,2,2-trifluoroethanol as the solvent at 30°C and 25°C, respectively. Size 50 Cannon-Fenske type viscometers were used to obtain efflux times ranging from 100 to 550 seconds. Kinetic energy corrections were used in accordance with the viscosity equation shown below:[28]

$$\eta = \alpha\rho\left(t - \frac{\beta}{\alpha t}\right)$$

where η is viscosity in mPa·s, ρ is density in g/ml, t is flow time in seconds, and α and β are calibration constants, β being the kinetic energy correction. For the size 50 viscometer, β=3.68 ml/m and α=4.23 x 10^{-3} ml/m·s^2, calculated using chloroform and acetone as standards. This leads to a correction of 18% compared to specific viscosities calculated by a simple flow time ratio when using chloroform as the solvent. Due to the higher viscosity of TFE, the correction is only a few percent, but was still used. Shear rates in the capillaries ranged from 1000 to 1500 sec^{-1} with chloroform as the solvent and from 250 to 400 sec^{-1} with TFE as the solvent.

As an alternative to capillary viscometers, a Bohlin Rheometer System VOR (Bohlin Reologi, Lund, Sweden) was used to investigate the possibility of shear thinning. The rotational viscometer was used with TFE at 25°C and a double-gap measuring system

to maximize sensitivity. Shear rates used were in the range from 1 to 80 sec^{-1}. Viscosities were extrapolated to zero shear for intrinsic viscosity calculations.

100 ml stock solutions were prepared from each sample, generally at a concentration of 0.1 g/dl, from which all dilutions were prepared to obtain specific viscosities in the range of 0.1 to 0.7. Dissolution was performed either by refluxing the sample in chloroform for 30 minutes to one hour or by dissolving without heat overnight. Samples were filtered using a 0.45 mm PVDF membrane prior to viscosity measurements. Intrinsic viscosity was determined by extrapolating Huggins' equation to infinite dilution:

$$\left(\frac{\eta_{sp}}{c}\right)_{\lim c \to 0} = [\eta] + k'[\eta]^2 c$$

where $[\eta]$ in the intrinsic viscosity, η_{sp} is the specific viscosity, c is the polymer concentration, and k' is Huggins' viscosity constant.

Intrinsic viscosity is related to the average molecular weight of a polymer through the Mark-Houwink equation:

$$[\eta] = KM^a$$

where M is the polymer molecular weight and K and a are constants for a given polymer-solvent system. The magnitude of a is related to the conformation of the polymer in the given solvent. A larger value of a indicates a relatively expanded polymer chain, while a smaller value indicates a compact conformation. The magnitude of K is related to the excluded volume effect of the polymer molecule in the given solvent.

Size Exclusion Chromatography

Size Exclusion Chromatography, more commonly called SEC or GPC for Gel Permeation Chromatography, is the most common method in use for the determination of the molecular weight of high polymers. Its wide spread use is due in large part to the minimal amount of sample and instrumentation required and because it reveals information concerning the molecular weight distribution of a polymer sample. SEC works on the principle of size-exclusion. A column packed with a highly cross-linked permeable matrix of polymeric beads is placed in a standard HPLC system. It differs from conventional liquid chromatography in that the mobile phase and the stationary phase are the same solvent. The smallest of the molecules in a given sample is able to permeate a greater portion of the matrix, leading to a more torturous path for smaller molecules which effectively leads to a longer retention time or a higher retention volume. By the same principle, larger molecules can permeate only a small portion of the matrix and are excluded from the remainder, giving the technique its name. As a sample proceeds through a column of this type, it is continuously separated by molecular size with the largest molecules eluding first and the smallest last.

Standard polymers of known molecular weight are used to construct a calibration curve for an SEC system. Molecular weight is plotted against retention volume. The retention volume of unknown samples is then recorded and converted to molecular weight through this curve. However, the fact that different molecular species possess different hydrodynamic volumes in a particular solvent means that the molecular weight standards

have to be of the same chemical composition or chain conformation as the unknown for this technique to be accurate. Because standard materials are available in very limited compositions, an accurate application of the technique is limited.

This problem was addressed by the development of the universal calibration method.[27] This method uses the principle of SEC to predict elution volumes for polymers of any composition, given that the relationship between molecular weight and intrinsic viscosity is known for the standard and the unknown. This technique uses the fact that the hydrodynamic volume of a polymer molecule is proportional to the product of its molecular weight and its intrinsic viscosity. According to Einstein's viscosity law:

$$[\eta] = \Phi\left(\frac{V_h}{M}\right)$$

where V_h is the hydrodynamic volume of a polymer molecule, M is its molecular weight, $[\eta]$ is its intrinsic viscosity, and Φ is a universal constant. Using the Mark-Houwink relationship, it can be manipulated to obtain:

$$V_h = \frac{1}{\Phi} M[\eta] = \Phi^{-1} K M^{a+1}$$

To obtain the universal calibration curve, the product $M[\eta]$ for a standard material is plotted against retention volume. The retention volume for an unknown is then measured and converted to molecular weight through this curve. In this manner a value proportional to the actual volume of individual molecules is used to predict the retention volume, which

is the principle SEC is built on. Measurements must be done using constant temperature, flowrate, sample concentration, and injection volume.

A concentration sensitive detector is typically used to detect retention volume. Using such a detector allows the calculation of both number average and weight average molecular weights, the ratio of which is known as the polydispersity index:

$$PDI = \frac{M_w}{M_n}$$

This ratio is an indication of the width of the molecular weight distribution. The larger the PDI, the broader the molecular weight distribution.

The averages M_n and M_w are calculated as follows. Detector response is proportional to polymer concentration, which is proportional to the product M_iN_i, where N_i is the number of molecules of molecular weight M_i. At each retention volume, M_i is known through the universal calibration curve. The detector response is divided by M_i for each retention volume to obtain a series of N_i's.

M_n and M_w are defined as:

$$M_n = \frac{\sum_i M_i N_i}{\sum_i N_i}$$

$$M_w = \frac{\sum_i W_i N_i}{\sum_i W_i} = \frac{\sum_i M_i N_i^2}{\sum_i M_i N_i}$$

where W_i is the total weight of the molecules of molecular weight M_i. Both averages can easily be calculated by manipulation of the detector response.

For this study, the chromatography system was composed of a Waters model 501 HPLC Pump, UK6 injector, column heater and model 410 differential refractometer. A pulse dampener was also in-line. The column set consisted of a Progel-TSK G7000H_{XL} GPC column (Supelco, Bellefonte, PA, USA) in series with a Shodex K-805 GPC column (Shodenko, LTD, Japan) with chloroform as the solvent at 30°C, the only temperature for which Mark-Houwink constants (K=1.18 x 10^{-4}, a=0.78) are reported for PHB in chloroform.[25] Flowrate was 1.0 ml/min. Narrow polystyrene standards ranging in molecular weight from $4x10^3$ g/mole to $3x10^7$ were used to generate the calibration curve. Sample concentration was generally 1 mg/ml, and injection volume was 100 µl, except for ultra-high molecular weight polystyrene standards for which the concentration was reduced to 0.25 mg/ml. All standards and samples were filtered through 0.45 µm membranes prior to injection. The refractometer signal was processed as previously described to obtain the molecular weight averages for each sample. No correction was made for band-broadening.

Light Scattering

Light scattering differs from the previous two methods for molecular weight determination in that it is an absolute technique. Both intrinsic viscosity and SEC are relative techniques. Intrinsic viscosity relies on constants generated by other experimentalists who used an absolute technique for their molecular weight determination.

SEC relies on many things, including retention times of known molecular weight standards and two sets of Mark-Houwink constants. SEC is also subject to band-broadening. Band-broadening occurs because of the simple fact that every molecule which is the same size cannot elude at exactly the same time. Even exceptionally narrow standards with polydispersities less than 1.01 must elude over a certain range.

In contrast, light scattering relies only on the physical properties of the molecule. Excess scattered light is directly proportional to the molar mass of the molecules causing the excess scattering.

Light scattering is based on the equation:

$$\frac{K^* c}{R_\theta} = \frac{1}{M_w P(\theta)} + 2A_2 c + \ldots$$

where c is concentration, R_θ is the Rayleigh scattering intensity at angle θ, M_w is the weight average molecular weight of the solute, and A_2 is the second virial coefficient accounting for solvent-solute interaction. Higher order virial coefficients may be used if necessary. K^* is an optical constant defined by:

$$K^* = 4\pi^2 n_o^2 (dn/dc)^2 \lambda_o^{-4} N_A^{-1}$$

where n_o is the refractive index of the solvent, dn/dc is the refractive index increment of the solute-solvent system, λ_o is the wavelength of the incident beam, and N_A is Avagadro's number. $P(\theta)$ is the scattering function which accounts for the angular dependence of scattered light for finite-sized molecules. Expanded to first order:

$$\frac{1}{P(\theta)} = \left[1 + \frac{16\pi^2}{3\lambda^2}\left\langle r_g^2 \right\rangle \sin^2\left(\frac{\theta}{2}\right) + \ldots\right]$$

where $\left\langle r_g^2 \right\rangle$ is the mean-square radius of gyration of the solute.

In a classical light scattering experiment, several solutions of known concentration are subjected to a light beam of known wavelength. The *dn/dc*, or the concentration dependence of the refractive index of the polymer-solvent pair, is determined in a separate experiment using a refractometer. The excess scattered light is measured at several angles θ from the incident beam. A Zimm plot is used for a dual extrapolation to zero angle θ and zero concentration c. Both extrapolations yield identical intercepts when the quantity K^*c/R_θ is plotted against a function of the scattering angle θ:

$$\left(\frac{K^* c}{R_\theta}\right)_{\theta \to 0} = \frac{1}{M_w} + 2A_2 c$$

and

$$\left(\frac{K^* c}{R_\theta}\right)_{c \to 0} = \frac{1}{M_w}\left[1 + \frac{16\pi^2}{3\lambda^2}\left\langle r_g^2 \right\rangle \sin^2\left(\frac{\theta}{2}\right)\right]$$

The intercept is equal to the inverse of the weight average molecular weight of the solute. This technique also yields the second virial coefficient A_2 in the slope of the zero angle extrapolation and the radius of gyration r_g in the extrapolation to infinite dilution. A_2 is a measure of how expanded the polymer coil is in the given solvent. A higher value of A_2 indicates a more expanded polymer, hence a "better" solvent. r_g determined in this manner can assist in determining molecular conformation.

Zimm's technique fails at very high molecular weights because of the inverse relationship between M_w and the zero concentration and zero angle limits. Alternatively, the Debye method can be used where R_θ/K^*c is plotted on the dependent axis. This method gives M_w directly from the intercept.

With the advent of SEC, light scattering as its founders knew it declined in use due to its difficult nature. A single dust particle in a solution can give erratic results because of the area of the spectrum being utilized. However, the two methods have been combined with excellent success due to the seperatory nature on the SEC method.

In the combined method, a light scattering detector is placed in-line following the GPC column. The sample is effectively divided up into slices by the column with light scattering intensities being measured for each slice. A calibrated concentration detector follows the scattering cell to measure the concentration of each molecular weight species. Using the Debye method, the molecular weight of each slice is calculated. M_n and M_w can then be calculated from these quantitative data rather than from the shape of the elution profile. This eliminates the band-broadening effect seen in conventional SEC. It also allows the freedom to work at different temperatures, flowrates, sample concentrations and injection volumes. Also, post-column concentrations are usually low enough to neglect the A_2 term in the M_w calculation, essentially assuming that experimental conditions are very close to infinite dilution.

In this report, light scattering analyses were performed using a Dawn-F multi-angle Laser Photometer (Wyatt Technology, Santa Barbara, CA) in series with the HPLC system used for the SEC analyses. The solvent used was 2,2,2-trifluoroethanol (Aldrich

Chemical Company, Milwaukee, WI, USA) and filtered through a 0.2 μm PTFE membrane. A Shodex K-807L GPC column (Shodenko, LTD, Japan) was used for sample separation. System temperature was maintained at 35°C and the flowrate at 1.0 ml/min. Sample concentrations ranged from 0.5 mg/ml for lower molecular weight samples to 0.01 mg/ml for higher molecular weight samples. Injection volumes varied from 25 μl to 200 μl depending on sample concentration to keep injected mass in the range from 20 μg to 50 μg. A Waters 410 differential refractometer was calibrated to measure both the *dn/dc* of the samples and the instantaneous concentrations of sample eluding from the column. Narrow polymethylmethacrylate standards were used for instrument normalization and calibration. Figure 4 on the following page verifies the calibration of the instrument. All samples were filtered through a 0.45 μm PVDF membrane prior to injection. ASTRA software (Wyatt Technology) was used for all molecular weight calculations.

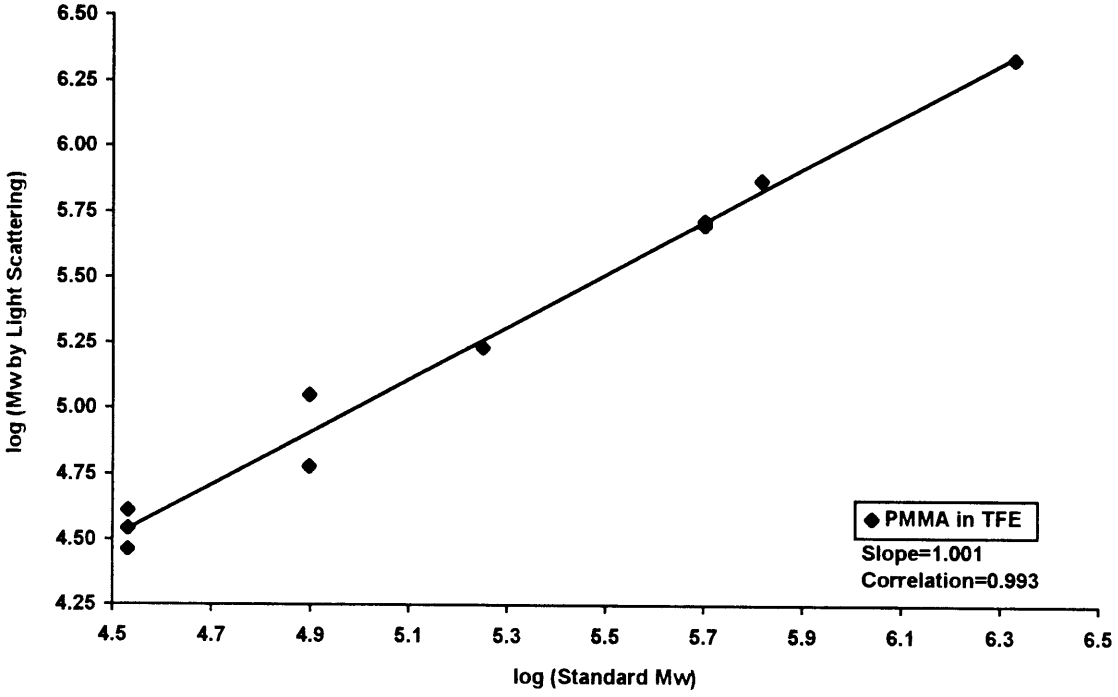


Figure 4. PMMA standards. *dn/dc*= 0.198

IV. RESULTS AND DISCUSSION

During the purification of PHB from the recombinant *E. coli* strains, it was noticed that the chloroform solution containing PHB produced by strain pAeT41 was much more viscous than the PHB isolated from pSP2. The filtration step lasted hours for pAeT41, but lasted only seconds for pSP2.

INITIAL DISCOVERY

PHB isolated from the initial shake flask cultures of DH5α/pAeT41 and DH5α/pSP2 were subjected to intrinsic viscosity analysis in chloroform (TABLE II). Demonstrated here is the order of magnitude difference in PHB chain length produced from the two different strains. Equivalent molecular weight is calculated from the Mark-Houwink constants reported later in this chapter. The difference in the strains is the optimized ribosome binding site in front of the synthase gene in the plasmid pSP2. This allows optimum expression of this gene in *E. coli*. As a result, more of the synthase enzyme should be produced by pSP2 than by pAeT41. Recall that the synthase enzyme acts in the final step in the biosynthesis of PHB. It "catalyzes" the polymerization of the hydroxybutyryl-CoA produced by the thiolase and reductase enzymes. From this initial experiment, it was hypothesized that high synthase levels leads to lower average molecular weights. This implies that the synthase enzymes present compete for the available

TABLE II. INTRINSIC VISCOSITY IN CHLOROFORM (30°C) OF PHB PRODUCED BY RECOMBINANT *E. COLI* (500 ml FLASK CULTURES)

Strain	[η] dl/g	Equivalent M_w
pAeT41	13.2	5.2×10^6
pSP2 (0.4 mM IPTG)	2.7	0.6×10^6

substrate; i.e. high synthase concentration would lead to many short chains being produced, and low synthase concentration would lead to few long chains being produced.

TWO LITER FERMENTATIONS

Initial Results

In an attempt to achieve a better controlled fermentation environment, the fermentation was scaled up to 2 liters in a batch-style fermentor. The molecular weight of the polymers produced by the two strains in this fermentation did not differ to the degree it did in the flask cultures (TABLE III).

IPTG Modulation

In the initial scale up, the difference in molecular weight was still apparent, but not as great as in the shake flask cultures. This can probably be attributed to the better controlled fermentation conditions increasing the cell density to levels higher than in the shake flask cultures, making the concentration of the induction agent isopropyl-β-D-thiogalactopyranoside (IPTG) per cell in the 2 liter pSP2 cultures effectively lower than it was in the 500 ml flask culture. In an effort to regain the order of magnitude difference seen previously, the molar concentration of the induction agent IPTG was increased from the nominal 0.4 mM to 1.4 mM in the pSP2 culture. As expected, the intrinsic viscosity of the resulting PHB decreased (TABLE IV and Figure 5).

Intrinsic viscosity decreased from 6.4 dl/g to 4.6 dl/g when IPTG concentration was increased. Also shown in Table IV are the results from changes in the IPTG concentration to 5.0 mM and 0.1 mM. Increasing the inducer concentration to 5.0 mM decreased the intrinsic viscosity to 3.5 dl/g. Decreasing IPTG concentration to below the

nominal 0.4 mM to 0.1 mM resulted in a slightly lower intrinsic viscosity. It should be noted that PHB production for this particular fermentation was severely diminished. This can be explained by the fact that the thiolase- and reductase-encoding genes are also inhibited with such a low induction level. In other words, not enough hydroxybutyryl-CoA was produced to yield a higher molecular weight. Assuming that increased IPTG levels increase the amount of the synthase enzyme produced, these results support the hypothesis that increased synthase concentration leads to lower molecular weight PHB.

TABLE III. INTRINSIC VISCOSITY IN CHLOROFORM (30°C) OF PHB PRODUCED BY RECOMBINANT *E. COLI* (2 LITER FERMENTATIONS)

Strain	[η] dl/g	Equivalent M_w
pAeT41	12.0	4.6×10^6
pSP2 (0.4 mM IPTG)	6.4	2.0×10^6

TABLE IV. INTRINSIC VISCOSITY IN CHLOROFORM (30°C) OF PHB PRODUCED BY RECOMBINANT *E. COLI* STRAIN DH5α/pSP2 WITH VARIOUS IPTG CONCENTRATIONS

IPTG Concentration (mM)	[η] (dl/g)	Equivalent M_w (Da)
0.1	5.8	1.7×10^6
0.4	6.4	2.0×10^6
1.4	4.6	1.3×10^6
5.0	3.5	0.9×10^5

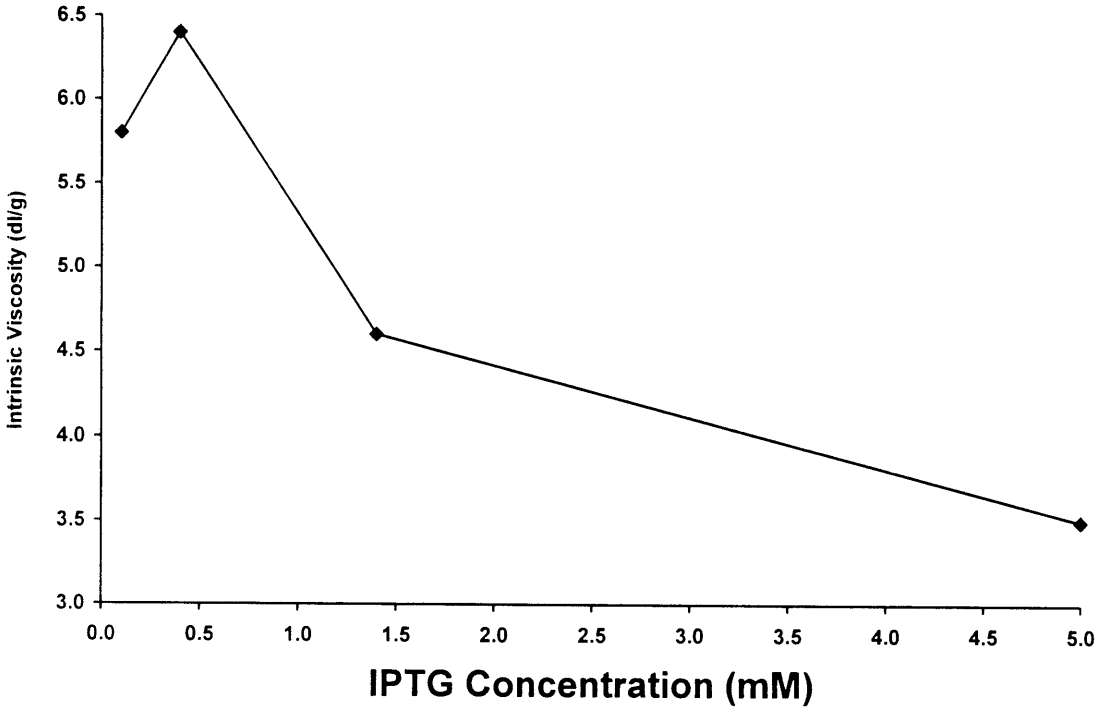


Figure 5. IPTG Dependence of Intrinsic Viscosity in Chloroform (30°C) of PHB Produced by Recombinant *E. coli* Strain DH5α/pSP2,

TEN LITER FERMENTATIONS

Doi, *et. al.* (1994) reported that the molecular weight of PHB produced in *A. eutrophus* reached a maximum molecular weight, then began degrading for the remainder of the fermentation. It was desirable to emulate such an experiment to determine if PHB produced in *E. coli* was degrading during the fermentation as it does in its native organism. No known PHA depolymerase enzyme is included in the construction of either of the two plasmids used in this study, and no PHA depolymerase is known to exist in *E. coli*. For these reasons, it was thought that the PHB was probably not degrading during fermentation.

The fermentation was scaled up to 10 liters in order to be able to collect sufficient samples for the entire fermentation and allow it to continue. For the pSP2 strain, two fermentations were carried out. The first was induced at the nominal 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) concentration and the second at an IPTG level of 5.0 mM.

Intrinsic Viscosity Analysis

All samples were first analyzed for intrinsic viscosity in chloroform to determine the approximate molecular weight (TABLE V and Figure 6). No samples were available prior to 6.5 fermentation hours due to only small amounts of PHB produced before this time. Equivalent molecular weights are calculated from Mark-Houwink constants reported later in this chapter.

TABLE V. INTRINSIC VISCOSITY IN CHLOROFORM (30°C) OF PHB PRODUCED BY RECOMBINANT *E. COLI* (10 LITER FERMENTATIONS)

Strain	time (hrs)	[η] (dl/g)	Equivalent M_w (Da)
pAeT41	6.5	6.5	2.0×10^6
	9.9	10.9	4.0×10^6
	14.6	9.8	3.5×10^6
	18.7	9.9	3.6×10^6
	23.3	10.0	3.6×10^6
	32.3	10.7	3.9×10^6
pSP2	6.9	5.9	1.8×10^6
0.4mM IPTG	8.4	5.4	1.6×10^6
	11.5	4.8	1.4×10^6
	13.3	5.1	1.5×10^6
	18.3	4.7	1.3×10^6
	23.6	4.6	1.3×10^6
	35.5	4.7	1.3×10^6
	40.0	4.7	1.3×10^6
pSP2	8.2	4.2	1.1×10^6
5.0 mM IPTG	11.4	4.2	1.1×10^6
	16.5	3.0	0.7×10^6
	23.4	2.8	0.7×10^6
	30.2	3.0	0.7×10^6
	37.7	3.0	0.7×10^6

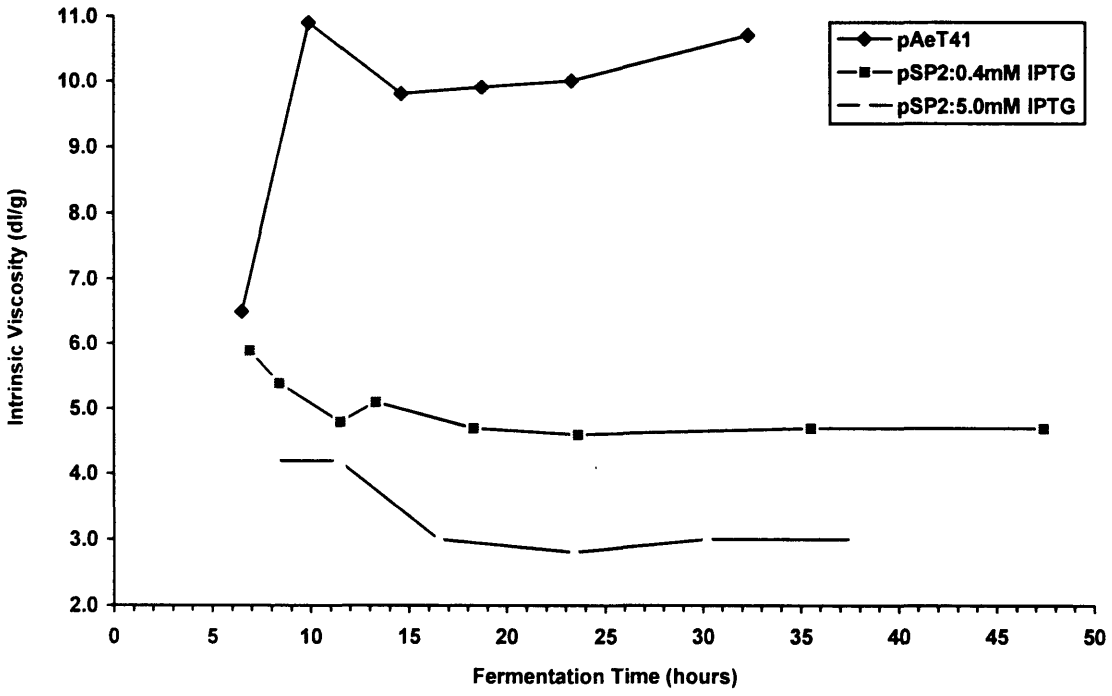


Figure 6. Intrinsic Viscosity in Chloroform (30°C) of PHB Produced by Recombinant *E. coli* (10 Liter Fermentations)

Since pAeT41 is a slower growing strain, it appears that the first data point at 6.5 hours was taken when the polymer chains were still in the early stages of development. The average molecular size then levels out at an intrinsic viscosity between 10 and 11 dl/g. At first glance, it appears that PHB from the pSP2 strain degraded in both cases. However, the decrease in average molecular weight could be due to increased synthase concentration during the fermentation if the proposed hypothesis is correct.

Several other factors indicate that degradation did not occur and that the decreases in molecular weight are due to increased synthase concentration. First, as previously mentioned, these strains are not encoded for any known depolymerase enzyme. Secondly, the PHB content of the fermentation broth, as determined by Dr. Sang Jun Sim, was steadily increasing during the molecular weight decrease (Figure 7), which occurred between 8 and 15 hours into the fermentations. Specific synthase activity measurements, also performed by Dr. Sim, show distinct peaks at the same time points at which the intrinsic viscosities sharply decrease (Figure 8). Another indicator is that after the dramatic drop in molecular weight during the pSP2 fermentations, the molecular weight did not continue to decrease, instead maintaining a new relatively constant level, which indicates that the bacteria is not using the PHB as a carbon or energy source even after the supplied carbon source is depleted.

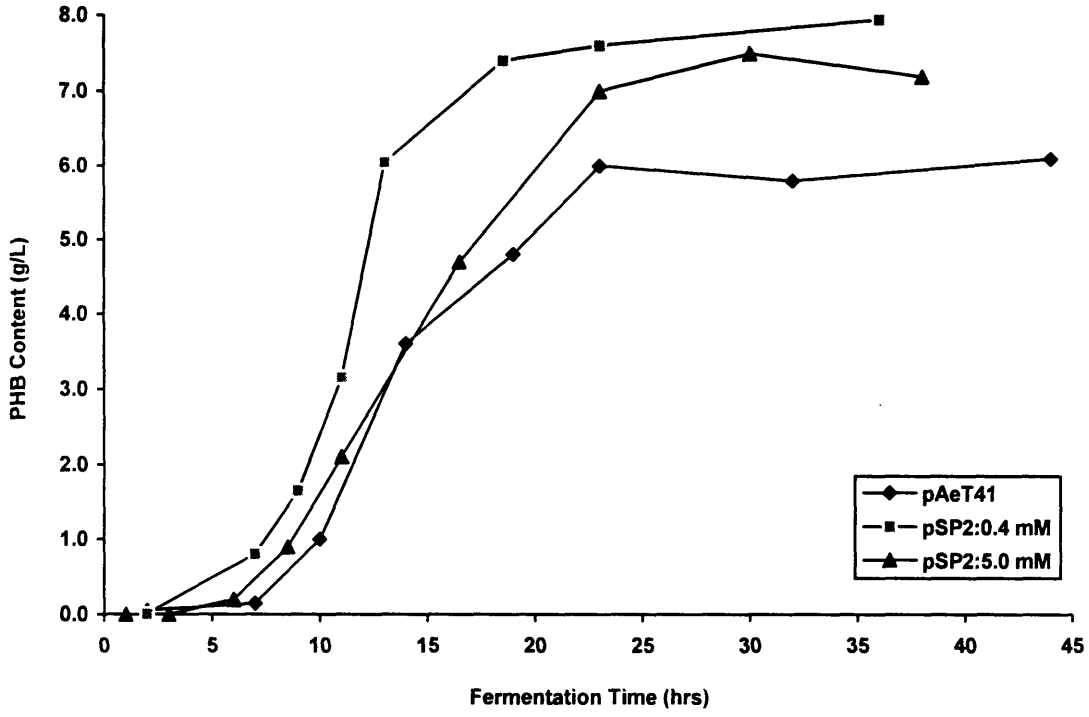


Figure 7. PHB Content of Recombinant *E. coli* (10 Liter Fermentations)
(Courtesy of Dr. Sang Jun Sim)

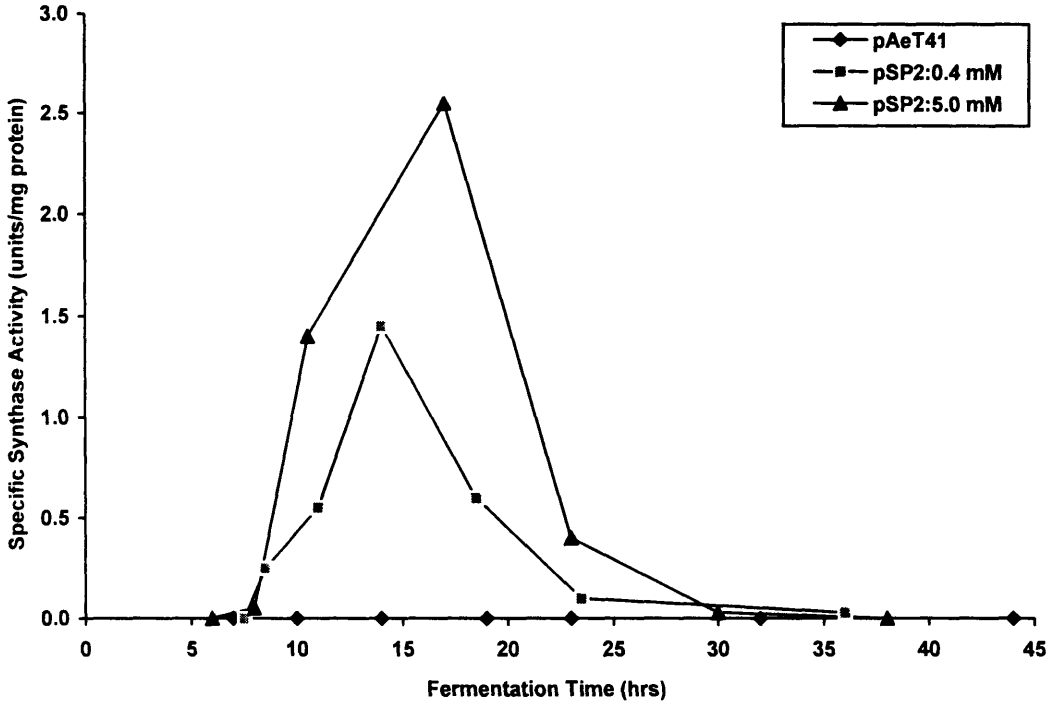


Figure 8. PHB Synthase Activity of Recombinant *E. coli* (10 Liter Fermentations)
(Courtesy of Dr. Sang Jun Sim)

SEC Analysis

Further evidence that increases in synthase concentration lead to decreases in the length of the PHB chains produced was attainable through SEC. If in fact the cells begin producing new shorter PHB chains when synthase levels rise, the decrease in average molecular weight should be accompanied by a broadening of the molecular weight distribution toward lower molecular weights. TABLE VI presents the molecular weight determined by SEC.

The same data is presented graphically in Figure 9. The high molecular weight of PHB from pAeT41 proved difficult to precisely determine. It varied between 3 and 5 million Da for the entire fermentation with no apparent trend. Lower concentration injections may have given better peaks to manipulate. However, adherence to GPC procedures necessitates constant concentration injections.

The PHB samples from pSP2 with 0.4 mM IPTG induction show weight average molecular weights with a slight downward trend starting at 1.8 million Da and ending at 1.3 million Da. This trend is magnified in the 5.0 mM IPTG induced pSP2 samples. A distinct downward shift in molecular weight is seen between 8 and 17 hours, which is the time in Figure 5 at which specific synthase activity peaks. The trends in the molecular weight by GPC agree well with the trends observed in the intrinsic viscosity measurements. The new information obtained using chromatography manifests itself in the shape of the chromatograms (Figures 10 and 11).

TABLE VI. MOLECULAR WEIGHT DETERMINED BY SEC OF PHB PRODUCED BY RECOMBINANT *E. COLI* (10 LITER FERMENTATIONS)

Strain	time (hrs)	M_w (Da)
pAeT41	9.9	3.9×10^6
	14.6	3.0×10^6
	18.7	5.5×10^6
	23.3	4.7×10^6
	32.3	4.8×10^6
	43.3	3.4×10^6
	55.3	3.5×10^6
	73.7	3.1×10^6
pSP2	6.9	1.8×10^6
0.4mM IPTG	11.5	1.5×10^6
	13.3	1.2×10^6
	18.3	1.4×10^6
	23.6	1.1×10^6
	35.5	1.3×10^6
	47.4	1.3×10^6
pSP2	5.8	1.3×10^6
5.0 mM IPTG	8.2	1.3×10^6
	11.4	1.0×10^6
	16.5	0.7×10^6
	23.4	0.7×10^6
	30.2	0.7×10^6
	37.7	0.7×10^6

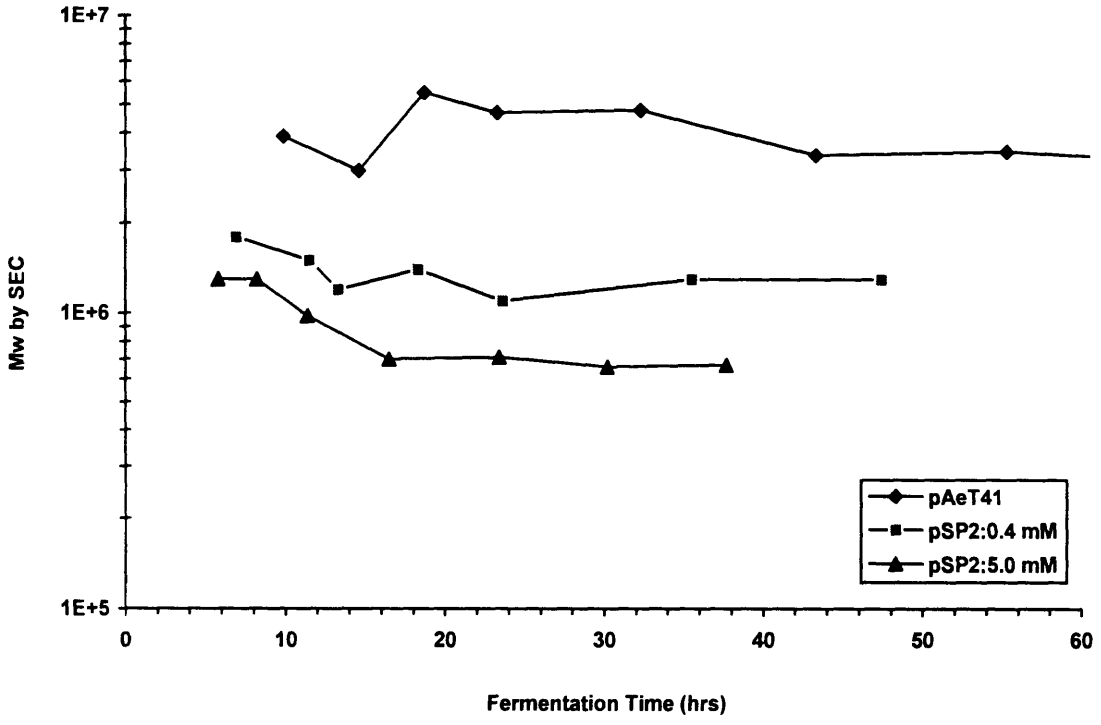


Figure 9. Weight-Average Molecular Weight of PHB Produced by Recombinant *E. coli* Determined by SEC (10 Liter Fermentations)

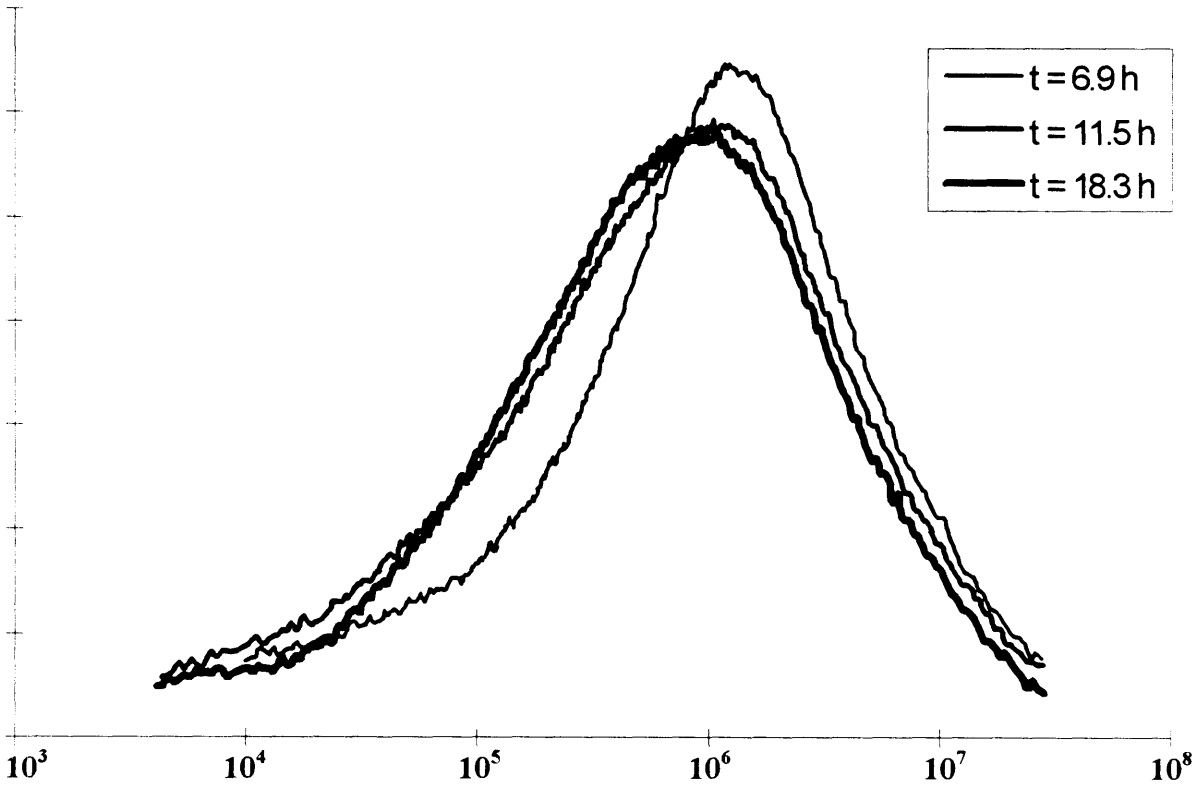


Figure 10. SEC Chromatograms of PHB Produced by pSP2
(10 Liter Fermentation, 0.4 mM IPTG)

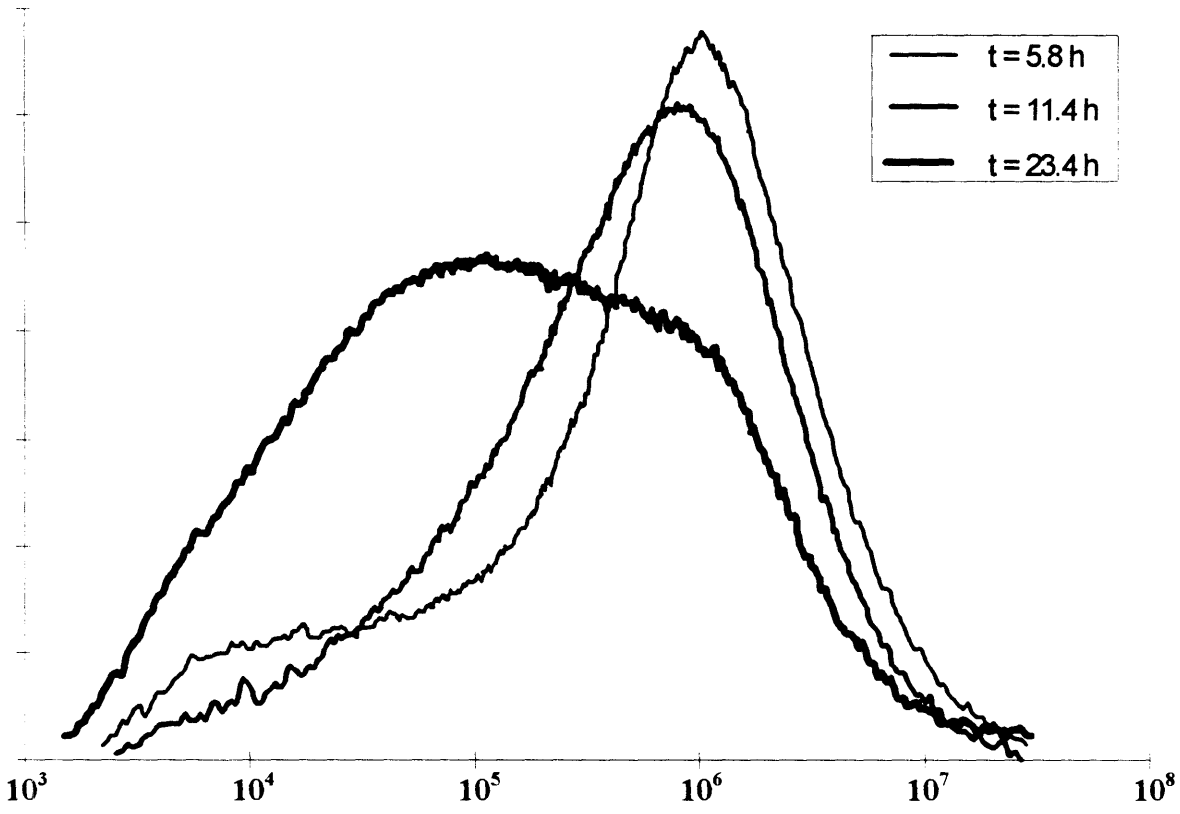


Figure 11. SEC Chromatograms of PHB Produced by pSP2
(10 Liter Fermentation, 5.0 mM IPTG)

Corresponding to the downward trend in molecular weight is a shift of the distribution toward the low molecular weight end of the scale. Coupling this with the fact that PHB content was increasing during this time period and that synthase activity peaked during this period, it is clear that increased synthase concentration produces shorter PHB molecules. The shift is much more dramatic in Figure 11 with the higher induction level of 5.0 mM IPTG. The fact that higher IPTG concentration leads to higher synthase activity, a lower average molecular weight, and a more dramatic broadening of the molecular weight distribution is further evidence to prove the hypothesis. A polydispersity index is not reported in this section due to the band-broadening effect. Without correction, it is largely over estimated. Quantitative results for the molecular weight distribution are arrived at using light scattering.

Light Scattering

Because light scattering is very dependent on *dn/dc*, chloroform is not a suitable solvent for light scattering analyses of PHB. The *dn/dc* of PHB in chloroform was determined to be between 0.03 and 0.05 ml/g by integration of the GPC RI peaks with a known mass, flowrate, and refractometer calibration constant. This value is too low to obtain light scattering peaks in a dilute regime. For this reason, all previous light scattering experiments reported on PHB have employed 2,2,2-trifluoroethanol as the solvent due to its exceptionally low refractive index. Reported in TABLE VII on the next page are the weight average molecular weights, polydispersity index, and z-average radius of gyration for each sample from the large scale 10 liter fermentations. *dn/dc* was determined to be 0.149 ml/g, agreeing well with Akita, *et. al.*[25]

TABLE VII. MOLECULAR WEIGHT DETERMINED BY LIGHT SCATTERING OF PHB PRODUCED BY RECOMBINANT *E. COLI* (10 LITER FERMENTATIONS)

Strain	time(hrs)	Mw (Da)	M_w/M_n	$\langle r_g^2 \rangle^{1/2}$
pAeT41	9.9	4.3×10^6	1.01	106.4
	14.6	4.3×10^6	1.03	103.5
	18.7	4.0×10^6	1.02	104.5
	23.3	3.1×10^6	1.08	99.9
	32.3	4.0×10^6	1.02	102.7
	43.3	4.1×10^6	1.04	103.4
	55.3	3.8×10^6	1.03	102.3
pSP2	6.9	2.2×10^6	1.15	89.7
0.4mM IPTG	11.5	1.7×10^6	1.33	90.1
	13.3	1.5×10^6	1.47	88.4
	18.3	1.4×10^6	1.57	86.9
	23.6	1.3×10^6	1.54	84.1
	35.5	1.3×10^6	1.55	83.1
	47.4	1.3×10^6	1.57	79.7
pSP2	5.8	2.2×10^6	1.31	95.7
5.0 mM IPTG	8.2	1.4×10^6	1.58	83.6
	11.4	1.3×10^6	1.43	80.6
	16.5	7.6×10^5	2.59	73.2
	23.4	7.4×10^5	2.81	76.6
	30.2	7.8×10^5	2.37	78.0
	37.7	7.3×10^5	2.29	72.0

As expected the same trends are seen in M_w as the pSP2 fermentation progresses (Figure 12), with a decrease in molecular weight between 10 and 15 hours, the time corresponding to the increase in synthase activity. pAeT41 again shows no apparent trend, the molecular weight staying between 3 and 4 million Da. Using light scattering with SEC, a value for the polydispersity index was attainable (Figure 13). Note the very sharp distribution in all pAeT41 samples. This is another indication that PHB does not degrade in a recombinant *E. coli* system; further evidence that the significant distribution broadening in pSP2 is due to synthase increases leading to the production of new, shorter chains. Again, the effect is much more distinct with the high induction level. Figure 14 charts the RMS radius as the fermentation progresses.

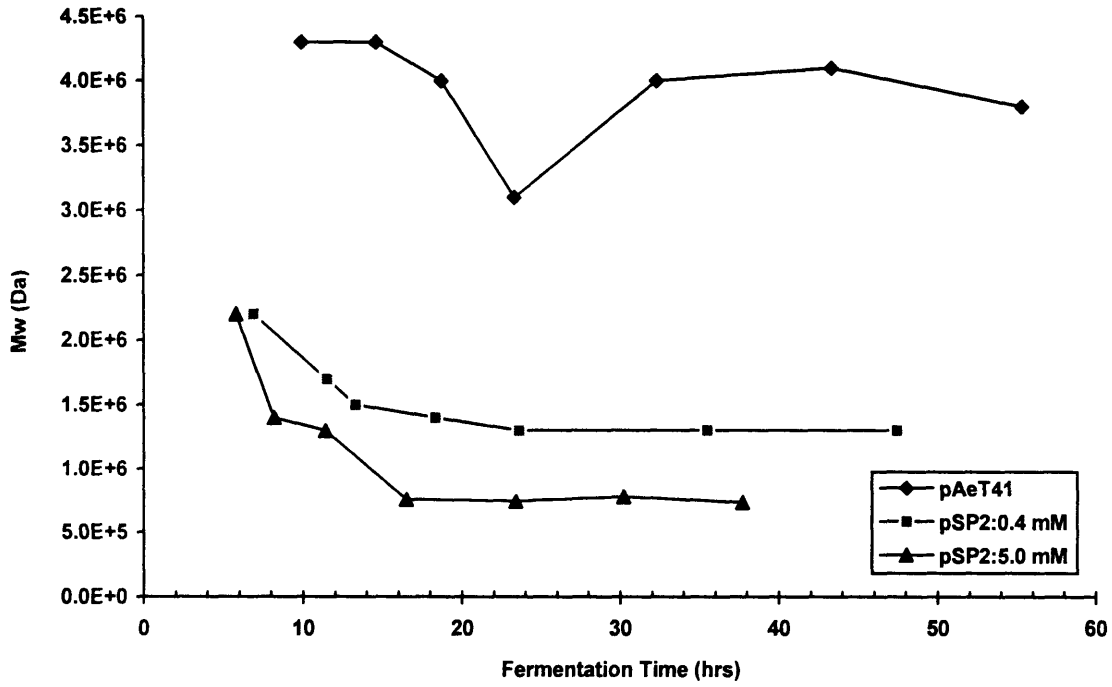


Figure 12. Weight-Average Molecular Weight of PHB Produced by Recombinant *E. coli* Determined by Light Scattering (10 Liter Fermentations)

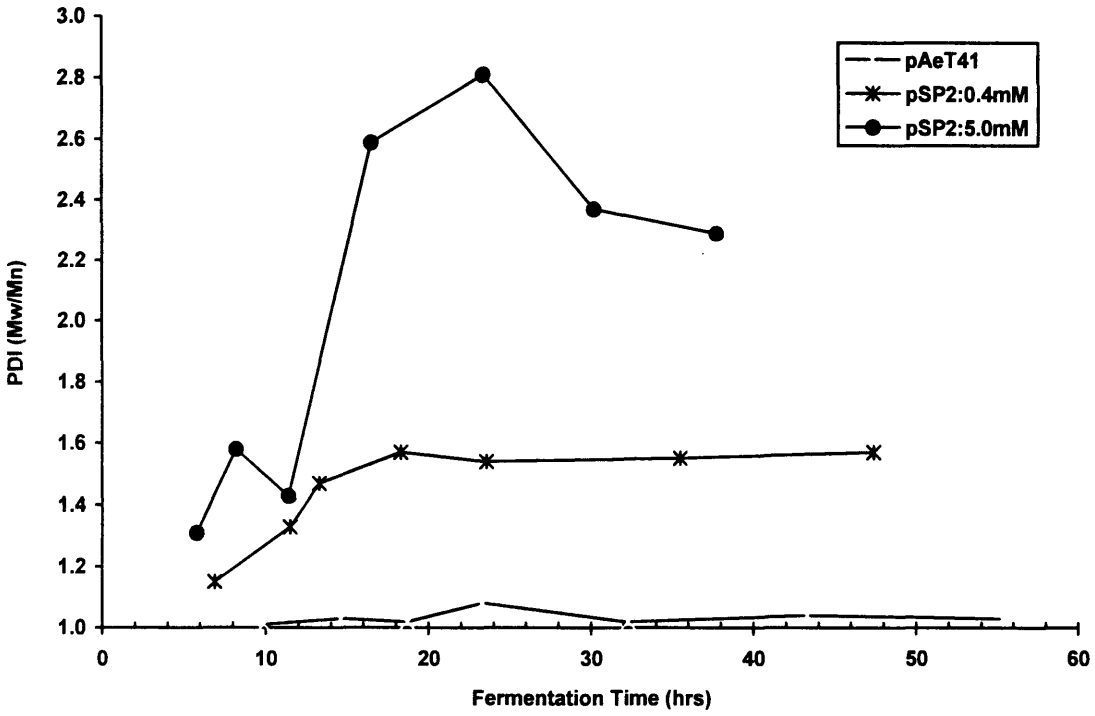


Figure 13. Polydispersity Index of PHB Produced by Recombinant *E. coli* (10 Liter Fermentations)

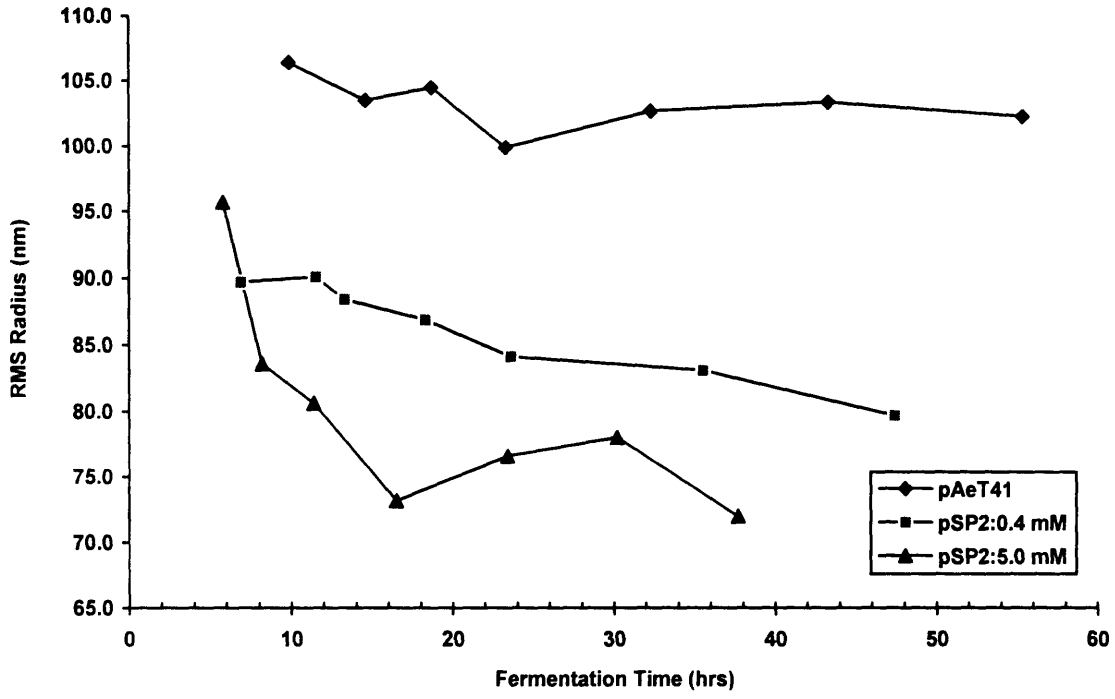


Figure 14. RMS Radius in TFE of PHB Produced by Recombinant *E. coli* (10 Liter Fermentations)

MARK-HOUWINK RELATIONSHIP

Mark-Houwink constants were derived from intrinsic viscosity in chloroform and light-scattering measurements (Figure 15). The following relationship was found for PHB in chloroform at 30°C:

$$[\eta] = 1.21 \times 10^{-4} M_w^{0.75}$$

The values of K and a seem close to those reported by Akita, *et.al.*[25] (K=1.18 x 10^{-4}, a=0.78). However, graphically the relationships reported by Akita, *et.al.*[25] and Marchessault, *et.al.*[23] (K=0.77 x 10^{-4}, a=0.82) are different from the relationship derived in this study (Figure 16). For a given molecular weight, the intrinsic viscosities were found to be significantly lower than those reported in the literature.

In an attempt to determine the reason for the difference, further experiments were carried out. These experiments investigated the possibility of shear thinning leading to lower intrinsic viscosity measurements and the possibility that synthase enzyme attached to the ends of PHB chains cause PHB molecules to assume a smaller conformation in solution.

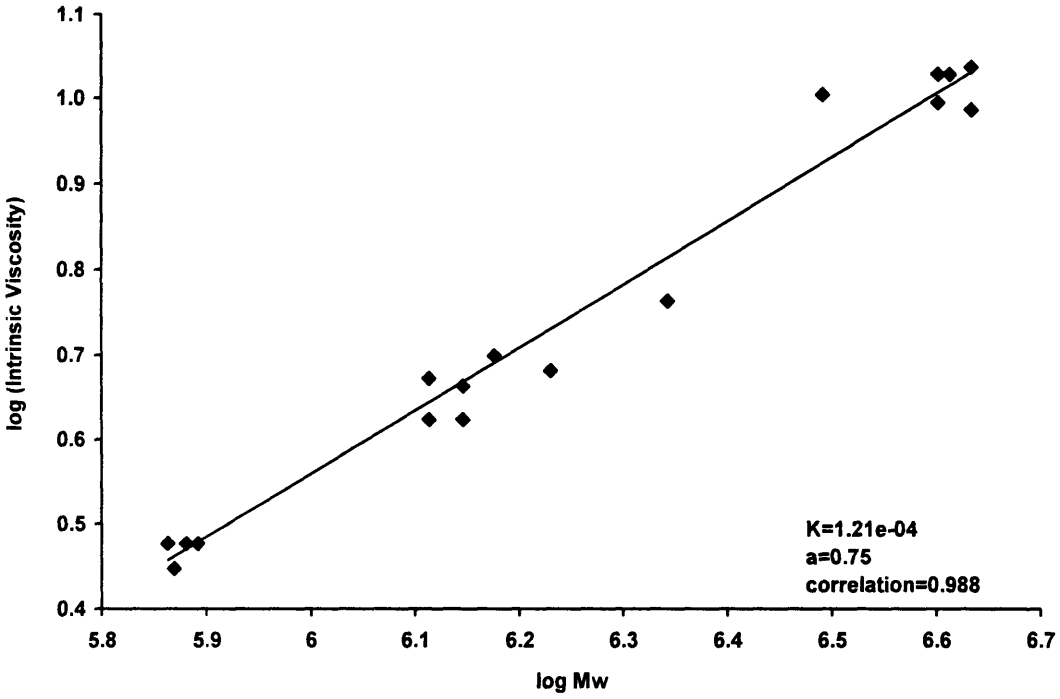


Figure 15. Intrinsic Viscosity-Molecular Weight Relationship for PHB in Chloroform at 30°C

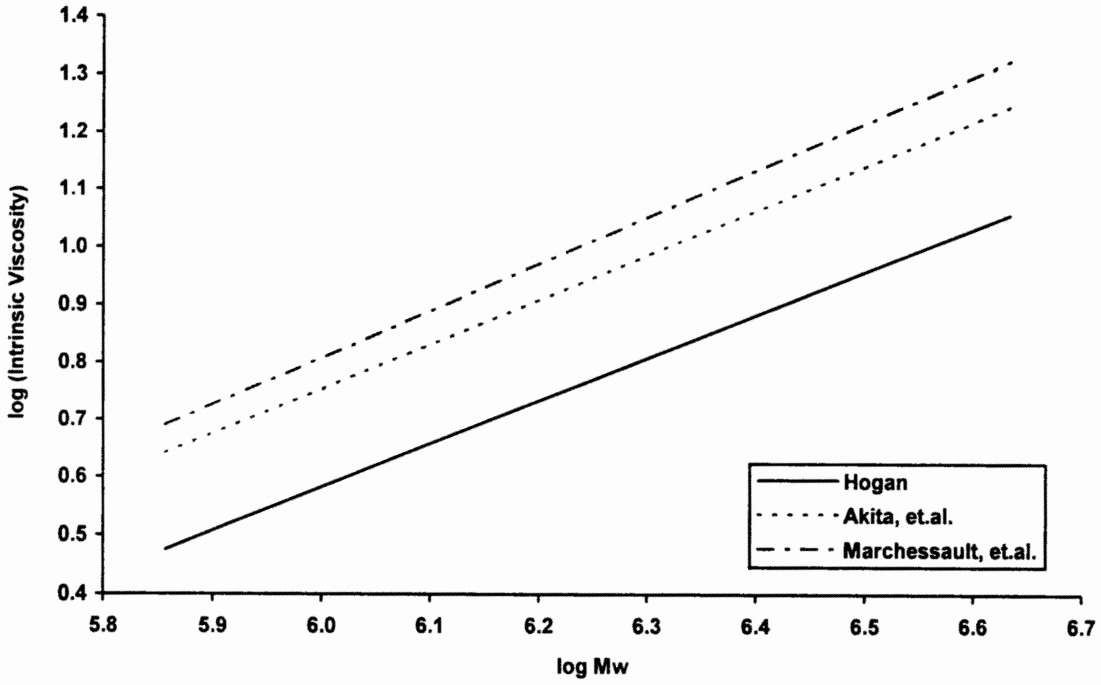


Figure 16. Mark-Houwink Relationship Comparison

Shear Thinning Effect

The fact that the light-scattering and the SEC molecular weight data agree well (Figure 17) led to the investigation of error in the measurement of intrinsic viscosity. Significant shear thinning has been noted by other research groups during viscosity determination.[24,26] To determine the shear rate in the size 50 capillary viscometer, laminar Poiseuille flow was assumed in the capillary, and the flow volume was measured. Using flow times for chloroform and acetone, the diameter of the size 50 capillary calculated to 0.47mm in both cases. . The Reynolds number in the capillary was determined to be *Re*=264 with pure chloroform, validating the assumption of laminar flow for all solutions measured.

For pure chloroform in the capillary with a viscosity of 0.514 mPa·s at 30°C, the average shear rate is $\dot{\gamma}$ =1660 sec^{-1}. For solutions with relative viscosities from 1.1 to 1.7, the average shear rate ranges from $\dot{\gamma}$ =1510 to 980 sec^{-1}. With TFE as the solvent at 25°C, for solutions with relative viscosities from 1.1 to 1.7, the average shear rate ranges from $\dot{\gamma}$ =405 to 260 sec^{-1}.

Rotational viscometry was used with TFE to determine the intrinsic viscosity of three samples from the 10 liter fermentations. These samples had weight-average molecular weights, determined by light-scattering, of 7.6 x 10^5, 1.7 x 10^6, and 4.0 x 10^6. Plots of viscosity vs. shear rate showed no shear thinning in the region from 1 to 80 sec^{-1} (Figure 18). Shear thinning would cause a decrease in the apparent viscosity with increasing shear rate. These three points were plotted against TFE intrinsic viscosity data

from Cornibert, *et.al.*[24], Akita, *et.al.*[25], and Miyaki, *et.al.*[26] (Figure 19). Even though viscosity determinations were extrapolated to zero shear, the measured intrinsic viscosities in TFE fall below those reported in the literature to the same degree which the chloroform intrinsic viscosity measurements do.

Time Dependence of Viscosity Determinations

Further evidence that shear thinning is not prevalent is the absence of time dependency in the viscosity measurements (Figure 20). For these experiments, the delay time between viscosity measurements at each shear rate was varied from 0.5 to 10 seconds. Plotted in Figure 20 are the shear stress and apparent viscosity of a sample as it was subjected to a shear sweep from 9 sec^{-1} to 600 sec^{-1} then back to 9 sec^{-1}. If shear thinning was occurring, the shear stress curves would show significant hysteresis, particularly at the short delay times. In other words, if the shape of the PHB molecules was changing due to shear, the short delay times would not allow the polymer chains time to return to their original shape before the measurement was complete. However, it is clearly evident that there is no change in the shear stress curve as the shear rate is decreased nor in the apparent viscosity as the shear rate is varied. Every delay time produced identical results.

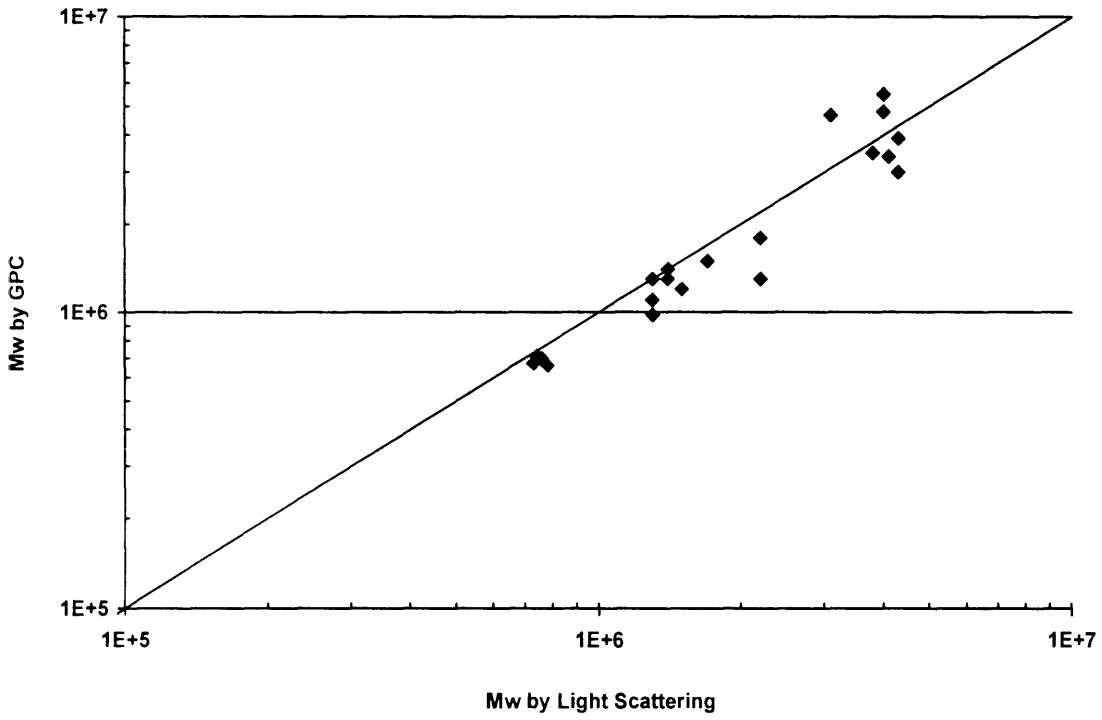


Figure 17. Comparison of Light Scattering and SEC Data
(Solid line represents perfect agreement)

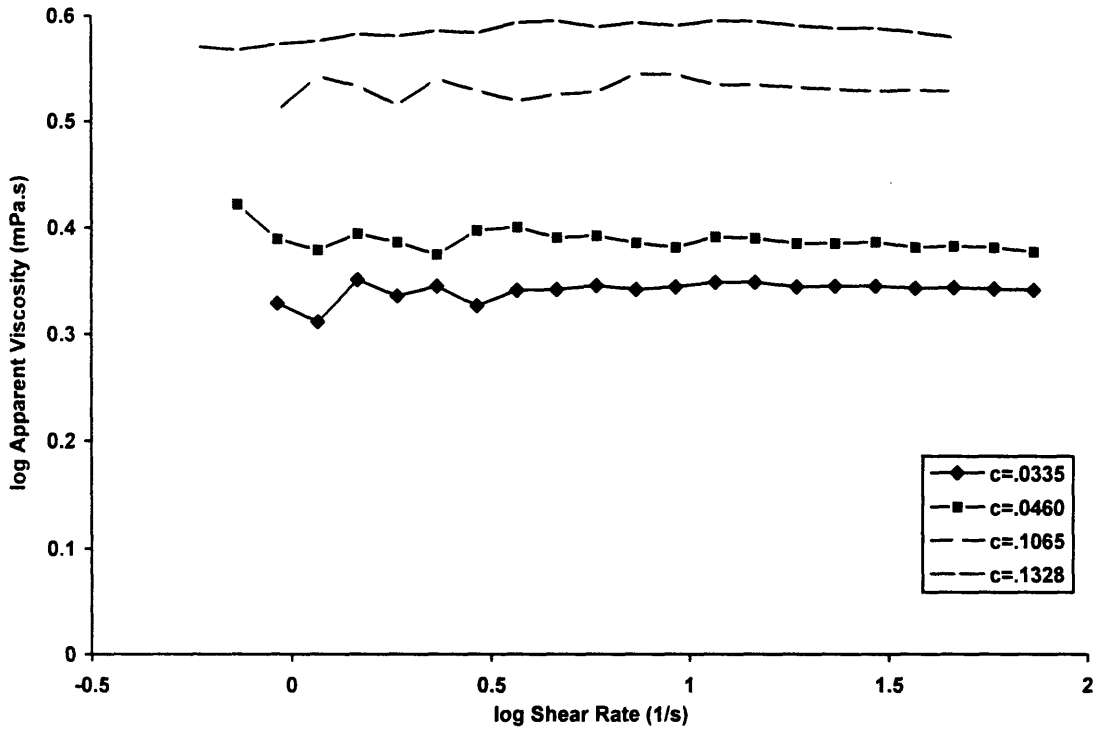


Figure 18. Viscosity as a function of Shear Rate for M_w=1.7 x 10^6 PHB. Concentrations from 0.0335 to 0.1328 g/dl

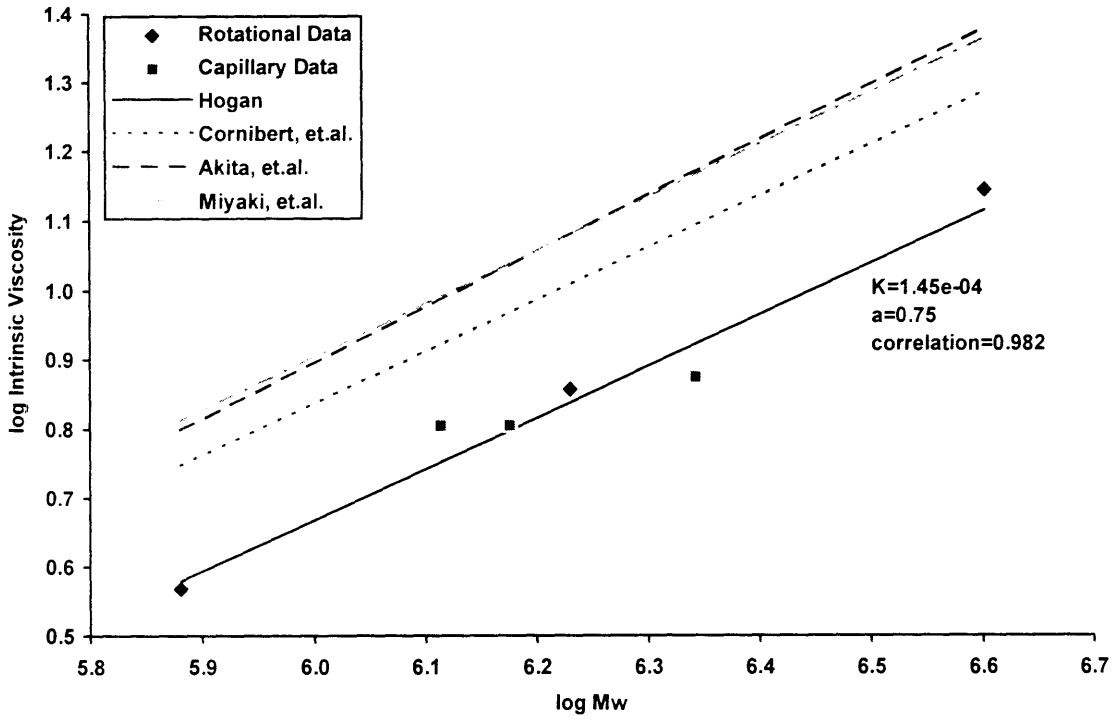


Figure 19. Intrinsic Viscosity-Molecular Weight Relationship for PHB in Trifluoroethanol at 25°C

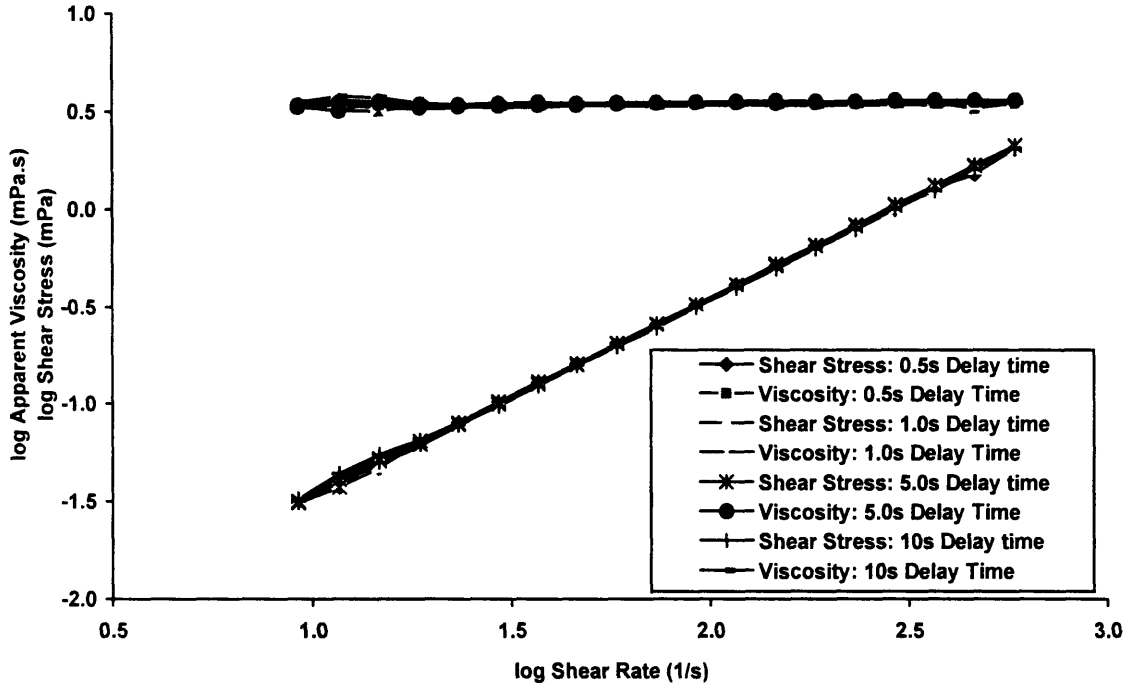


Figure 20. Time Dependency of Viscosity of PHB in TFE at 25°C
M_w=1.7 x 10^6 and Concentration=0.1396 g/dl

To compare Mark-Houwink constants in TFE, three more points were measured using the size 50 capillary viscometer, since shear-thinning did not exist in the samples subjected to rotational viscometry. The fact that these capillary measurements fall on the same line as the rotational measurements (Figure 19) is further proof than shear thinning is not a factor. If it were, the rotational data would be significantly shifted on the [η] axis.

The following relationship between weight-average molecular weight and intrinsic viscosity in TFE at 25°C was observed (Figure 19 and TABLE VIII):

$$[\eta] = 1.45 \times 10^{-4} M_w^{0.75}$$

It may be concluded then, that a shear thinning effect is not responsible for falsely low intrinsic viscosity measurements. The disparity exists even when zero shear measurements are used.

TABLE VIII. INTRINSIC VISCOSITY IN TRIFLUOROETHANOL (25°C) OF PHB PRODUCED BY RECOMBINANT *E. COLI*

M_w by Light Scattering	[η] dl/g
7.6×10^5	3.7
1.3×10^6	6.4
1.5×10^6	6.4
1.7×10^6	7.2
2.2×10^6	7.5
4.0×10^6	13.9

Removal of Synthase

From previous unpublished work in Dr. Anthony J. Sinskey's Laboratory at MIT, there is evidence that the synthase enzyme remains attached to the PHB chains through polymer extraction and purification. The enzyme itself, in its purified form, has proven to be difficult to work with by adhering to various HPLC column materials and other lab instruments.[57] It was suspected that perhaps the enzyme itself was responsible for altering the conformation of PHB molecules in solution to a size smaller than that seen by previous research groups.

In attempt to remove the synthase from the PHB, the same three PHB samples used for rotational viscometry experiments were treated with Proteinase-K (Sigma Chemical Company, St. Louis, MO, USA), a protein removal agent, by Dr. Kristi D. Snell. The PHB samples were ground to a fine powder to maximize surface area and treated for 6 hours at 50°C in an aqueous suspension. Each 100 mg sample of ground polymer was treated with a 1 ml solution containing 25 mM Tris-Cl (pH 7.4), 0.5% (w/v) SDS, 0.2 M EDTA (pH 8), and 1 mg/ml proteinase-K. Intrinsic viscosities were then determined in chloroform at 30°C. The results are shown in TABLE IX and Figure 21.

After treatment with Proteinase-K, intrinsic viscosities increased by an average of ten percent, bringing the values slightly closer to those previously published. A 60 to 70 percent increase would be necessary to match the data of Akita, *et.al.*[25], and an 80 to 100 percent increase to match the data of Marchessault, *et.al.*[23] This raises the issue of whether or not all of the synthase was removed. It is highly unlikely that all synthase was removed due to the insoluble nature of PHB in water. Though the PHB samples were

ground to a fine powder, it is unlikely that the ends of each PHB molecule was on the surface of each granule for treatment. To remove 100 percent of the protein, the removal must take place in solution. PHB is soluble only in strong organic solvents such as chloroform which is not compatible with proteinase-K or other protein removers.

TABLE IX. INTRINSIC VISCOSITY IN CHLOROFORM (30°C) OF PHB PRODUCED BY RECOMBINANT *E. COLI*, , BEFORE AND AFTER PROTEINASE-K TREATMENT

[η] dl/g Before ProteinaseK	[η] dl/g After ProteinaseK	%Increase
3.0	3.2	6.6
4.8	5.5	14.6
9.9	10.8	9.1

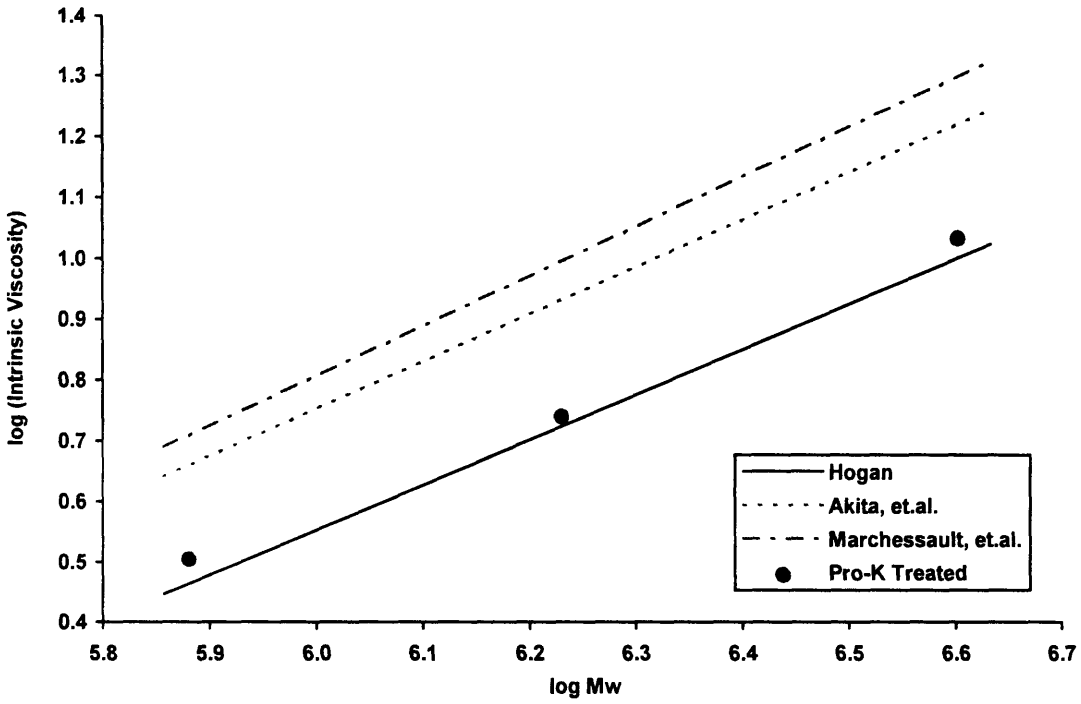


Figure 21. Intrinsic Viscosity-Molecular Weight Relationship for PHB in Chloroform at 30°C, Including Proteinase-K Treatment

REPRODUCIBILITY OF EXPERIMENTS

Several of the intrinsic viscosity measurements and light scattering measurements were repeated to verify their reproducibility. TABLE X shows the results of the intrinsic viscosity verifications, and TABLE XI shows the results of the light scattering verifications.

Intrinsic viscosities differed by an average of 2.9% and were overall very reproducible. The correlation coefficient calculated between the two trials is 0.997. Light scattering measurments differed by an average of 4.7%. The correlation coefficient for the two light scattering trials is 0.982. The reproducibility of GPC experiments was not calculated. GPC was used primarily to verify the other two techniques and to study the shape of the chromatograms. Each sample was measured only once.

The reproducibility of the evidence that higher synthase concentration causes lower molecular weight PHB to be produced is inherent in the three different sizes of fermentations. Although each fermentation was only carried out one time, the same trends were apparent in the shake flask cultures, the 2 liter fermentations, and the ten liter fermentations. pSP2 always produced PHB with a lower average molecular weight than did pAeT41.

TABLE X. REPRODUCIBILITY OF INTRINSIC VISCOSITY MEASUREMENTS

Trial 1 (dl/g)	Trial 2 (dl/g)	% Difference
5.1	5.1	0
13.2	13.0	1.5
13.6	13.6	0
10.0	10.9	8.6
9.9	9.8	1.0
9.9	9.9	0
4.6	4.4	4.4
4.1	4.2	2.4
2.8	3.0	6.9
2.6	2.7	3.7

Correlation coeffiecient = 0.997

TABLE XI. REPRODUCIBILITY OF LIGHT SCATTERING MEASUREMENTS

Trial 1 (Da x 10^{-6})	Trial 2 (Da x 10^{-6})	% Difference
4.4	4.3	2.3
3.7	3.7	0
3.1	3.1	0
4.0	4.1	2.5
4.7	4.7	0
3.8	3.8	0
2.2	3.0	30.1
1.4	1.4	0
1.4	1.4	0
1.6	1.5	6.5
1.3	1.4	7.4

Correlation coeffiecient = 0.982

V. CONCLUSIONS

It has clearly been shown that in recombinant *E. coli* systems expressing PHB biosynthetic enzymes, the molecular weight of PHB is regulated by the concentration of the synthase enzyme. High synthase concentrations lead to lower molecular weights. It appears that the synthase molecules present compete for the available substrate, hydroxybutyryl-CoA, and effectively distribute it among a corresponding number of polymer chains. This suggests that once a synthase molecule starts a chain, it stays with that chain until the substrate is exhausted. The conclusion is that the synthase enzyme does not behave like a traditional catalyst. It behaves as an initiator of a continuing polymerization reaction.

Further evidence that a synthase enzyme remains with the polymer chain which it intitiated for the duration of the polymerization lies in the exceptionally narrow molecular weight distributions in the pAeT41 samples. The result of randomly dividing up x number of repeat units into y chains, y being the number of synthase molecules present, results in a Poisson-type distribution, assuming rapid initiation and $x>>y$.[56] A Poisson distribution gives a molecular weight distribution very close to 1, in stark contrast to Flory's most probable distribution[43] which PHB emulates in *A. eutrophus*.[3] If a transfer reaction was present, a most probable distribution would result.[56] The broadening of the distributions in the pSP2 fermentations is a result of increasing the synthase concentration midway in

the fermentation. This results is a continuous distribution of Poisson distributions. The final distribution width is a complex function of synthase activity and time.

Using the recombinant *E. coli* system DH5α/pSP2, the final molecular weight of the PHB can be controlled by controlling the induction level. Increasing isopropyl-β-D-thiogalactopyranoside (IPTG) concentration has been shown to increase synthase activity and reduce the molecular weight of the PHB produced. This marks the first method available to control the molecular weight of PHB *in vivo*. There have been claims that the molecular weight can be controlled *in vitro*.[53] It has also been claimed that the molecular weight can be controlled by varying the time of exposure to sodium hypochlorite during extraction.[31] This method actually relies on producing a high molecular weight PHB, then degrading it to the level desired. The desired molecular weight could in theory be obtained from *A. eutrophus* as well by waiting until the depolymerase degrades the PHB to the desired level, which occurs when all of the initial carbon source has been used.[32-33] Neither of these methods offer a way to control the length of polymer chains produced. Instead they offer ways to reduce the molecular weight of larger chains already produced.

Also shown in this study is a large discrepancy in the Mark-Houwink relationship for PHB in chloroform and in trifluoroethanol relative to prior publications on the subject. The following relationships were observed for PHB from recombinant *E. coli* encoded for production of the enzymes responsible for the biosynthesis of PHB in *A. eutrophus*:

$$[\eta] = 1.21 \times 10^{-4} M_w^{0.75} \qquad \text{chloroform, 30°C}$$
$$[\eta] = 1.45 \times 10^{-4} M_w^{0.75} \qquad \text{trifluoroethanol, 25°C}$$

Molecular weight and intrinsic viscosity were both measured using two techniques to verify these relationships. Neither shear thinning nor viscosity time dependency was observed using rotational viscometry.

It was also shown that Proteinase-K treatment increased the intrinsic viscosity of PHB from recombinant *E. coli*. Whether or not synthase removal was responsible for this increase is an area for future research. If indeed the synthase enzyme is responsible for PHB assuming an unusually small conformation in solution, this would explain the disparity in the Mark-Houwink relationships between this report and other studies in the literature. This observation also relies on the fact that in previous studies different bacterial strains were used to produce the PHB and on the assumption that the *A. eutrophus* synthase enzyme is unique; i.e., this enzyme was not present in the studies performed by other groups. Protein content was not determined for the samples studied in this report due to the fact that commercially available protein assays rely on aqueous solutions. Purified PHB granules have previously been shown to contain approximately 2% proteins and lipids.[38]

VI. FUTURE RESEARCH

The idea presented in this thesis is that the synthase enzyme acts as an initiator of a polymerization reaction and not as a typical catalyst. Polymer scientists have used initiator concentration to control molecular weight of the resulting polymer since they began to understand free-radical polymerization. A more recent development in polymeriztion techniques is the anionic polymerization. While a free-radical polymerization involves initiation, chain-propagation, and chain-termination, an anionic polymerization involves only initiation and propagation. An example of the process is shown below:[56]

$$R^{-}Na^{+} + CH_2 = CHR \rightarrow R - CH_2 - CHR^{-}Na^{+}$$

which is shortened to:

$$I^{-} + M \rightarrow M_1^{-} \qquad \text{(initiation)}$$

followed by:

$$RM_n^{-}Na^{+} + M \rightarrow RM_{n+1}^{-}Na^{+}$$

which is shortened to:

$$IM_n^{-} + M \rightarrow M_{n+1}^{-} \qquad \text{(propagation)}$$

Anionic polymerizations proceed to completion by using up the available monomer. There is no termination step, leading to the alternate name "living polymers."

This name comes from the fact that after completion of the reaction, each polymer chain still contains an active site. Addition of more monomer will result in these chains propagating further. This type of reaction scheme is used to produce very narrow distributions for applications such as polymer standards. Molecular weight can be controlled by initiator concentration since, assuming rapid initiation, the number average degree of polymerization is equal to:

$$P_n = \frac{[\Delta M]}{[I]}$$

where P_n is the number average degree of polymerization and *[ΔM]/[I]* is the mole ratio of converted monomer to initiator.

This analog can be used with the PHB polymerization. In the preceeding initiation reaction, PHA synthase is analogous to the initiator, *I*, and HBCoA is analogous to the monomer, *M*. They combine to form $M_1^{\cdot}$ and propagate to form $M_n^{\cdot}$. In both cases, an active site is retained for the next repeat unit to be added.

The similarities in the reaction mechanisms can be exploited in future research. The polymerization kinetics have already been studied, along with predicted molecular weights and molecular weight distributions. The challenge of the PHB research is the fact that initiator concentration is a function of time, leading to a complex distribution formed by the addition of several Poisson distributions. An *in vitro* analysis, for which the technology exists at MIT, seems to be ideal for such experiments.

In vitro experiments are also ideal for finally determining whether the synthase enzyme has an effect on the conformation of PHB in solution. It has been shown that the

synthase enzyme is localized at the surface of PHB granules.[58] Proteinase-K treatment of the granules would ensure complete removal of the synthase enzyme. Intrinsic viscosity analysis of treated and untreated granules would reveal any conformation differences.

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