

FOCUS REVIEW

Fundamental factors determining the molecular weight of polyhydroxyalkanoate during biosynthesis

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This review focuses on the factors that determine the molecular weight of polyhydroxyalkanoate (PHA), an aliphatic polyester synthesized by bacteria for carbon and energy storage. PHA is a polymer with good thermoplastic, biodegradable, biocompatible and renewable properties. Therefore, it has attracted considerable attention as an environmentally friendly and biomedical material. Because the mechanical strength of PHA increases with its molecular weight, high-molecular-weight PHA polymers are preferred. The synthesis of high-molecular-weight PHA should consider the following factors: the concentration of PHA synthase, the occurrence of a chain transfer reaction, the catalytic activity of PHA synthase and the simultaneous degradation of PHA during biosynthesis. These factors have a direct impact on the molecular weight of PHA. Furthermore, the factors that affect the molecular weight of PHA during *in vitro* PHA polymerization and cultivation of PHA native and non-native bacteria are discussed. *Polymer Journal* (2016) 48, 1051–1057; doi:10.1038/pj.2016.78; published online 7 September 2016

INTRODUCTION

Many bacterial species synthesize the aliphatic polyester polyhydroxyalkanoate (PHA) to store carbon and its reducing equivalents.^{1–3} Accumulated PHA accounts for ~30–50% of the dry weight of most bacterial cells. However, the well-known PHA producer *Ralstonia eutropha* (also known as *Cupriavidus necator*) is capable of accumulating PHA at over 90% of the dry cell weight. PHA accumulation is triggered by imbalances in nutrient conditions, such as excess carbon but limited nitrogen availability. PHA is degraded under starvation conditions to maintain cellular energy homeostasis. PHA has natural thermoplasticity in addition to biodegradability and biocompatibility, and it is produced from renewable biomass such as sugars and plant oils. Therefore, PHA has attracted attention not only as an environmentally friendly alternative to petroleum-based plastics but also as a biomedical material for surgical sutures or a scaffold for tissue engineering.

Microbial cells typically accumulate poly[(*R*)-3-hydroxybutyrate] (P(3HB)), a PHA that contains a repeating C4 monomeric unit. P(3HB) is a highly crystalline material with similar thermal properties to those of polypropylene, a commodity plastic produced from petroleum. The melting and glass-transition temperatures of P(3HB) are 177 and 4 °C, respectively.⁴ However, P(3HB) is typically quite brittle and less elastic (elongation at break = 5%) than polypropylene (elongation at break = 400%). The weight-average molecular weight (M_w) of P(3HB) synthesized by bacteria is generally in the range of $0.1\text{--}2.0 \times 10^6$. *Ralstonia eutropha* utilizes only three enzymes to synthesize P(3HB) from acetyl-coenzyme A (acetyl-CoA): 3-ketothiolase (PhaA, encoded by *phaA*), NADPH-dependent acetoacetyl-CoA reductase (PhaB, encoded by *phaB*) and PHA synthase (PhaC, encoded by *phaC*; Figure 1).⁵

PHA with a high molecular weight is preferred because its mechanical strength is positively correlated with its molecular weight.⁶ Alternatively, the mechanical strength of ultrahigh-molecular-weight P(3HB) [UHMW-P(3HB)], which has a defined $M_w > 3.0 \times 10^6 \text{ g mol}^{-1}$,⁷ is much higher than that of P(3HB) because the long polymer chains are entangled; therefore, UHMW-P(3HB) can be used to develop high-strength fibers and films.⁶ The tensile strength and Young's modulus of UHMW-P(3HB) fibers are 1.3 and 18.1 GPa, respectively, both of which are significantly higher than those of P(3HB) (Table 1). These high-molecular-weight PHAs are synthesized by controlling the factors involved in PHA polymerization; however, the precise mechanism remains unclear.

The production and applicability of PHA have been comprehensively reviewed by several researchers.^{1–4,6} The purpose of this review is to highlight recent advances in elucidating the factors affecting the molecular weight of PHA. Here, I propose four fundamental factors that are directly responsible for regulating the molecular weight of PHA.

PHA SYNTHASES

PHA synthases play a central role in PHA polymerization in the cell without the use of a template, unlike other biological macromolecules such as proteins and nucleotides. 3-Hydroxyacyl-CoA is the primary substrate of PHA synthase; specifically, only the *R*-enantiomer of 3-hydroxyacyl-CoA is polymerized by PHA synthase. PHA synthases polymerize the acyl moiety of the CoA substrate by releasing free CoA and generating long polyester chains that are stored as water-insoluble inclusions of PHA in cells.^{3,8}

PHA synthases are classified into four groups according to their substrate specificities and subunit compositions.⁸ Class I and II PHA

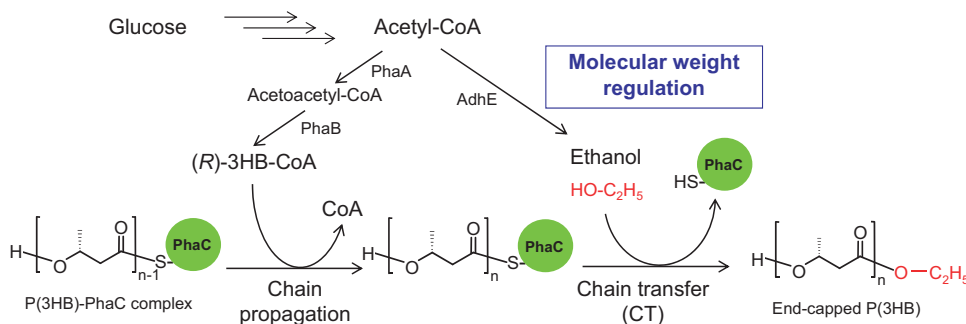


Figure 1 Poly[(*R*)-3-hydroxybutyrate] (P(3HB)) biosynthesis from acetyl-coenzyme A and molecular-weight regulation with endogenous ethanol in *E. coli*.¹⁸

Table 1 Mechanical properties of fibers produced from poly[(*R*)-3-hydroxybutyrate] (P(3HB))

| Polymer | $M_w (\times 10^6)$ | Tensile strength (MPa) | Elongation at break (%) | Young's modulus (GPa) |
|-------------|---------------------|------------------------|-------------------------|-----------------------|
| P(3HB) | 0.6 | 190–740 | 26–60 | 3.8–10.7 |
| UHMW-P(3HB) | 5.3 | 1320 | 35 | 18.1 |

Data from ref. 6.

synthases are composed of single subunits of PhaC (molecular mass = 60–70 kDa) and contain a PhaC box sequence ([GS]-X-C-X-[GA]-G) in the active site.⁹ Class I PHA synthases, represented by the *R. eutropha* enzyme, mainly polymerize short-chain-length monomers (C3–C5), whereas class II PHA synthases, represented by the *Pseudomonas oleovorans* enzyme, polymerize medium-chain-length monomers (C6–C14). Although most class II PHA synthases do not accept the C4 monomer, some atypical synthases such as those produced by *Pseudomonas* sp. 61-3 and *Pseudomonas stutzeri* can polymerize the C4 monomer.¹⁰ Class III and IV PHA synthases, represented by the *Allochroamatium vinosum* and *Bacillus megaterium* enzymes, respectively, consist of two heterosubunits. The catalytic subunit PhaC of class III and IV synthases (~40 kDa) requires the secondary subunits PhaE (~40 kDa) and PhaR (~20 kDa), respectively, for the full expression of the activity of PhaC.⁹

The molecular basis of PHA synthase has been extensively studied for the *R. eutropha* and *A. vinosum* PHA synthases. However, the X-ray crystal structure of PHA synthase remains unavailable because of difficulties in crystallizing the synthase.

MODEL OF PHA POLYMERIZATION

The PHA synthase-catalyzed polymerization reaction, referred to as a model for the chain polymerization system, is thought to include initiation, propagation and termination steps.^{11–13} The proposed polymerization reaction is described using class I PHA synthase as a model as follows (Scheme 1).

The PhaC subunit exists in an inactive (for PHA polymerization) water-soluble single form immediately after translation of the PhaC protein in the cell. During the initiation step, two PhaC subunits are dimerized to form an active enzyme when an (*R*)-3HB-CoA monomer approaches. The active site of this dimer reacts with (*R*)-3HB-CoA before undergoing a chain propagation reaction.^{14–16} The active site of PHA synthase consists of two thiol groups, the origins of which remain unclear. Two theories have been proposed: the thiol groups are

provided by the cysteine residue of the PhaC box in each PhaC subunit, or the thiol groups are provided by the PhaC subunit and CoA, respectively.¹⁶ In the propagation step, one thiol group binds to the incoming 3HB monomer, while the other binds to the propagating polymer chain. Propagation proceeds by transferring the growing chain between the thiol groups. In the termination step, PHA synthase loses its capacity to polymerize. Alternatively, a chain transfer (CT) reaction occurs when the polymer chain is transferred to a CT agent, resulting in covalent binding of the CT agent to the carboxy terminal of the P(3HB) chain. Water, 3HB and some hydroxy compounds have been proposed as potential naturally occurring CT agents.^{11,13}

EVIDENCE OF A CHAIN TRANSFER REACTION

A significant amount of effort has been devoted to understanding the CT reaction with respect to the polymer chain kinetics during PHA biosynthesis.¹¹ Because the CT reaction occurs at the end of the polymerization reaction, it is one of the factors responsible for determining the molecular weight of PHA. The CT reaction decreases the average molecular weight of the polymer and increases the number of polymer chains synthesized. Thus, the PHA chain number N_P (l^{-1}) increases as a result of an increase in the CT reaction as follows:

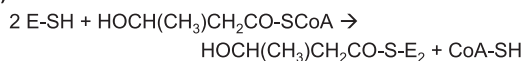
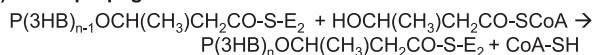
$$N_P = N_E + N_{CT} \quad (1)$$

where N_E and N_{CT} are the number of active PHA synthase sites involved in PHA polymerization (l^{-1}) and number of CT reactions (l^{-1}), respectively. The PHA chain number, N_P , can also be calculated using the following equation:¹⁷

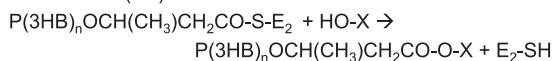
$$N_P = Y/M_n \times N_A \quad (2)$$

where Y , M_n and N_A denote the yield of synthesized PHA ($g\ l^{-1}$), number average molecular weight of synthesized PHA ($g\ mol^{-1}$) and Avogadro's constant (mol^{-1}), respectively. Therefore, accurate estimation of N_E facilitates kinetic analysis of the CT reaction.^{5,11,17,18}

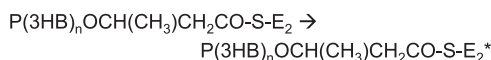
The polymer chain is transferred to a CT agent in the CT reaction, resulting in covalent binding of the CT agent to the carboxy terminus of the PHA chain. Therefore, structural analysis of the carboxylic end of PHA may provide detailed information regarding the CT reaction. Nuclear magnetic resonance (NMR) is a powerful tool that can be used to determine the chemical structure of polymers. However, end-structure analysis of high-molecular-weight polymers by ¹H- and ¹³C-NMR faces several limitations such as weak signals from the polymer end. Madden *et al.*¹³ introduced a method wherein end-group analysis of PHA could be conducted using ³¹P-NMR by derivatizing the PHA with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane, which enabled the selective detection of the hydroxy and carboxylic ends of PHA. This analysis demonstrated that

(1) Initiation**(2) Chain propagation****(3) Chain termination**

•Chain transfer (CT)



•Deactivation



Scheme 1 Proposed model of poly[(*R*)-3-hydroxybutyrate] (P(3HB)) polymerization. CoA-SH, free coenzyme A; E-SH, PHA synthase (enzyme) subunit; E₂-SH, dimerized PHA synthase (active); E₂^{*}-SH, dimerized PHA synthase (inactive); HO-X, chain transfer (CT) agent; 3HB, 3-hydroxybutyryl monomer.

the carboxylic end of the P(3HB) produced by *R. eutropha* cells grown on glycerol was capped by glycerol, suggesting that glycerol is the target agent of the CT reaction.

Low-molecular-weight polyethylene glycols (PEGs) are well-known artificial CT agents.^{19–22} The use of PEG as a CT agent causes a reduction in PHA molecular weight; this has been extensively studied in many PHA-producing bacteria such as *R. eutropha*^{19,20} and *P. oleovorans*.^{21,23} Moreover, ¹H-NMR analysis of lower-molecular-weight PHA suggested that PEG is incorporated at the carboxy terminus of the polymer via the CT reaction between the growing PHA chain and PEG hydroxy group.^{20,21} Because PEG is a synthetic polymer and not a bacterial compound, it can be used to study the CT reaction as an exogenous CT agent.²⁴

PROPERTIES OF CHAIN TRANSFER AGENTS

The CT agent must contain at least one hydroxy group, as the CT reaction is a transesterification reaction between the hydroxy group of the CT agent and carboxy group of the growing polymer chain-PHA synthase complex. As mentioned previously, water, 3HB and some hydroxy compounds are potential naturally occurring CT agents.^{11,13} There is little evidence regarding the direct involvement of water in the CT reaction. In particular, the effect of water on changes in the chain-termination reaction rate in an aqueous environment and the differences between the CT reaction and hydrolysis of the polymeric chain after biosynthesis remain unclear. 3HB is another potential CT agent, but it shows low efficacy.¹³ The frequency of the CT reaction can be increased by increasing the concentrations of CT agents.^{13,24,25}

The efficacy of a CT agent is largely dependent on its molecular properties rather than on its concentration. A quantitative structure-activity relationship study revealed that hydroxy compounds with higher hydrophobicity exhibit higher efficacies as CT agents.²⁶ The efficacies of less hydrophobic CT agents such as glycerol and PEG are relatively low compared with those of hydrophobic alcohols such as ethanol, propanol and butanol. The quantitative structure-activity relationship study also revealed that the efficacies of CT agents are not affected by the types of hydroxy groups (primary or secondary).²⁶ However, the cellular uptake of exogenous CT agents depends on the hydrophobicity of the component; moreover, PHA synthases have structural limits for their acceptance of CT agents.²⁶

ESCHERICHIA COLI AS A NON-NATIVE PHA PRODUCER

PHA-native bacteria such as *R. eutropha* and *P. oleovorans* possess inherent PHA depolymerases that contribute to the mobilization of PHA; however, PHA degradation complicates the analysis of the molecular weight of synthesized PHA. *Escherichia coli*, a non-native PHA producer, has been used to study the CT reaction *in vivo*. The use of *E. coli* for studying the CT reaction *in vivo* is extremely advantageous because of the absence of PHA degradation enzymes²⁷ and its well-developed genetic background. *E. coli* transformed with the biosynthetic genes *phaCAB* allows for the accumulation of P(3HB).^{5,27}

Unlike P(3HB) synthesized by native bacteria, P(3HB) synthesized by recombinant *E. coli* generally has a very high *M_w* of ~20 × 10⁶ g mol⁻¹.^{27,28} This can be attributed to the lack of the CT reaction and PHA degradation during PHA biosynthesis. Recent studies have demonstrated that intracellular degradation of PHA affects the molecular weight of PHA in the native PHA producer *R. eutropha*. Deletion of several PHA depolymerase genes allows the production of high-molecular-weight PHA (*M_w* = 3 × 10⁶ g mol⁻¹) in *R. eutropha*²⁹ as well as in recombinant *E. coli*. The simultaneous degradation of PHA may be a major factor determining the molecular weight of PHA in PHA-native bacteria.

Under CT reaction- and PHA degradation-free conditions, the concentration of PHA synthase controls the molecular weight of PHA, as observed for P(3HB) biosynthesis in recombinant *E. coli*.^{30–32} The molecular weight of PHA decreases with increasing PHA synthase concentrations. The same dependency has been observed in other studies using various types of recombinant *E. coli*.^{33,34} PhaC likely forms an inclusion body when overexpressed in an *E. coli* host. Therefore, co-expression of molecular chaperones is effective for producing the active form of PHA synthase and can be used to control the synthase concentration.³⁵

To improve the understanding of the CT reaction in *E. coli*, which harbors the minimum number of genes required for P(3HB) biosynthesis, the occurrence and progression of CT was evaluated using PEG as an exogenous CT agent.²⁴ As a result, the carboxylic end of P(3HB) produced in the *E. coli* transformant was capped with PEGs, despite *E. coli* being a non-native PHA producer.²⁴ This *in vivo* result indicates that PHA synthase directly reacts with the CT agent (PEG) without mediation by other native proteins involved in PHA metabolism. Furthermore, the yield of PEGylation (covalent attachment rate of the P(3HB) carboxy terminus to the PEG chain) was estimated to be as high as 88%. This suggests that the CT reaction in *E. coli* culture was mediated mainly by the external CT agent.

In contrast, kinetic analysis of P(3HB) revealed that the molecular weight of *E. coli*-synthesized P(3HB) remained nearly unchanged, whereas the number of polymer chains increased over time during P(3HB) accumulation.⁵ If P(3HB) were synthesized under CT reaction- and PHA degradation-free conditions, the molecular weight of the synthesized P(3HB) would increase over time, while the number of polymer chains would be independent of time. Therefore, termination with or without a CT agent occurred in *E. coli* cells under these culture conditions.⁵

ENDOGENOUS ETHANOL AS CT AGENT IN *E. COLI*

As described above, the concentration of PHA synthase does not entirely account for the molecular weight of PHA synthesized in *E. coli*. Some studies have suggested that the CT reaction occurs in *E. coli* in the absence of exogenous CT agents.^{5,18,24,33} The molecular weight of PHA is thought to be *E. coli* strain-dependent. For example, the molecular weights of PHA have been shown to differ when the

derivative strains of *E. coli* K-12 used as hosts for PHA production were cultured under the same conditions.³³ The XL1-Blue strain of *E. coli* is likely to produce high-molecular-weight PHA compared with the other previously reported strains.^{27,33,36}

E. coli inherently produces relatively large amounts of ethanol under hypoxic and anaerobic conditions to maintain the cellular redox balance. Therefore, the *E. coli* strain-dependency of the PHA molecular weight may be explained by considering endogenous ethanol as a potential CT agent (Figure 1). High-molecular-weight PHA can be synthesized by ensuring sufficient culture aeration to facilitate cell growth while preventing ethanol production. This also demonstrates that the ethanol-producing ability of strain XL1-Blue was low and comparable to that of the alcohol dehydrogenase gene-deletion strain.¹⁸ Because of its low ethanol-producing ability, XL1-Blue tends to synthesize higher-molecular-weight PHA compared to other strains of *E. coli*.

Even when *E. coli* strains are cultured in the same concentration of ethanol, the molecular weights of PHA vary depending on the PHA synthase employed for PHA production. Among the PHA synthases examined to date, *A. caviae* synthase (PhaC_{Ac}) is the most sensitive to the effect of ethanol on the molecular weight of PHA,³⁷ producing low-molecular-weight PHA even at low ethanol concentrations, although it has the potential to produce very high-molecular-weight PHA.^{29,38} In contrast, *Delftia acidovorans* and *Halomonas elongata* synthases are less sensitive to ethanol, producing high-molecular-weight PHA in normal *E. coli* strains.^{34,37,39}

Endogenous ethanol induces random alcoholytic cleavage of the PHA chain other than the CT reaction in the presence of class IV synthases such as *B. cereus* and *B. megaterium* synthases, in turn reducing the molecular weight of PHA and broadening the molecular-weight distribution during post-PHA synthesis.^{40–43} This unique property of class IV synthases enables the synthesis of low-molecular-weight and end-structure-regulated PHA.⁴⁴

IN VITRO POLYMERIZATION OF PHA

In 1994, Gerngross *et al.* overexpressed *R. eutropha* PHA synthases in *E. coli* to produce sufficient amounts of pure enzyme in a soluble form.¹⁴ This opened up new avenues for the *in vitro* synthesis of PHA. Subsequently, they polymerized P(3HB) *in vitro* using purified *R. eutropha* PHA synthase and 3HB-CoA.⁴⁵ This study revealed that P(3HB) synthesized *in vitro* had a significantly higher M_w ($> 10 \times 10^6$) than P(3HB) synthesized *in vivo*. In addition, the concentration of PhaC was an important factor regulating the molecular weight of

P(3HB).⁴⁵ This was attributed to the lack of the CT reaction and PHA degradation during *in vitro* polymerization, as observed in recombinant *E. coli*. Thus, the induction of a CT reaction *in vitro* will aid in understanding the nature of the CT reaction. However, the low yield of PHA synthesized *in vitro* hindered the characterization of its end structures by NMR. High yields of P(3HB) could not be achieved by *in vitro* polymerization, as free CoA released after polymerization is known to inhibit PhaC activity. This limitation was overcome by developing a new system for *in vitro* PHA synthesis by recycling CoA.^{46–48}

In our previous study, P(3HB) was efficiently polymerized *in vitro* by recycling CoA using a transesterification reaction between free CoA and the monomer precursor (crotonic anhydride).⁴⁹ The M_w and polydispersity index (M_w/M_n) of the synthesized P(3HB) were determined to be $6.4 \times 10^6 \text{ g mol}^{-1}$ and 1.6, respectively, by gel permeation chromatography. Furthermore, the absolute molecular weight, $M_{w(\text{MALLS})}$, of P(3HB) was measured to be $5.8 \times 10^6 \text{ g mol}^{-1}$ by gel permeation chromatography-multi-angle laser light scattering (MALLS). The molecular weight of P(3HB) synthesized *in vitro* was very high. The stoichiometry of *in vitro* P(3HB) polymerization is shown in Table 2. Assuming that a PhaC dimer forms an active site, PhaC dimer produced the P(3HB) polymer chain at a stoichiometry of approximately 1:1. This observation strongly suggests that the CT reaction is minimal during *in vitro* polymerization. Interestingly, the molecular-weight distribution of P(3HB) synthesized *in vitro* was identical to that of P(3HB) synthesized in *E. coli*, as shown in Figure 2, indicating the similarity of these two polymerization systems.

If the rate of initiation of *in vitro* polymerization was much higher than the rate of chain propagation (fast initiation), the polydispersity index of synthesized PHA would be similar to that of ideal living polymerization with a Poisson distribution of molecular weight according to equation (3):⁵⁰

$$M_w/M_n \approx 1 + 1/P_n \quad (3)$$

where P_n denotes the degree of polymerization. The M_w/M_n and P_n of *in vitro* synthesized PHA were 1.6 and 45 000, respectively (Table 2). The high value of M_w/M_n suggests that the rate of initiation of *in vitro* polymerization is very low (slow initiation). Slow initiation may be closely related to the mechanism with which PhaC changes the active form of the enzyme; however, this remains to be verified.

Induction of the CT reaction *in vitro* was confirmed by adding PEGs (molecular weight = 200–400) to the *in vitro* polymerization system.⁴⁸ ¹H-NMR analysis of the resultant P(3HB) revealed that the carboxylic end of P(3HB) was covalently linked to PEGs, providing

Table 2 Stoichiometry of PHA synthase to the synthesized P(3HB) chain during *in vitro* polymerization

| Synthase for <i>in vitro</i> polymerization | <i>R. eutropha</i> (PhaC) ^a | <i>R. eutropha</i> (PhaC) ^b | <i>A. vinosum</i> (PhaEC) ^c |
|---|--|--|--|
| M_n | 4.0×10^6 | 0.6×10^6 | 0.9×10^6 |
| M_w | 6.4×10^6 | — | 1.6×10^6 |
| M_w/M_n | 1.6 | — | 1.8 |
| $M_{w(\text{MALLS})}$ | 5.8×10^6 | — | — |
| P_n | 4.5×10^4 | 0.7×10^4 | 1.0×10^4 |
| $N_E: N_P$ ^d | 1:1.2 | 1:1.1 | 1:25 |

Abbreviation: —, not determined or not described.

^aData from ref. 49.

^bData from ref. 15.

^cData from ref. 51.

^d N_E : number of PHA synthases (dimer form for *R. eutropha*), N_P : number of synthesized P(3HB) chains.

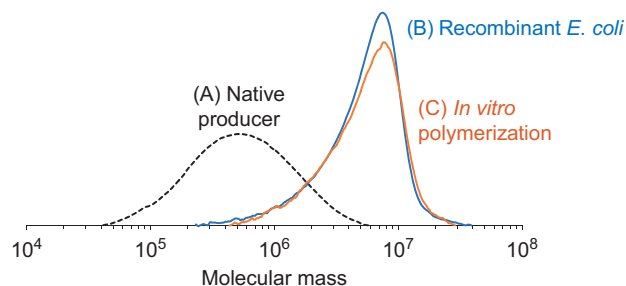


Figure 2 Molecular-weight distribution of poly[(R)-3-hydroxybutyrate] (P(3HB)) synthesized by (A) *R. eutropha* (PHA native bacteria) ($M_w=0.77 \times 10^6$, $M_w/M_n=2.2$), (B) recombinant *E. coli* XL1-Blue harboring *phaCAB* genes ($M_w=6.1 \times 10^6$, $M_w/M_n=1.7$), and (C) under *in vitro* conditions ($M_w=6.4 \times 10^6$, $M_w/M_n=1.6$).⁴⁹

Table 3 Guidelines for the efficient biosynthesis of UHMW-P(3HB)

| Factors | Preferred conditions ^a |
|--|--|
| Concentration of PHA synthase | Maintain at a low concentration by tuning the expression levels |
| CT reaction | Does not occur when alcohol production is blocked |
| Catalytic activity of PHA synthase | Employ PHA synthase or a mutant capable of polymerizing at a high rate |
| Degradation of PHA during biosynthesis | Does not occur when using PHA depolymerase genes-deleted strain or a non-native PHA producer as a host |

Abbreviations: CT, chain transfer; PHA, polyhydroxyalkanoate.

^aSelect the culture pH, temperature, aeration and medium to maintain the preferred conditions.

direct evidence of the *in vitro* CT reaction. In contrast, PEG, with an average molecular weight of 1000, was not incorporated at the carboxylic end of P(3HB) because of the structural limits of PHA synthase for accepting CT agents.⁴⁸

As an exception, during *in vitro* polymerization of P(3HB) with PHA synthase from *A. vinosum*, CT to water or the hydrolysis of the polymer chain was observed (Table 2).⁵¹ In support of this *in vitro* observation, *A. vinosum* synthase has been proposed to catalyze the hydrolytic cleavage of ester bonds.^{52–54}

CATALYTIC ACTIVITY OF PHA SYNTHASE

The catalytic activity of PHA synthase also affects the molecular weight of PHA. The relationship between the molecular weight of PHA and catalytic activity of PHA synthase was investigated using various mutants of the *R. eutropha* PHA synthase.³³ The results suggested that PhaC(F420S), a mutant with a high catalytic activity, can synthesize higher-molecular-weight P(3HB) compared with wild type or mutants with lower catalytic activities.

Investigations of the PHA synthases from *D. acidovorans* and *H. elongata* indicated that these synthases produced higher-molecular-weight P(3HB) ($M_{w(MALLS)} = 4.2\text{--}8.0 \times 10^6 \text{ g mol}^{-1}$) compared with the *R. eutropha* PHA synthase ($M_{w(MALLS)} = 2.1\text{--}2.7 \times 10^6 \text{ g mol}^{-1}$) in an *E. coli* host.^{34,37,55} The catalytic activities of these synthases may be very high; however, this was not observed in *in vitro* kinetic analysis. *In vitro* kinetic analysis of the *D. acidovorans* synthase revealed that this enzyme had a lower substrate affinity and slightly higher maximum reaction rate compared with the *R. eutropha* synthase.³⁴

The differences between the *in vivo* and *in vitro* polymerizations is the lag time before the start of polymerization *in vitro* shown by purified PhaC homodimer-type synthase. This lag time may be attributed to the dimerization of PhaC subunits to create an active form of the enzyme.¹⁴ Therefore, lag eliminators such as Hecameg and TritonX-100 must be added to the assay mixture for *in vitro* kinetic analysis.^{45,56} The lag-eliminating efficiency and optimal concentrations of lag eliminators differ with the type of synthase used, which complicates the comparison of kinetics between these synthases. In addition, PHA granule-binding proteins such as PhaP and PhaM modulate the activity of PHA synthase *in vivo*.^{57–59} It is important to note that the catalytic activity of PHA synthase is easily influenced by such environmental factors.

FACTORS AFFECTING THE MOLECULAR WEIGHT OF PHA

In conclusion, various factors affecting the molecular weight of PHA are summarized in this review. The studies mentioned above suggest that the molecular weight of PHA is determined by four fundamental factors: the concentration of PHA synthase, occurrence of the CT reaction, catalytic activity of PHA synthase and simultaneous degradation of PHA during biosynthesis (Table 3).

In vitro PHA synthesis occurs under CT reaction- and PHA degradation-free conditions; therefore, the molecular weight of the

resultant PHA is very high. The molecular weight is influenced mainly by the concentration of PHA synthase. In addition, the CT reaction can be induced *in vitro* in the presence of CT agents.

The polymerization of PHA in non-native *E. coli* also occurs under CT reaction- and PHA degradation-free conditions, similarly to its occurrence under *in vitro* conditions. Therefore, the molecular weight of the resulting PHA is very high and is influenced by the concentration of PHA synthase. However, endogenous ethanol can also function as a CT agent depending on the cellular conditions. Moreover, the sensitivity of PHA synthase towards the CT agent can vary according to the type of enzyme employed, resulting in PHA with different molecular weights, even under similar culture conditions. Notably, to obtain a sufficient yield of UHMW-P(3HB), PHA synthase should have high catalytic activity *in vivo* while maintaining the concentration at a low level.

The factors described above are involved in regulating the PHA molecular weight in PHA native bacteria. Simultaneous degradation of PHA is the largest contributor to this regulation, as the deletion of PHA depolymerase genes facilitates the production of high-molecular-weight PHA.²⁹

The molecular weight of PHA has been controlled by regulating the culture conditions, such as pH and temperature, for PHA-producing bacteria.^{27,28,33,36} These environmental conditions affect the four factors that are directly involved in molecular-weight regulation. However, little is known about the hydrolytic activity of PHA synthase, such as that shown by *A. vinosum* PHA synthase^{51–54} (Table 2). A further understanding of molecular-weight regulation will facilitate the biosynthesis of PHAs with desired molecular weights for a variety of applications.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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