Development of novel methods for controlling the molecular weight of microbial polyesters

A Dissertation for the Degree of Doctor of Engineering

Seiya Tanaka

Graduate School of Chemical Sciences and Engineering
Hokkaido University
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Chapter 1

General Introduction

1.1. Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHAs) are a class of aliphatic polyesters synthesized intracellularly by microorganisms[1]. PHAs are produced from biomass feedstocks and serve as energy storage materials. Under starvation conditions, microorganisms degrade PHA to utilize it as an energy source. Figure 1-1 illustrates the chemical structure of naturally occurring PHAs. They possess a backbone consisting of three carbon atoms and side chains ranging from 0 to 9 carbon atoms in length, exclusively in the (*R*)-configuration.

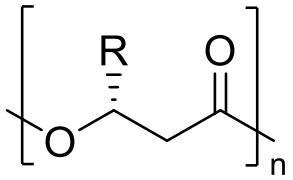


Figure. 1-1. General structure of naturally occurring PHAs. R: alkyl group

Poly(3-hydroxybutyrate) [P(3HB)] is a representative example of PHAs. In 1926, Dr. Lemoigne at the Pasteur Institute identified a substance found intracellularly in *Bacillus megaterium* as poly[(*R*)-3-hydroxybutyrate] [P(3HB)], a homopolymer consisting of (*R*)-3-hydroxybutyrate (3HB) monomer units [2]. Since then, research has been conducted on the utilization of PHAs as biodegradable plastics. Through these studies, it has been revealed that approximately 200 types of microorganisms biosynthesize and intracellularly accumulate PHAs consisting of (*R*)-3-hydroxyalkanoate monomer units [3].

1.2. PHA biosynthesis

PHA synthase (PhaC) plays a pivotal role in the microbial synthesis of PHAs. As a key enzyme in PHA biosynthesis, PhaC dictates the structure of the polymer, including its monomer composition and molecular weight [4]. PhaC catalyzes the polymerization reaction by recognizing coenzyme A (CoA) thioesters of 3-hydroxyalkanoic acids (3HA-CoAs), which typically possess a hydroxyl group at the C3 position, as substrates[5]. While PhaC exhibits strict recognition for the CoA moiety, its substrate specificity regarding the chain length and hydroxyl group position (e.g., C4 or C5 positions) of the acyl moiety varies among different enzyme types [6]. Therefore, the *in vivo* synthesis of PHAs with desired compositions requires not only the construction of a supply pathway for CoA-activated monomers but also the selection of a PhaC with appropriate substrate specificity for those monomers.

To date, more than 60 types of PhaCs have been isolated and categorized into four major classes (Class I to Class IV) based on their substrate specificity and subunit structure (Table 1-1) [7].

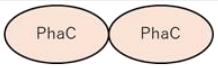
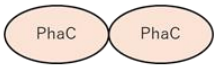
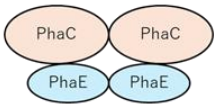
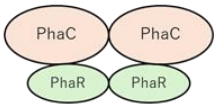
Class I synthases exhibit substrate specificity primarily for short-chain-length (SCL) monomers containing 3 to 5 carbon atoms. Representative enzymes in this class include those from *Ralstonia eutropha* and *Aeromonas caviae*; these PhaCs, with molecular weights ranging from 60 to 73 kDa, exhibit activity by forming a homodimer.

Class II synthases primarily polymerize medium-chain-length (MCL) monomers with 6 to 14 carbon atoms [8]. This class includes enzymes derived from *Pseudomonas* species, such as *Pseudomonas aeruginosa* and *P. putida*, which function as homodimers of proteins with shorter N-terminal regions compared to Class I enzymes.

Class III and Class IV synthases share the SCL monomer specificity of Class I but are characterized by the requirement of multiple subunits for activity. Class III enzymes (e.g., from *Allochromatium vinosum*) show activity by forming heterotetramers composed of a 40 kDa PhaC and PhaE subunits, while Class IV enzymes (e.g., from *Bacillus megaterium*) form heterotetramers composed of PhaC and an approximately 22 kDa PhaR subunit. These enzymes exhibit negligible polymerization activity unless the subunits are assembled.

While the general classification is described above, flexible substrate specificity has been reported regarding the synthesis of SCL-MCL copolymers. For instance, Class I enzymes, such as those from *R. eutropha*, have demonstrated the potential to incorporate MCL monomers like 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), and 3-hydroxydecanoate (3HD) into PHA chains (Ref. 9). Similar capabilities have been confirmed in *A. caviae*, *Rhodospirillum rubrum*, *Rhodocyclus gelatinosus*, and *Rhodococcus ruber* [4]. These findings serve as significant motivation for the development of engineered enzymes with expanded capabilities for MCL unit incorporation, thereby enabling the controlled synthesis of SCL-MCL PHA copolymers.

Table1-1 Class of PHA synthase

Class	Subunits	Representative species	Substrate
Ⅰ	 PhaC: -60 – 73 kDa	*Cupriavidus necator* *Sinorhizobium melioti* *Burkholderia sp.*	3HA-CoA (~C3-C5) 4HA-CoA 5HA-CoA
Ⅱ	 PhaC: -60 – 65 kDa	*Pseudomonas aeruginosa* *P. Putida*	3HA-CoA (~≥C5) 3HAscl-CoA
Ⅲ		*Allochromatium vinosum* *Thiocapsa pfennigii* *Synechocystis sp. PCC6803*	(3HAmcl-CoA [~C6-C8], 4HAscl-CoA, 5HAscl-CoA)
Ⅳ	 PhaC: -40 kDa PhaR: -20 kDa	*Bacillus megaterium* *Bacillus sp. INT005*	3HAscl-CoA

PHAs are produced by microorganisms that utilize various carbon sources. These sources range from monosaccharides and fatty acids derived from plant oils to inexpensive waste-derived components, such as beet and sugarcane molasses. The types of monomers incorporated into PHA synthesis depend on the carbon sources and their metabolic pathways. Based on the monomer composition of the synthesized PHA, wild-type PHA-producing bacteria are generally categorized into two major groups. One group is represented by *Ralstonia eutropha*, which produces SCL PHAs. The other group is represented by *Pseudomonas* species, which produce MCL PHA[7].

Intracellular monomer supply pathways vary depending on the carbon source used. When carbohydrates serve as the carbon source (Figure 1-2, Pathway I), acetyl-CoA generated by glycolysis serves as the starting material. Acetyl-CoA is converted to

acetoacetyl-CoA by β-ketothiolase (PhaA), which catalyzes dimerization, and is subsequently reduced by NADPH-dependent acetoacetyl-CoA reductase (PhaB) to form (*R*)-3-hydroxybutyryl-CoA (3HB-CoA), a C4 monomer substrate [8,9]. Alternatively, when acetyl-CoA enters the fatty acid synthesis (de novo synthesis) pathway, it can be converted into (*R*)-3-hydroxyacyl-CoA (3HA-CoA) with 4 to 12 carbon atoms via the action of (*R*)-3-hydroxyacyl-ACP-CoA transferase (PhaG) (Figure 1-2, Pathway III).

On the other hand, when fatty acids such as plant oils are used as carbon sources (Figure 1-2, Pathway II), the β-oxidation pathway becomes the main energy source. Enoyl-CoA, an intermediate, is converted by (*R*)-specific enoyl-CoA hydratase (PhaJ) into (*R*)-3HA-CoAs with various side-chain lengths. These 3HA-CoAs are recognized by PhaC and accumulated in the cell as PHA after polymerization. Thus, the monomer composition, which determines PHAs' physical properties, depends on two factors: the types of monomers supplied by the pathway and the substrate specificity of the PhaC enzyme in each microorganism[8,9].

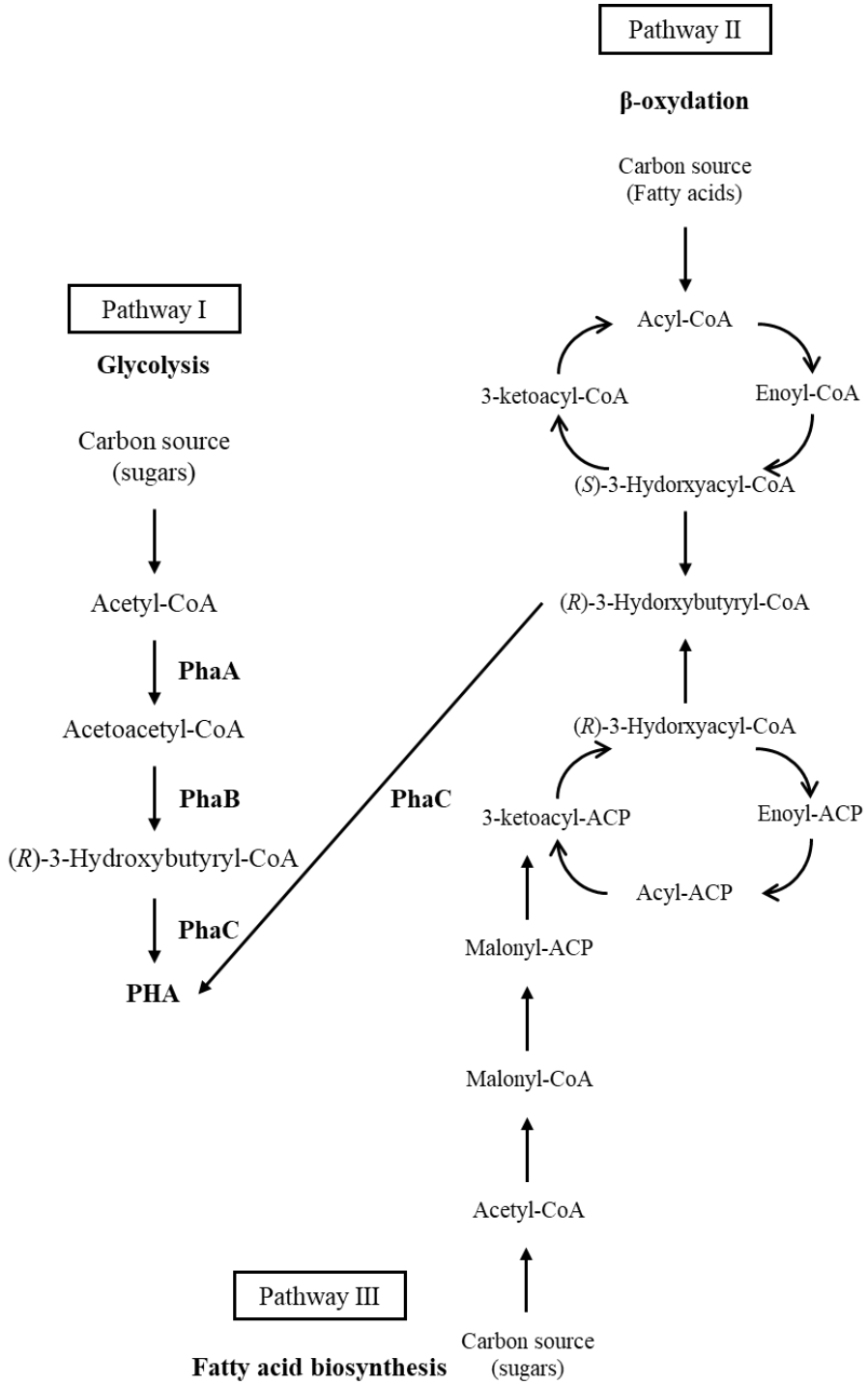


Figure 1-2. Metabolic pathways for P(3HB) biosynthesis.

1-3 Physical properties of PHA

PHA accumulates intracellularly as amorphous granules; however, upon extraction using organic solvents such as chloroform, it is obtained as highly crystalline thermoplastic polyesters. The thermal properties of P(3HB) are similar to those of polypropylene (PP). Although it falls short of polyethylene terephthalate (PET), P(3HB) exhibits excellent heat resistance (Table 1-1)[10]. However, as a material, P(3HB) suffers from being hard and brittle compared to PP and PET, a drawback attributable to its high crystallinity, which results in an extremely low elongation at break of approximately 5%. Moreover, since its glass transition temperature T_g is below room temperature, P(3HB) undergoes secondary crystallization over time after molding, leading to further embrittlement. Consequently, improving its flexibility is essential for practical applications. To address these issues, strategies of synthesizing copolymers using secondary substrates and increasing the molecular weight are expected to improve physical properties.

Table 1-2 Thermal properties of some polymers

Polymer	Melting temperature T_m (°C)	Glass-transition temperature, T_g (°C)	Crystallization temperature(°C)	Elongation at break(°C)
P(3HB)	180	4	70	5
Polypropylene	176	176	50-70	400
Polyethylene	267	267	30-50	100

1-3-1 Modification of PHA Properties via Copolymerization

In contrast to highly crystalline SCL-PHAs, MCL-PHAs possess elastomeric properties characterized by low melting temperatures T_m and low T_g, as well as high elongation at break, although their tensile strength is relatively low. Therefore, copolymerizing MCL units with SCL units is an effective strategy to overcome the brittleness of homopolymers and to control thermal and mechanical properties.
A representative example is P(3HB-*co*-3HHx), a copolymer of 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HHx). The introduction of monomers with alkyl side chains, such as 3HHx, disrupts the regular packing within the 3HB crystal lattice, thereby inhibiting crystallization[11]. In fact, P(3HB-*co*-25 mol% 3HHx) exhibits significantly lower thermal properties (T_m of 52°C) compared to the melting point of P(3HB) (approx. 180°C).

By controlling the monomer fraction in this manner, P(3HB-*co*-3HHx) exhibits a wide range of physical properties, from hard to soft, while demonstrating excellent biodegradability, hydrolytic resistance, and water vapor barrier properties. Thus, by controlling the monomer fraction, P(3HB-*co*-3HHx) exhibits a wide range of physical properties.

1-3-2 Influence of molecular weight on the physical properties of PHA

The molecular weight of PHAs is a critical factor determining the material properties of the polymers. Although the molecular weight of P(3HB) depends on production conditions, such as the host microorganisms and PHA synthases employed, it typically

falls within the range of 10^5 to 10^6 in commercially available products and numerous research reports[12–14]. PHA exhibits distinct properties as the molecular weight varies from this general range. In the low-molecular-weight regime, the glass transition temperature T_g tends to decrease, a trend observed in general polymers[15]. Leveraging this property, low-molecular-weight polymers are utilized as plasticizers to impart flexibility to materials[16]. In particular, oligomeric PHAs with molecular weights on the order of 10^3 have been reported as building blocks for polyurethane synthesis[17]. Conversely, in the high-molecular-weight regime, tensile strength improves with increasing molecular weight but exhibits a saturation behavior at a certain molecular weight threshold[12]. Furthermore, PHAs with a weight-average molecular weight (M_w) exceeding 3×10^6 are defined as "ultra-high-molecular-weight polymers" and are known to exhibit exceptionally high Young's moduli[18].

1-4 Molecular weight control of PHA

1-4-1 Action of chain-transfer agents

There are limited reports in terms of controlling molecular weight of PHA. Chain-transfer (CT) agents is known to reduce the molecular weight of PHA. CT agents used in PHA production are exogenous alcohols, such as diethylene glycol (DEG) and polyethylene glycol (PEG), which facilitate the CT reaction—specifically, a transesterification reaction between the growing polymer chain and the CT agent[19–22]. Furthermore, water and endogenous alcoholic compounds, such as ethanol, can also function as CT agents[19]. The supplementation of the culture medium with CT agents leads to a reduction in the molecular weight of the resulting PHAs. This phenomenon

has been consistently observed in several *in vitro* studies as well[23]. Thus, the use of CT agents represents an effective strategy for downregulating molecular weight.

Moreover, the CT reaction generates polymers in which the carboxyl terminus is covalently bound to the CT agent[17]. This phenomenon has been exploited for the terminal modification of PHAs. For instance, DEG-bound PHA oligomers have been utilized as building blocks for polyurethane synthesis[24].

1-4-1 Expression level of PHA synthase

In several PHA production systems, expression level of PHA synthase has been known to be a factor determining the molecular weight of PHAs. For instance, Sim et al. constructed synthetic operons in *E. coli* harboring the PHA synthase gene from *Ralstonia eutropha*, designing them to enhance PhaC expression by modulating plasmid promoter strength and ribosome binding site sequences. Their experiments revealed that P(3HB) synthesized under conditions of elevated PhaC expression exhibited a lower molecular weight compared to that produced using the native operon. Based on these findings, it was reported that the concentration of PhaC alters the frequency of polymerization initiation events, thereby directly influencing the molecular weight of the polymer [25]. Furthermore, it has been reported that the specific gene arrangement within the PHA synthesis operon is critical for determining both the molecular weight and the production yield of P(3HB)[26].

1-5 Aim of this study

This study aimed at developing new methods to control molecular weight of PHAs. To meet this goal, in Chapter 2, I focused on the action of CT agents. The molecular weight reduction induced by CT agents is generally hypothesized to result from a competition between chain elongation and the CT reaction. To validate this hypothesis, I investigated the effects of aprotic polyethers on PHA synthesis. Surprisingly, the experiments demonstrated that hydroxy groups are not essential to the downregulation of PHA molecular weight.

In Chapter 3, I further explored the structure of aprotic polyethers that influence the molecular weight of PHAs. Through the screening, I found that a crown ether upregulates PHA molecular weight. To my best knowledge, this is the first case of cultivation additive that increases the molecular weight of PHAs.

Overall, in this thesis, I worked on new approaches to control the molecular weight of PHAs and obtained new insights on the molecular weight-determining mechanism of the polymers.

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Chapter 2

Insights into the molecular weight reduction of polyhydroxyalkanoates by aprotic polyethers

2.1. Introduction

The molecular weight of P(3HB) varies depending on production conditions, such as the host organism and the PHA synthase employed, typically falling within the range of 10^5 to 10^6.[1,2] While numerous conditions for synthesizing polymers with varying molecular weights have been empirically established, methodologies for precisely controlling the molecular weight of PHAs remain limited.

As mentioned in Chapter 1, CT agents exhibit critical effect on molecular weight of PHAs. The molecular weight reduction induced by CT agents is generally hypothesized to result from a competition between chain elongation and the CT reaction. To validate this hypothesis, I investigated the effects of aprotic polyethers on PHA synthesis. If chain-transfer reactions induced by CT agents solely contribute to the reduction in molecular weight, aprotic polyethers are not expected to exhibit similar effects. The polyethers selected for this study were diethylene glycol dimethyl ether (DGDM) and triethylene glycol dimethyl ether (TGDM), which are structurally analogous to DEG but lack hydroxyl groups. DGDM was selected due to its lack of hydroxyl groups and similarity in chain length to DEG, while TGDM was included to assess whether the chain length of the compound influences the effect, as it possesses a longer chain. For P(3HB) synthesis, recombinant *Escherichia coli* strains expressing a 3HB-CoA-supplying enzyme along with either the Class I synthase $PhaC_{AR}$ or the Class II synthase $PhaC1_{Ps}STQK$ were employed. I compared the effects of these polyethers on the molecular weight of PHAs synthesized by the Class I and Class II enzymes.

2.2. Materials and methods

2.2.1 Bacterial strains and plasmids

In this study, *Escherichia coli* JM109 was employed as the host strain for the biosynthesis of poly(3-hydroxybutyrate) [P(3HB)]. For *in vivo* P(3HB) production, two distinct plasmids were utilized: pBSP$_{Re}$*phaC*$_{AR}$(opt)*pctalkK*, which expresses the Class I synthase PhaC$_{AR}$,[3] and pBSP$_{Re}$*phaC*1$_{Ps}$STQK*pct*, which expresses the Class II synthase PhaC1$_{Ps}$STQK.[4] To facilitate monomer supply, genes encoding CoA-activating enzymes were introduced into these plasmids; specifically, the pct gene encoding propionyl-CoA transferase from *Megasphaera elsdenii* and the *alkK* gene encoding medium-chain-length hydroxyalkanoate CoA ligase from *Pseudomonas putida* were employed. The coding sequence for PhaC$_{AR}$ in the expression vector was replaced with a codon-optimized fragment to enhance expression efficiency. For the in vitro analysis, the pQE30-phaCAR plasmid was constructed to express purified PhaC$_{AR}$.[5] This plasmid encodes the N-terminal His6 tag and a TEV protease cleavage site.

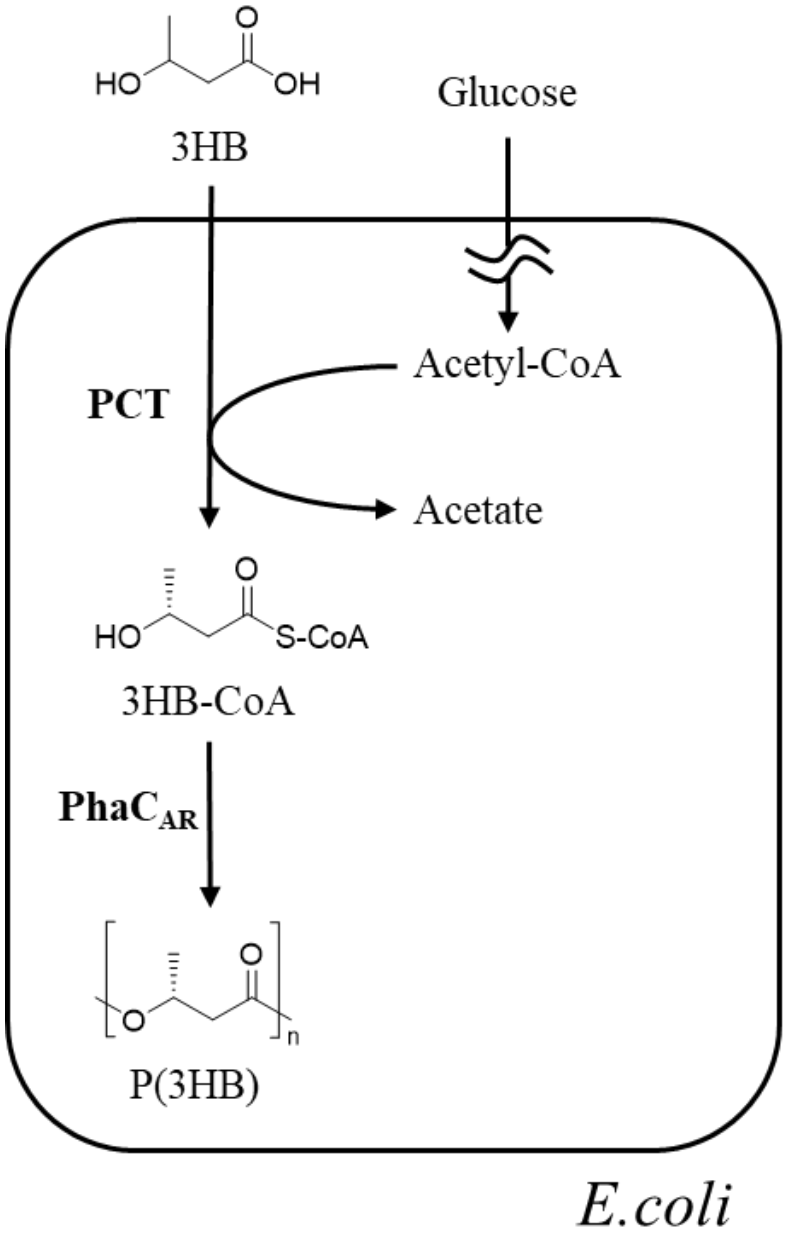
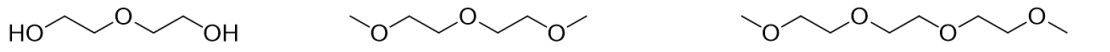


Figure 2-1 Metabolic pathway used in this study. 3HB precursor for monomer synthesis is activated by PCT to yield 3HB-CoA.

2.2.2. Culture conditions and polymer extraction

Transformed *E. coli* cells were precultured in 1.5 mL of Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 100 μg/mL ampicillin) with shaking at 180 rpm at 30°C for 12 h. The optical density at 600 nm (OD600) of the preculture reached 4.5 ± 0.1. Next, a 1-mL aliquot of the pre-culture was inoculated into a 500-mL shake flask containing 100 mL of LB medium supplemented with 20 g/L glucose, 100 μg/mL ampicillin, and 2.5 g/L sodium (*R/S*)-3HB as a monomer precursor. At this point, the specific ester compounds indicated in Figure 2-2 were added at final concentrations of 2, 5, 10, and 100 mM. Main cultivation was carried out at 30°C with shaking at 120 rpm for 48 h. Following cultivation, cells were harvested by centrifugation at 5000 × *g* and 4°C for 10 min. The cell pellets were then washed twice with distilled water and subsequently lyophilized. The Cell Dry Weight (CDW) was determined by weighing the lyophilized biomass. For polymer extraction, the lyophilized cells were suspended in chloroform and incubated at 60°C for 48 h. Finally, the polymer contained in the chloroform solution was recovered and purified by precipitation into an excess of ethanol.



Diethylene glycol (DEG) | Diethylene glycol dimethyl ether (DGDM) | Triethylene glycol dimethyl ether (TGDM)

Figure 2-2. Ester compounds used in this study.

2.2.3. NMR analysis

To determine the chemical structure, polymer samples (2–5 mg/mL) were dissolved in 1 mL of deuterated chloroform at 60°C. Upon cooling the solution to room temperature (approximaterly 25°C) and filtration was performed using a DISMIC-13HP PTFE filter (0.2 μm pore size). ^{1}H NMR spectra were recorded on a JEOL JNM-ECS400 spectrometer. The monomer composition was calculated from the integration values of characteristic signals in the ^{1}H NMR spectra, following the protocol described in previous literature.[6]

2.2.4. Molecular weight of polymer

The molecular weights of the polymers were determined by size exclusion chromatography (SEC). Polymer samples were dissolved in chloroform at 60°C to a final concentration of approximately 5 mg/mL. Before analysis, the polymer solutions were filtered through a PTFE membrane filter with a pore size of 0.2 μm. The SEC system was equipped with two tandem K-807L columns (Shodex). The analysis was conducted under the following conditions: mobile phase, chloroform; flow rate, 0.8 mL/min; column temperature, 40°C; and sample injection volume, 100 μL. Molecular weights were calculated based on a calibration curve constructed using commercially available polystyrene standards (Shodex) with peak molecular weight (M_p) ranging from 1,180 to 3,210,000 g/mol. All molecular weight values are reported relative to polystyrene.

2.2.5. Measurement of polymer production and medium components

Polymer production was determined by converting the intracellular polymers into ethyl esters through ethanol degradation. Approximately 10 mg of lyophilized cells were treated with 500 μL of chloroform and 0.5 mL of a 15% (v/v) sulfuric acid/ethanol solution. The reaction mixture was heated at 100°C for 2 h. Following the reaction, 4 mL of ultrapure water was added to induce phase separation. The lower organic layer was recovered by centrifugation (1,000 × *g*, 4°C, 1 min) and subsequently dehydrated using dried molecular sieves for 30 min.

The resulting 3-hydroxybutyrate (3HB) ethyl esters were analyzed using a Shimadzu GC-2030 gas chromatography (GC) system (Shimadzu, Japan) equipped with a Flame Ionization Detector (FID) and an InertCap1 capillary column (0.25 mm I.D. × 30 m, film thickness 0.25 μm; GL Sciences, Japan). The GC analysis conditions were set as follows: the column oven temperature was initially held at 45°C, ramped to 230°C at a rate of 2°C/min, and maintained at 230°C for 10 min. Both the injector and detector temperatures were maintained at 250°C.

To analyze the concentrations of ether compounds and ethanol in the culture supernatant, 1-mL aliquots of the culture medium were sampled every 12 h during cultivation. The concentrations of these components were quantified using a GC system equipped with a ZB-WAX capillary column (0.25 mm I.D. × 30 m, film thickness 0.25 μm; GL Sciences).

2.2.6. *In vitro* assay of PHA synthesis

The recombinant His_6-tagged $PhaC_{AR}$ was purified via a Ni-NTA column as described previously. For the *in vitro* assay, the purified enzyme (final concentration: 100 nM) was added to a reaction mixture (200 μL total volume) containing 5 mM ammonium acetate (pH 7.0), 200 μM 3HB-CoA, and polyether compounds (200, 500, or 1000 μM). Prior to enzyme addition, the mixture was pre-equilibrated at 30°C. The reaction was allowed to proceed at 30°C for 30 min. During incubation, 20-μL aliquots were periodically sampled and mixed with an equal volume (20 μL) of 1% trichloroacetic acid to terminate the reaction.

The samples were subsequently filtered and analyzed by LC-MS using an LCMS-2020 system (Shimadzu) equipped with a Mastro C18 column (150 mm). The analysis employed electrospray ionization and single quadrupole mass spectrometry, following previously established methodology.[7] 3HB-CoA was identified and quantified based on the M-H ion at m/z 852.2, eluting at a retention time of 11.8 min.

2.2.7. Immunoblot analysis

For $PhaC_{AR}$ expression analysis, transformed *E. coli* cells were cultured without substrate precursors (30°C, 12 h) and harvested. The cell pellets were resuspended in 0.2 mL of lysis buffer (20 mM HEPES, 10% glycerol, 300 mM NaCl; pH 7.5) and subjected to ultrasonication for 10–20 min. After centrifugation (15,000 × *g*, 4°C, 3 min), the supernatant was collected as the crude extract. Protein concentrations were determined using the Bradford assay (Bio-Rad, USA).

Samples were diluted to equalize the total protein concentration, mixed with 4 × sample buffer [200 mM Tris–HCl (pH 6.8), 8% SDS, 0.4% bromophenol blue, 40% glycerol, and 20% 2-mercaptoethanol], and heated at 95°C for 5 min. Samples (2 μg total protein) were subjected to SDS-PAGE on a 12% acrylamide gel, with Precision Plus Protein All Blue Prestained Protein Standards (Bio-Rad) used as markers. Proteins were transferred to a PVDF membrane using the iBlot™ system (Invitrogen, USA). Immunoblotting was conducted as described previously, utilizing Anti-Rabbit IgG, HRP-linked Whole Ab Donkey (Cytiva, Japan) as the secondary antibody[7]. Signals were detected using ECL Prime reagents (Cytiva), and band intensities were quantified using ImageJ software. Band intensities for the control (0 mM) condition were used as reference values for normalization.

2.3. Results

2.3.1. Culture results of *E. coli* expressing $PhaC_{AR}$

The influence of polyethers and DEG on cell growth and polymer synthesis was investigated (Figure 2-3A). Within the tested concentration range, DEG addition did not significantly inhibit cell growth, although a slight decrease in P(3HB) production was observed (Figure 2-3A and Table 2-1). Compared with DEG, the aprotic polyethers exhibited a slightly more pronounced inhibitory effect on both cell growth and polymer production.

Consistent with previous findings,[8] the molecular weight of P(3HB) decreased in a concentration-dependent manner upon the addition of DEG. Interestingly, a similar reduction in molecular weight was also induced by the addition of aprotic polyethers (Figure 2-3B and Table 2-1). Furthermore, due to the inhibitory effect of polyethers on cell growth and the nearly constant polymer production, the cellular polymer content increased, particularly under TGDM-supplemented conditions. Although the underlying mechanism remains to be clarified, this observation aligns with previously reported inverse correlations between cell growth and intracellular P(3HB) content.[9]

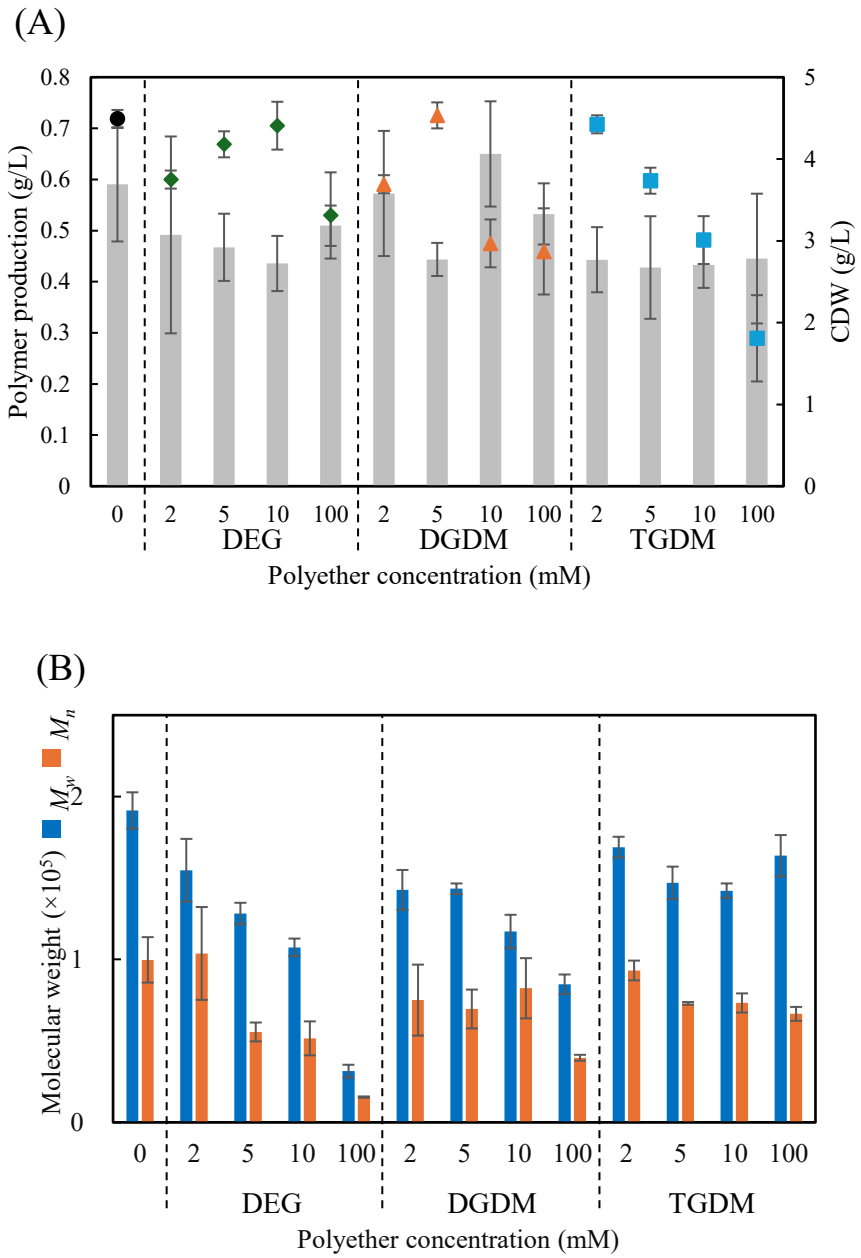


Figure 2-3. Effect of polyether compounds on the production and molecular weight of the polymer synthesized by *E. coli* expressing PhaC$_{AR}$. (A) The bars represent polymer production, and plots represent CDW (black circle: no addition, green diamond: DEG, orange triangle: DGDM, blue square: TGDM). (B) Blue and orange bars represent the weight-average (M_w) and number-average (M_n) molecular weights, respectively. Data represent the mean ± SD of three independent experiments.

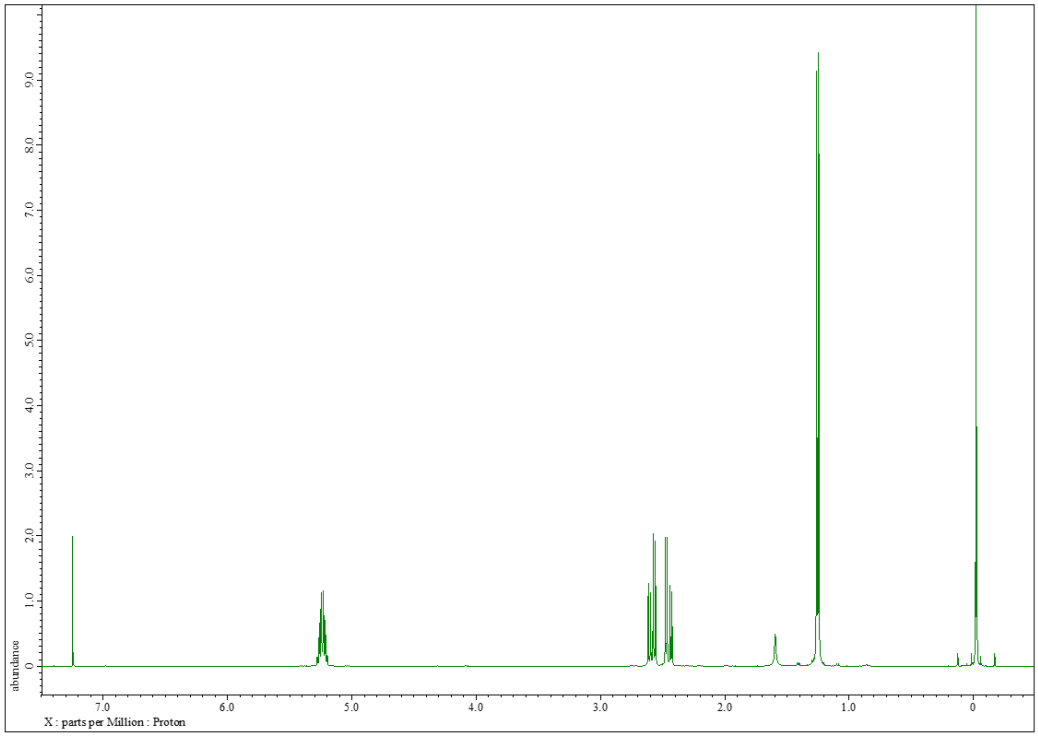
Table 2-1 P(3HB) production in *E. coli* expressing $PhaC_{AR}$

	Concentration (mM)	CDW (g/L)	Polymer production (g/L)	M_n ($\times 10^5$)	M_w ($\times 10^5$)	M_w/M_n
Control	0	4.49±0.11	0.59±0.06	1.00±0.14	1.91±0.11	1.95±0.23
DEG	2	3.75±0.16	0.49±0.01	1.04±0.29	1.55±0.19	1.60±0.57
	5	4.18±0.29	0.47±0.05	0.56±0.06	1.28±0.07	2.32±0.17
	10	4.41±0.53	0.44±0.14	0.52±0.10	1.07±0.05	2.06±0.32
	100	3.31±0.09	0.5±0.07	0.16±0.01	0.31±0.04	2.12±0.54
DGDM	2	3.69±0.38	0.57±0.07	0.75±0.22	1.43±0.12	2.31±0.29
	5	4.53±0.14	0.44±0.07	0.70±0.12	1.43±0.03	1.52±0.37
	10	2.97±0.05	0.65±0.12	0.82±0.18	1.17±0.10	1.82±0.18
	100	2.87±0.01	0.53±0.04	0.40±0.02	0.85±0.06	2.01±0.14
TGDM	2	4.42±0.64	0.44±0.05	0.93±0.06	1.69±0.06	1.95±0.14
	5	3.73±0.34	0.43±0.02	0.73±0.01	1.47±0.10	1.92±0.11
	10	3.01±0.27	0.43±0.03	0.73±0.06	1.42±0.04	2.06±0.25
	100	1.81±0.13	0.4±0.12	0.67±0.04	1.64±0.13	2.04±0.02

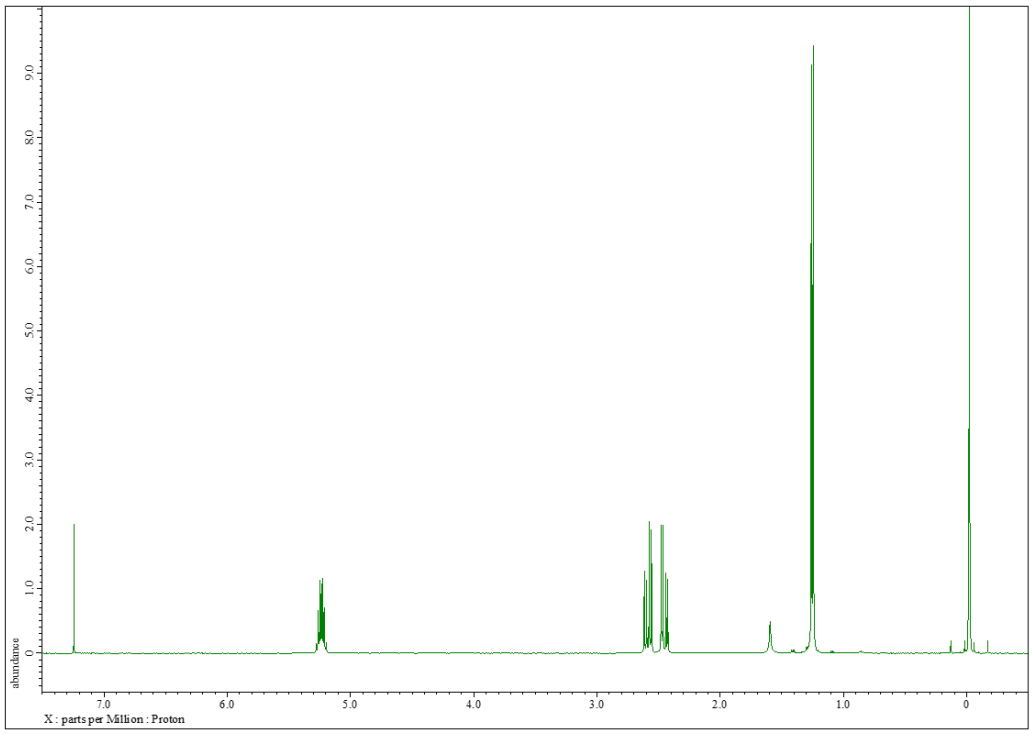
2.3.2. Terminal structure analysis using ^{1}H NMR

^{1}H NMR analysis was employed to elucidate the terminal structure of the synthesized P(3HB). The full spectra are shown in Figure 2-4A~D. The spectra revealed that DEG was covalently bound to the carboxyl terminus of P(3HB) synthesized in the presence of 100 mM DEG (Figure 2-4E). In contrast, for polymer samples synthesized with DGDM and TGDM, no corresponding signals attributable to polyether fragments were detected, despite the observed reduction in molecular weight.

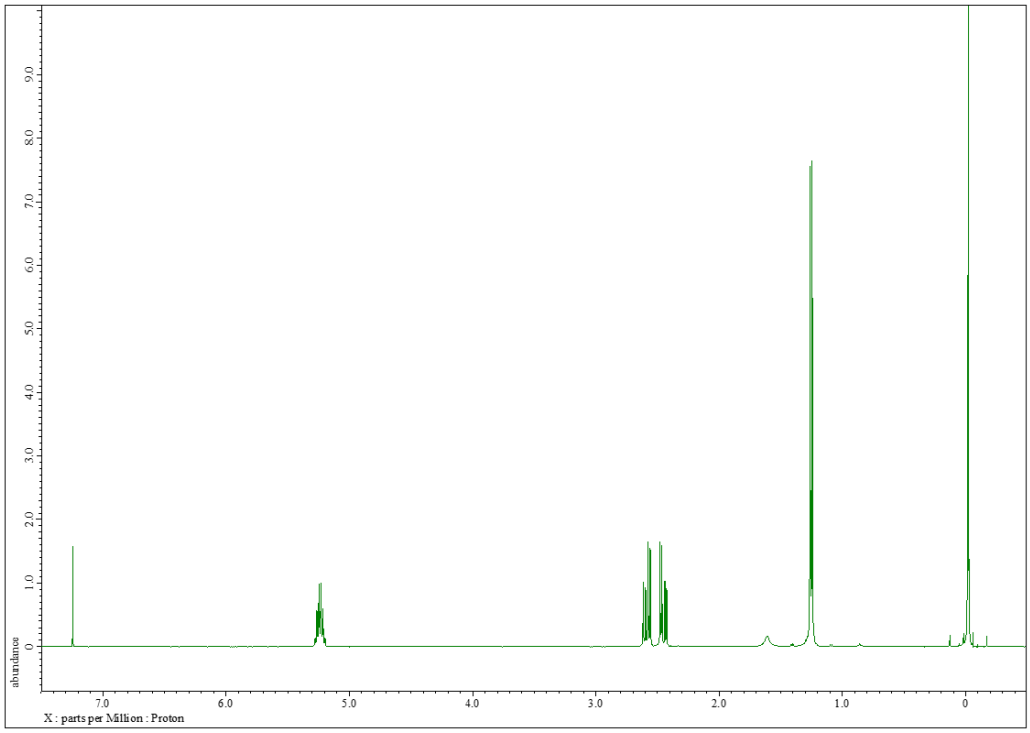
(A)


abundance
X : parts per Million : Proton

(B)


abundance
X : parts per Million : Proton

(C)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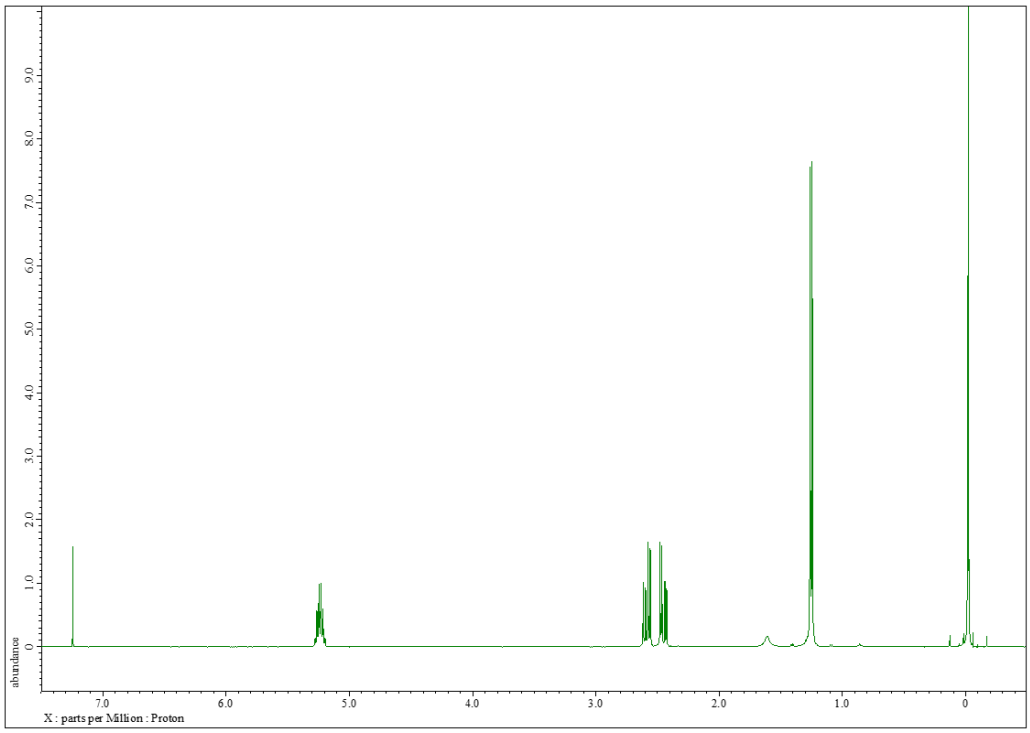
(D)


abundance
X : parts per Million : Proton

(E)

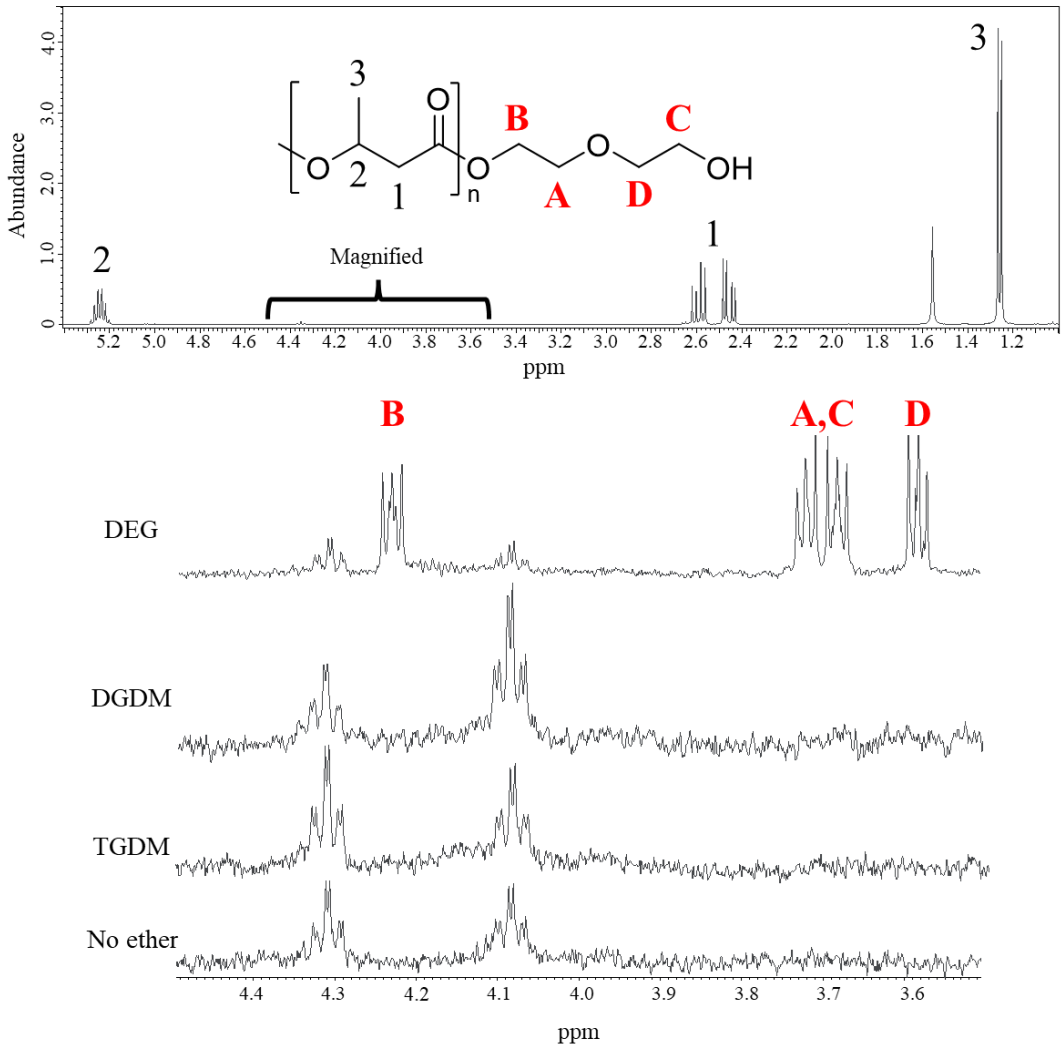


Figure 2-4. ^{1}H NMR spectra of P(3HB) synthesized by *E. coli* expressing PhaC$_{AR}$ with addition of DEG and polyethers at 100 mM. (A), Control (0 mM); (B), DEG; (C), DGDM; (D), TGDM. (E) Expanded ^{1}H NMR spectra in the range of 3.5–4.5 ppm

Therefore, it is unlikely that the aprotic polyethers functioned as CT agents via ester exchange reactions. Additionally, the concentrations of aprotic polyethers in the culture medium did not decrease significantly during cultivation (Figure 2-5).

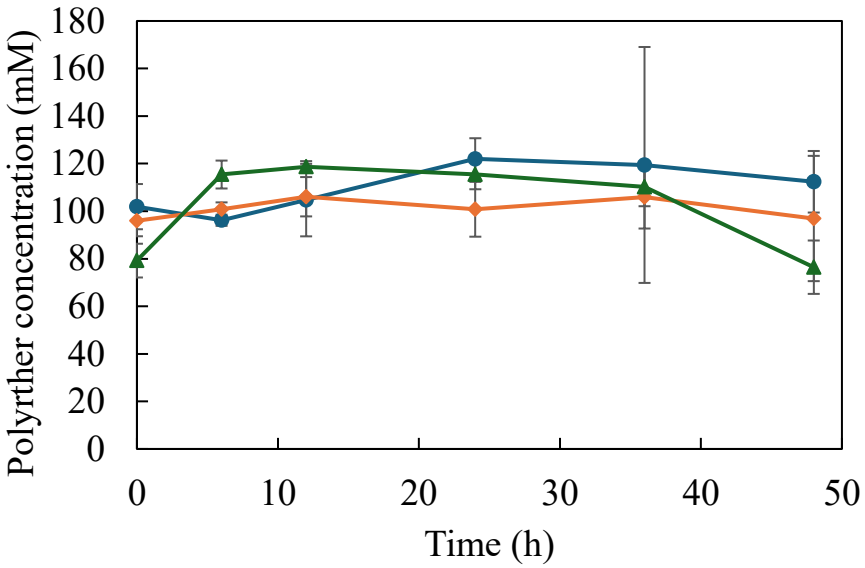


Figure 2-5. Variation in the concentration of aprotic polyether in the culture medium with incubation time. Data represent the mean ± SD of three independent experiments. *Blue circle*, 100 mM DEG; *orange diamond*, 100 mM DGDM; *green triangle,* 100 mM TGDM. Data represent the mean ± SD of three independent experiments.

Furthermore, I investigated the potential influence of endogenous ethanol, a known CT agent, on molecular weight reduction. It was hypothesized that the addition of polyethers might stimulate ethanol production in *E. coli*. To test this, ethanol concentrations in the culture medium were quantified in both the presence and absence of polyethers. The results indicated no significant difference in ethanol concentrations across all tested conditions (Figure 2-6). Consequently, it was concluded that the observed reduction in molecular weight was not attributable to an increase in ethanol-mediated chain transfer.

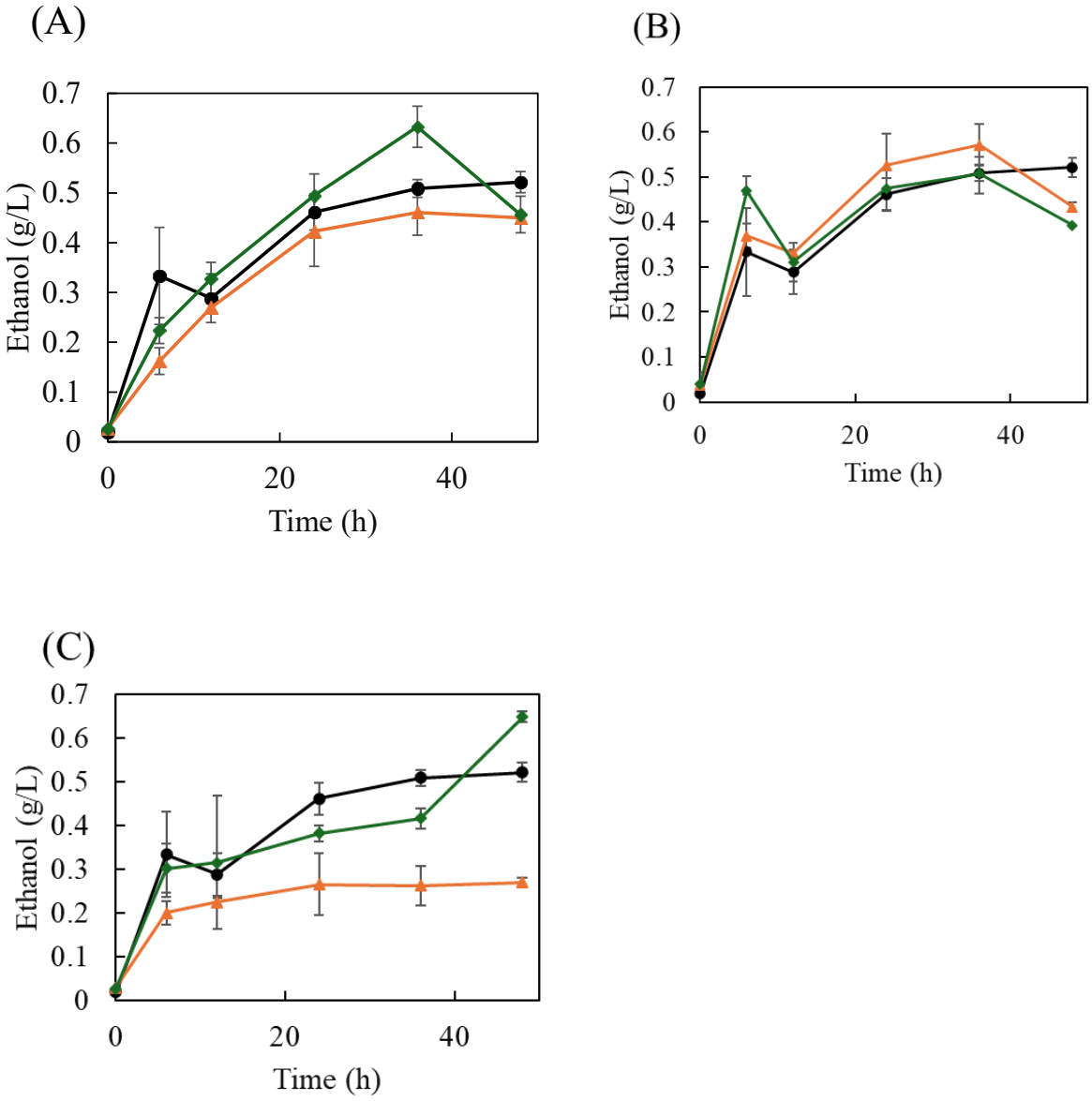


Figure 2-6. Ethanol content in culture medium for P(3HB) production conditions by *E. coli* expressing PhaC$_{AR}$. DEG (A), DGDM (B), and TGDM (C). Data represent the mean ± SD of three independent experiments. *Black circle*, 0 mM; *orange triangle*, 10 mM; *green diamond*, 100 mM. Data represent the mean ± SD of three independent experiments.

The molecular weight of P(3HB) synthesized in the presence of ethanol (0.25–10 mM) was determined (Figure 2-7, Table 2-3). No significant reduction in molecular weight was observed at ethanol concentrations of 1 mM or lower. However, the addition of ethanol at concentrations of 2 mM or higher resulted in a concentration-dependent decrease in molecular weight, the extent of which was comparable to that observed with DEG. Additionally, a slight reduction in CDW was noted due to ethanol supplementation.

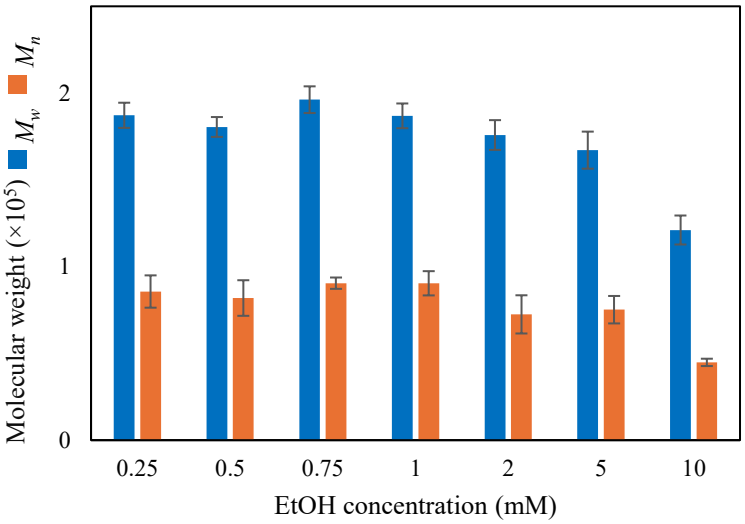


Figure 2-7. Effect of ethanol on the production and molecular weight of the polymer synthesized by *E. coli* expressing $PhaC_{AR}$. Blue and orange bars represent the weight-average (M_w) and number-average (M_n) molecular weights, respectively. Data represent the mean ± SD of three independent experiments.

Table 2-2. P(3HB) production in *E. coli* expressing $PhaC_{AR}$ under ethanol-supplemented conditions

EtOH concentration (mM)	CDW (g/L)	M_n ($\times 10^5$)	M_w ($\times 10^5$)	M_w/M_n
0	4.49±0.11	1.00±0.14	1.91±0.19	1.95±0.23
0.25	3.78±0.19	0.86±0.09	1.87±0.07	2.20±0.17
0.5	3.72±0.52	0.82±0.10	1.80±0.06	2.22±0.22
0.75	3.99±0.34	0.90±0.03	1.96±0.08	2.17±0.01
1	3.70±0.16	0.90±0.07	1.87±0.07	2.08±0.25
2	3.77±0.31	0.72±0.11	1.76±0.09	2.46±0.21
5	3.5±0.18	0.75±0.08	1.67±0.11	2.30±0.11
10	3.78±0.03	0.45±0.02	1.21±0.08	2.7±0.27

2.3.3. Culture results of *E. coli* expressing $PhaC1_{Ps}STQK$

Using the same method as $PhaC_{AR}$, the effect of DEG and polyethers on polymer biosynthesis by $PhaC1_{Ps}STQK$ was investigated. DEG addition did not exhibit significant inhibition of cell growth and polymer production, and a decrease in M_w and M_n was detected (Figure 2-8 and Table 2-3). Aprotic polyethers showed no inhibition of cell growth and polymer production. In contrast to $PhaC_{AR}$, P(3HB) of $PhaC1_{Ps}STQK$ is a moderate decrease in M_w and M_n. These results indicate that the effectiveness of the addition of polyethers on the molecular weight of PHA depends on the class of PhaC.

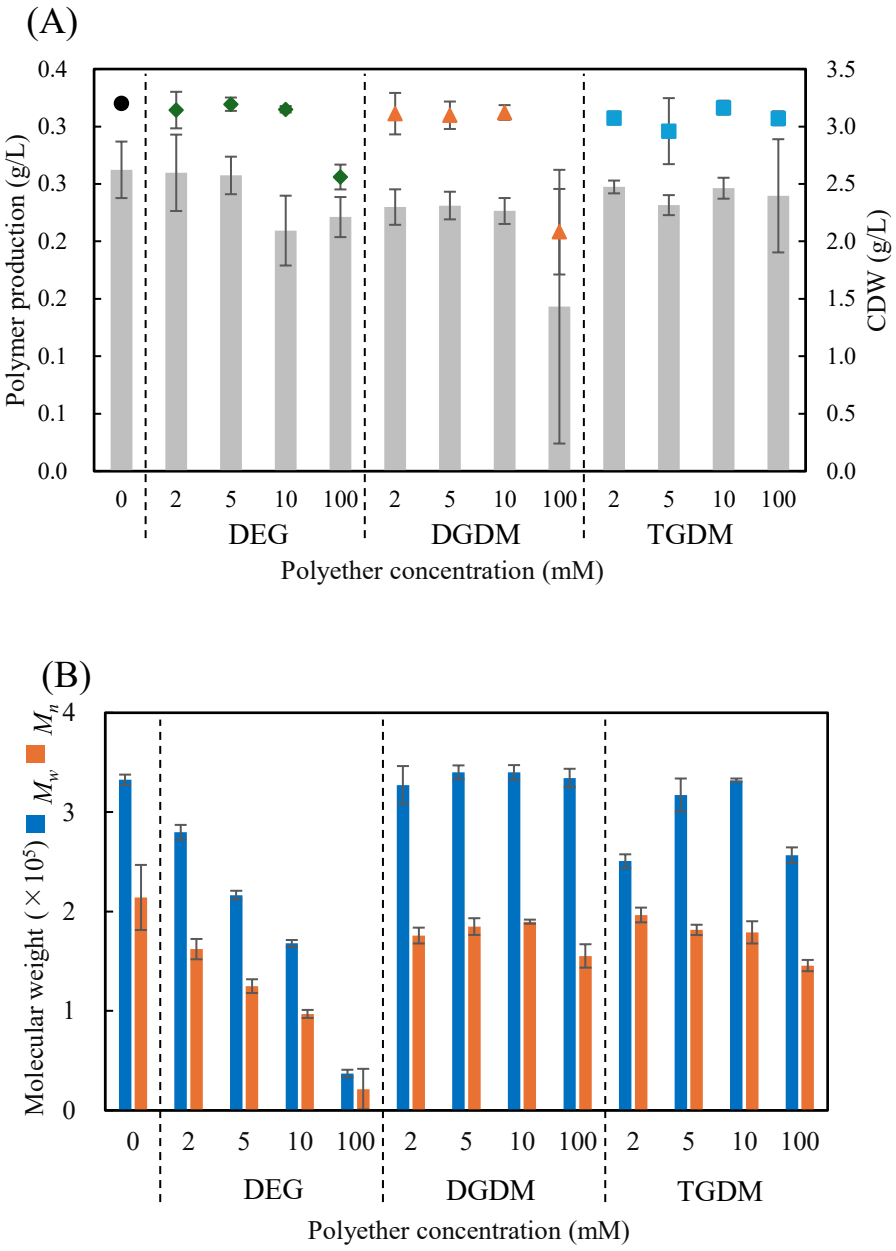


Figure 2-8. Effect of ether compounds on the production and molecular weight of the polymer synthesized by *E. coli* expressing PhaC1$_{Ps}$STQK. (A) The bars represent polymer production, and the plots represent CDW (black circle: no addition, green diamond: DEG, orange triangle: DGDM, blue square: TGDM). (B) Blue and orange bars represent M_w and M_n, respectively. Data represent the mean ± SD of three independent experiments.

Table 2-3. P(3HB) production in *E. coli* expressing PhaC1$_{Ps}$STQK

	Concentration (mM)	CDW (g/L)	Polymer production (g/L)	M_n ($\times 10^5$)	M_w ($\times 10^5$)	M_w/M_n
Control	0	3.20±0.06	0.26±0.02	2.06±0.27	3.50±0.05	1.73±0.31
DEG	2	3.14±0.16	0.26±0.03	1.62±0.10	2.80±0.07	1.73±0.09
	5	3.19±0.06	0.26±0.02	1.25±0.07	2.17±0.04	1.74±0.06
	10	3.15±0.03	0.21±0.03	0.97±0.04	1.68±0.03	1.74±0.06
	100	2.56±0.11	0.22±0.02	0.21±0.07	0.37±0.04	1.64±0.48
DGDM	2	3.11±0.18	0.23±0.01	1.76±0.20	3.27±0.19	1.87±0.12
	5	3.10±0.12	0.23±0.01	1.85±0.08	3.40±0.07	1.84±0.04
	10	3.12±0.06	0.23±0.01	1.90±0.08	3.34±0.07	1.69±0.07
	100	2.08±0.37	0.14±0.12	1.55±0.34	2.51±0.09	1.63±0.20
TGDM	2	3.07±0.06	0.25±0.01	1.97±0.02	3.54±0.07	1.80±0.02
	5	2.96±0.29	0.23±0.01	1.82±0.12	3.17±0.17	1.75±0.03
	10	3.16±0.06	0.25±0.01	1.79±0.20	3.10±0.02	1.85±0.06
	100	3.07±0.06	0.24±0.05	1.46±0.06	2.57±0.08	1.76±0.02

2.3.4. *In vitro* analysis of PhaC$_{AR}$ enzymatic activity

To elucidate the direct influence of polyethers on the enzymatic activity of PhaC$_{AR}$, in vitro polymerization assays were conducted. The reaction was monitored by measuring the rate of substrate (3HB-CoA) consumption (Figure 2-9). The results demonstrated that the addition of DEG or aprotic polyethers did not significantly alter the substrate consumption rate compared to the no addition. These findings suggest that neither hydroxyl-free polyether compounds nor DEG act as activators or inhibitors of the polymerization reaction catalyzed by PhaC$_{AR}$.

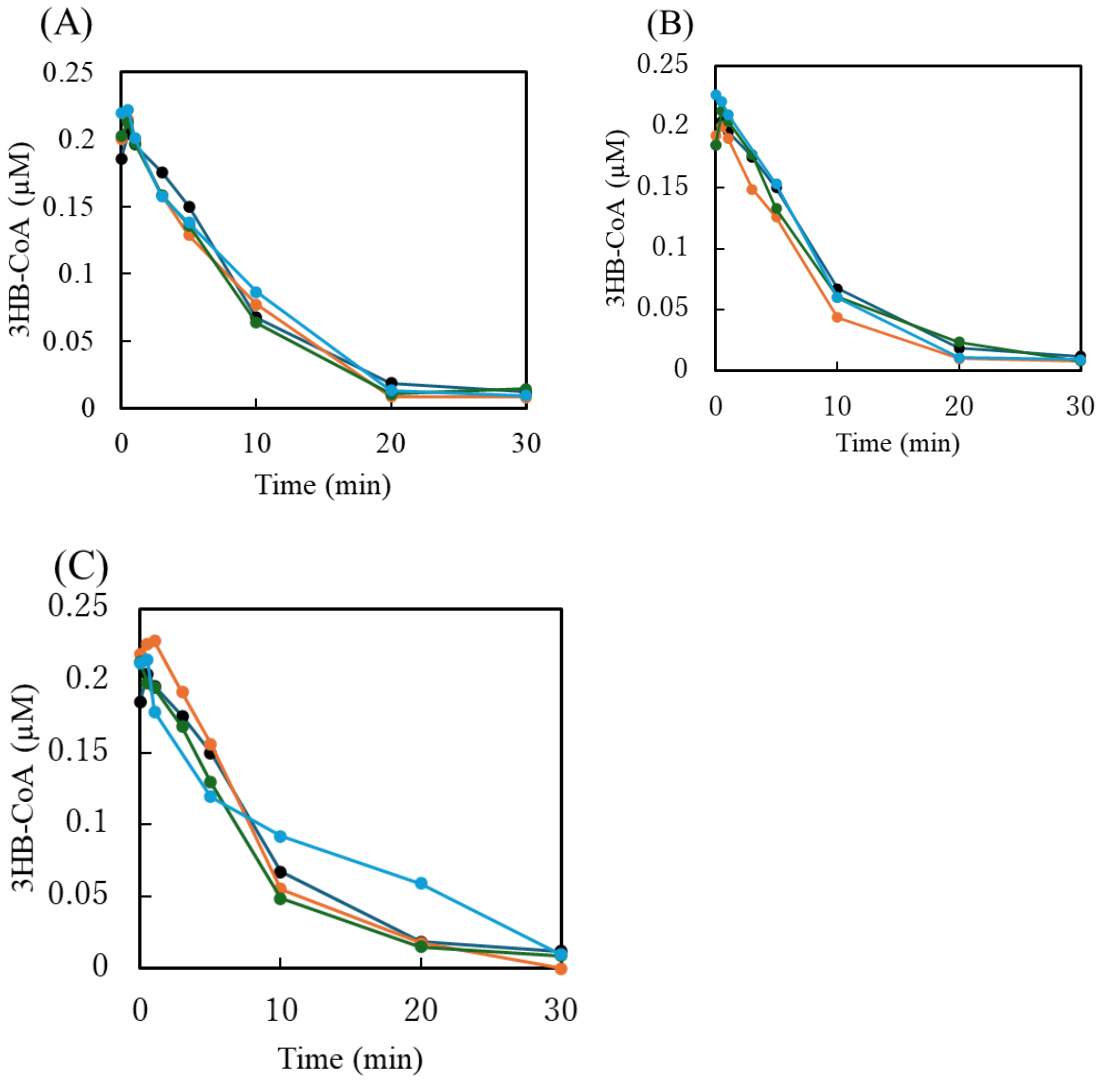


Figure 2-9. *In vitro* PhaC$_{AR}$ activity assay with the addition of polyether compounds DEG (A), DGDM (B), and TGDM (C). *Black circle*: no addition, *orange triangle*: 200 μM, *green diamond*: 500 μM and *blue square*: 1,000 μM. Data represent the mean ± SD of three independent experiments.

Polymer synthesis was performed under conditions identical to the activity assay, with the medium supplemented with either DEG or DGDM (Figure 2-10). Subsequent measurement of molecular weights revealed a decrease in polymer molecular weight in the presence of these additives. However, no significant difference in molecular weight was observed between the 10 mM and 100 mM concentrations.

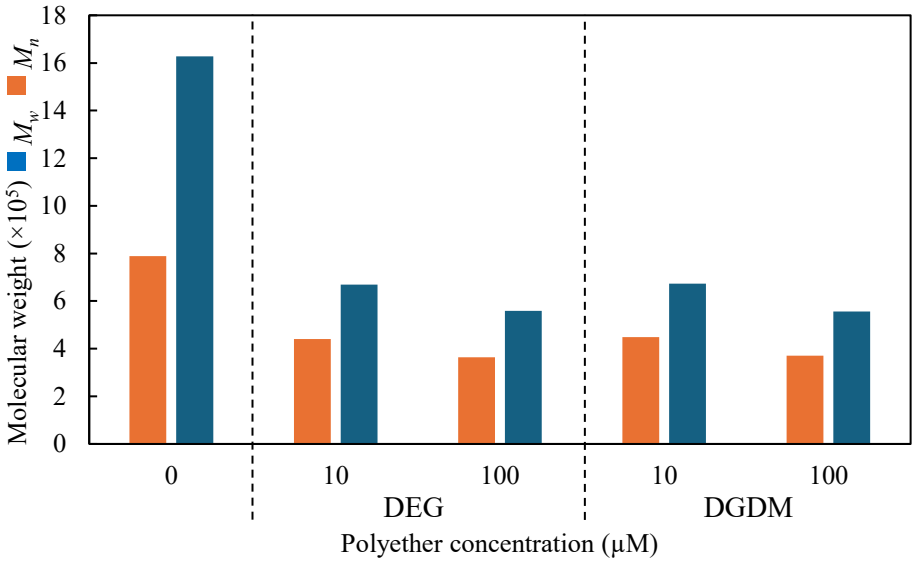


Figure 2-10. Effect of polyethers on the molecular weight of *in vitro* synthesized P(3HB) by $PhaC_{AR}$

2.3.5. The expression level of $PhaC_{AR}$ in *E. coli* cells

Given that the molecular weight of PHA is dependent on the intracellular concentration of PhaC, immunoblot analysis was performed to assess whether the addition of DEG and polyethers affected the expression levels of $PhaC_{AR}$ (Figure 2-11) To ensure the preparation of $PhaC_{AR}$ in its soluble form, cells were cultured under non-polymer-producing conditions (i.e., in the absence of precursor). Densitometric analysis revealed no significant differences in $PhaC_{AR}$ expression levels across all conditions tested with DEG and aprotic polyethers. Consequently, the observed reduction in molecular weight induced by these additives cannot be attributed to fluctuations in $PhaC_{AR}$ expression levels.

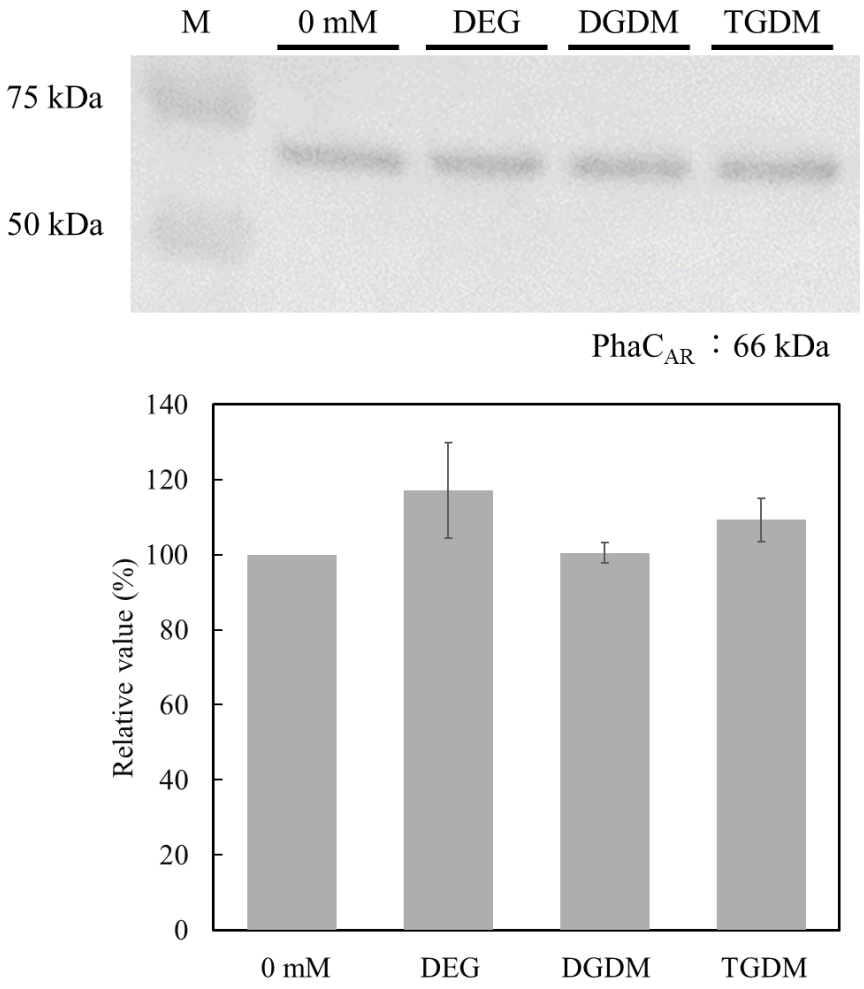


Figure 2-11. PhaC$_{AR}$ expression levels with the addition of DEG and aprotic polyether. The soluble fractions were analyzed. (A) Immunoblot image. M: size marker. The molecular weight of PhaC$_{AR}$ is approximately 66 kDa. The full image is shown in Figure S4. (B) Quantification of the relative expression levels. The values are presented as the mean ± SD of the biological triplicates.

3.2. Discussion

It is generally believed that the primary function of CT agents is to accelerate this transesterification reaction. The model is based on the observation that CT agents covalently attach to the carboxyl terminus of the growing PHA chain, thereby presumably playing a decisive role in the termination step of PHA biosynthesis. This termination event directly leads to a reduction in the molecular weight of the polymer. However, in this study, I observed an intriguing phenomenon.

Aprotic polyethers, which are structurally analogous to the efficient DEG but lack hydroxyl groups, exhibited a similar ability to decrease the molecular weight of P(3HB). In addition, ^{1}H NMR analysis revealed that these aprotic polyethers were not covalently bound to the polymer chain ends. This finding strongly suggests that the observed reduction in molecular weight is not caused solely by the classical CT reaction but involves an unknown "secondary mechanism."

To identify this secondary mechanism, several hypotheses were tested. First, the possibility of specific structural recognition was examined. I evaluated polyethers with varying chain lengths and observed similar effects on molecular weight, suggesting that the action of polyethers is likely independent of specific structural recognition by the enzyme.

Second, we investigated whether DEG and polyethers act as stabilizers for PHA synthase. Previous studies have demonstrated that higher concentrations of active PHA synthase correlate with decreased polymer molecular weight, presumably due to reduced substrate availability per synthase molecule[10]. However, my immunoblot analysis revealed that the addition of DEG or aprotic polyethers had no significant

effect on intracellular PHA synthase expression. Furthermore, in vitro enzymatic assays showed that these compounds did not significantly alter the substrate consumption rate of $PhaC_{AR}$. Collectively, these results provide no evidence to support the hypothesis that aprotic polyethers act as stabilizers or activators of PHA synthase. It has been proposed that the rate of monomer supply influences the molecular weight of PHAs. Given this, it cannot be ruled out that the monomer concentrations in the in vitro system used in this study differ from physiological conditions in *E. coli*, which might obscure the potential effects of polyethers on the enzyme *in vivo*.

Third, I examined the influence of ethanol, a major endogenous CT agent under standard cultivation conditions. It is well established that the use of ethanol-synthesis-deficient E. coli strains as hosts increases polymer molecular weight[11]. However, my data showed that the addition of polyethers did not significantly alter ethanol production (Figure 2-6). Therefore, the reduction in molecular weight induced by aprotic polyethers is unlikely to be attributable to an indirect enhancement of ethanol production.

Notably, the effect of aprotic polyethers was found to be weaker than that of DEG. For instance, while DEG effectively reduced the molecular weight of polymers synthesized by both $PhaC_{AR}$ and $PhaC1_{Ps}$STQK, aprotic polyethers did not efficiently decrease the molecular weight of polymers synthesized *in vivo* using $PhaC1_{Ps}$STQK. A related study by Shi et al.[7] reported that methyl-capped polyethers had no effect on the molecular weight of PHA synthesized in *Cupriavidus necator*. This finding contrasts with my results obtained using *E. coli*. These discrepancies suggest that the effect of aprotic polyethers on molecular weight may be enzyme- or host-specific, although the underlying mechanism remains to be fully elucidated. Furthermore, the fact that neither DEG nor aprotic polyethers affected substrate consumption in my *in vitro* $PhaC_{AR}$

activity assay provides additional evidence that their impact on molecular weight does not stem from direct modulation of $PhaC_{AR}$ kinetic activity. Consequently, the precise nature of the secondary mechanism remains an open question.

To account for the mechanism of aprotic polyethers, I focused on the action of “decoy” molecules proposed recently. It has been reported that the hydroxylation of non-natural substrates by Cytochrome P450BM3 (P450BM3) can be achieved using "decoy molecules" with shorter chain lengths than natural substrates[12–15]. Although P450BM3 is an enzyme that catalyzes the hydroxylation of the terminal position of long-chain fatty acids (Figure 2-12A), its application as a biocatalyst has historically been hindered by its extremely high substrate specificity. To address this limitation, Osami et al. focused on the binding affinity of substrates and their distance to the active center where hydroxylation occurs. They developed decoy molecules that bind to P450BM3 but are not themselves hydroxylated. The resulting P450BM3–decoy complex gained the ability to hydroxylate non-natural substrates, such as alkanes and benzene (Figure 2-12B), thereby expanding the scope of P450BM3 as a biocatalyst.

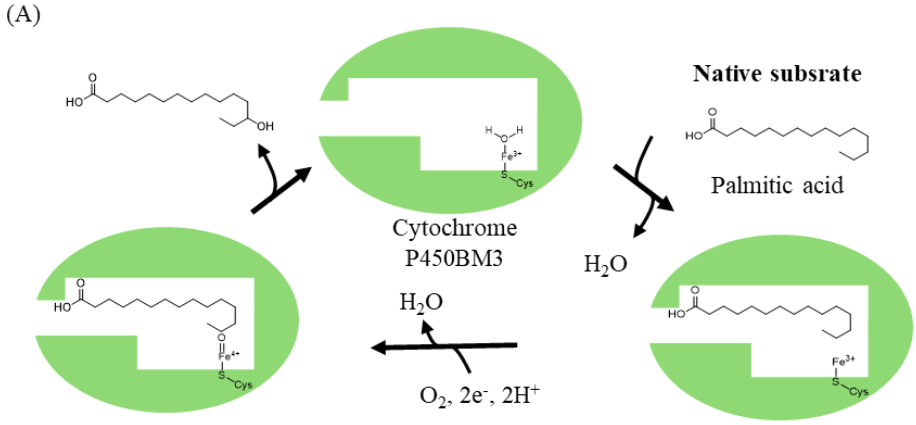
Drawing on this analogy, I propose the hypothesis that the aprotic polyethers used in this study function as decoy molecules in PHA polymerization. In the PHA polymerization system, termination is preferentially induced by CT agents possessing hydroxyl groups, such as DEG, despite the vast excess of water present in the medium. This suggests that DEG, which has a larger structure than freely diffusing water molecules, is immobilized within the substrate-binding pocket of PhaC through specific interactions. Consequently, the residence time of DEG near the active center is prolonged, thereby facilitating DEG-mediated chain termination (Figure 2-12C). Similarly, it is hypothesized that DGDM, which bears structural resemblance to DEG,

also binds within the substrate-binding pocket. By occupying the pocket, DGDM may restrict the molecular motion of water molecules, thereby prolonging their residence time near the active center. (Figure 2-12D).

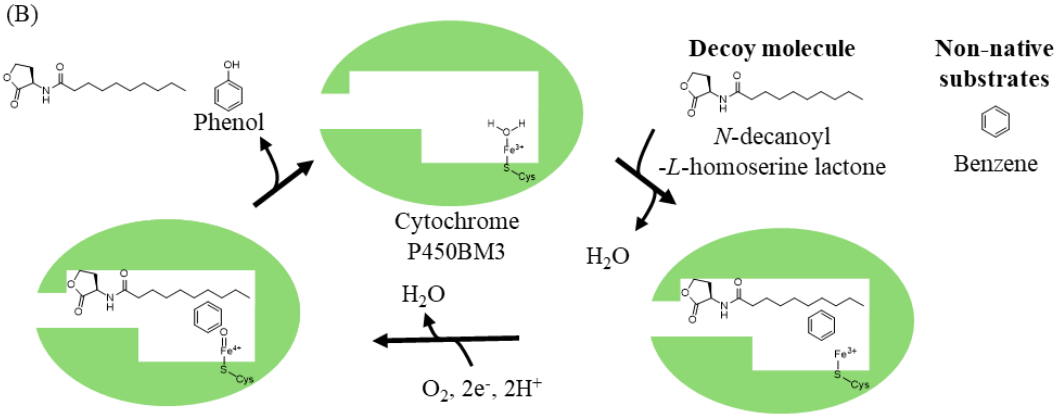
From an application standpoint, the findings of this study offer a novel strategy for controlling PHA properties. Conventionally, CT agents are used to downregulate molecular weight; for example, DEG-derived PHA oligomers are utilized as building blocks for polyurethane synthesis[16]. Diol-type CT agents introduce hydroxyl groups at the polymer terminus, resulting in oligomers with bifunctional hydroxy ends. In contrast, the use of aprotic polyethers enables molecular weight reduction without altering the terminal structure. Therefore, aprotic polyethers can be utilized to downregulate the molecular weight of PHAs without introducing unintended terminal modifications.

The mechanism determining molecular weight remains one of the key unknowns in PHA biosynthesis. The termination of polymerization is generally thought to be triggered by the interaction between PhaCs and CT agents. In this regard, this study provides significant clues, specifically the proposal of a secondary mechanism for CT agents and the varied effects of aprotic polyethers depending on the PhaC class. These insights are expected to contribute to a deeper understanding of the PHA synthesis mechanism at the molecular level.

(A)


Native subsrate
Palmitic acid
Cytochrome
P450BM3
H_2O
H_2O
O_2, $2e^-$, $2H^+$

(B)


Phenol
Decoy molecule
N-decanoyl
-L-homoserine lactone
Non-native
substrates
Benzene
Cytochrome
P450BM3
H_2O
H_2O
O_2, $2e^-$, $2H^+$

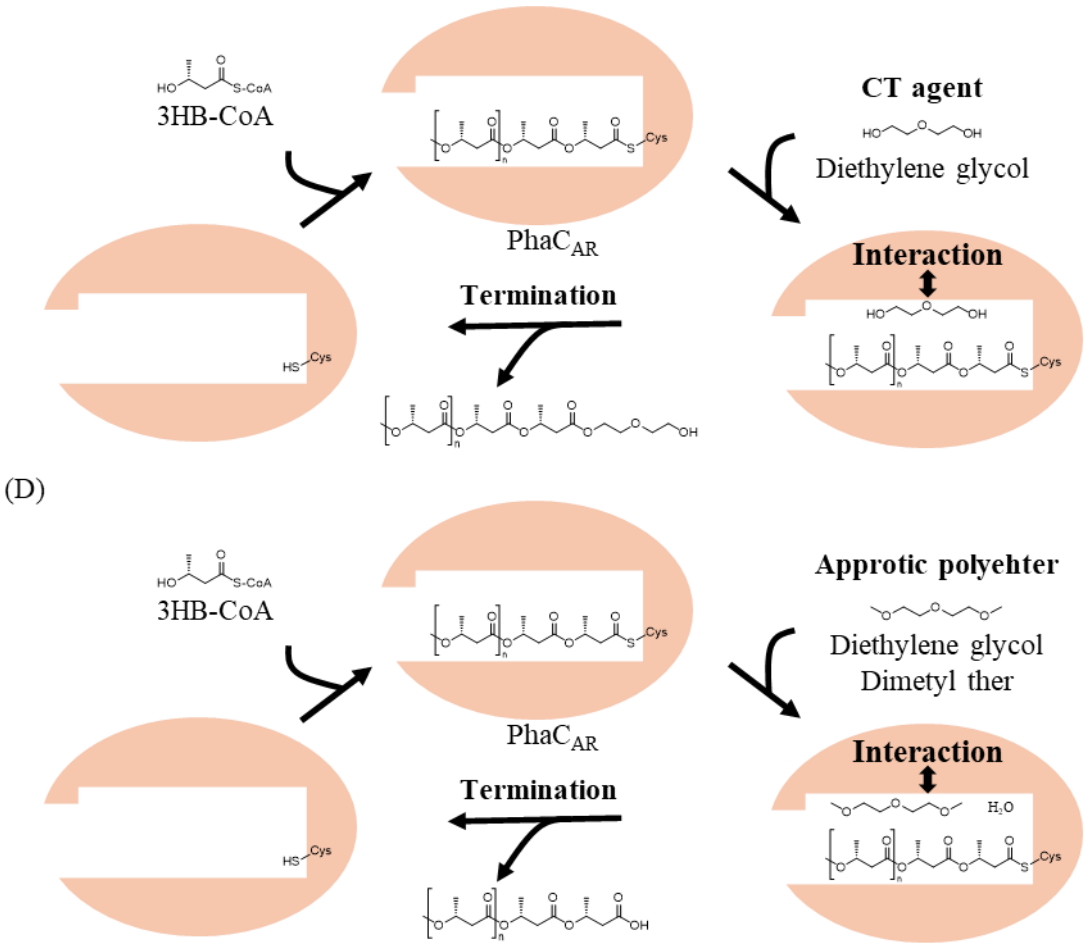


Figure 2-12. Proposed models of action of aprotic polyethers in chain-termination step of PHA synthesis. (A) Catalytic cycle for the hydroxylation of natural substrates by P450BM3. (B) Catalytic cycle for the hydroxylation of the non-natural substrate benzene by P450BM3 in the presence of decoy molecules. (C) Proposed catalytic cycle for PHA chain termination mediated by the hydroxyl group of DEG. (D) Proposed catalytic cycle for PHA chain termination by aprotic polyethers structurally similar to DEG.

3.3. Reference

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10, 829134. https://doi.org/10.3389/fbioe.2022.829134.

Chapter 3

Effect of 18-crown-6 ether on the molecular weight of polyhydroxyalkanoates

3.1. Introduction

PHAs possessing a weight-average molecular weight (M_w) exceeding 3×10^6 are defined as ultra-high-molecular-weight polymers and are characterized by a high Young's modulus[1]. Conversely, oligomeric PHAs with molecular weights on the order of 10^3 have been utilized as building blocks for polyurethane synthesis[2]. Thus, controlling the molecular weight is essential for tailoring the material properties of PHAs.

To downregulate the molecular weight of PHAs, the use of chain-transfer (CT) agents is an established and effective strategy. CT agents are typically alcohols that bind to the carboxyl terminus of the PHA chain; their addition to the culture medium is known to reduce the molecular weight via a transesterification reaction[3–5].

In contrast, methodologies for increasing the molecular weight are currently limited. The synthesis of high-molecular-weight poly(3-hydroxybutyrate) [P(3HB)] has primarily been achieved through genetic engineering strategies, such as the reduction of intracellular ethanol levels, modulation of PhaC expression, and disruption of P(3HB) degradation pathways. For instance, *Escherichia coli* XL1-Blue expressing PHA biosynthetic genes from *Cupriavidus necator* H16 has been reported to produce ultra-high-molecular-weight P(3HB)[6]. Additionally, the gene order within the PHA synthesis operon is crucial for determining both the molecular weight and the production yield of P(3HB)[7]. Furthermore, it has been demonstrated that a nitrogen-fixing bacterium with a deleted P(3HB) depolymerase gene produces a polymer with a higher molecular weight compared to the wild-type strain[8,9].

In Chapter 2, I reported that the addition of aprotic polyethers, such as diethylene

glycol dimethyl ether, to the culture medium of P(3HB)-producing *E. coli* resulted in a decrease in the molecular weight of the synthesized P(3HB)[10]. In those experiments, *E. coli* expressing an engineered PHA synthase ($PhaC_{AR}$) and propionyl-CoA transferase was cultivated in a medium supplemented with 3HB as a monomer precursor[11]. Since aprotic polyethers lack hydroxyl groups and therefore cannot function as CT agents, this finding implies that polyether compounds influence PHA synthesis through a mechanism distinct from the conventional CT reaction.

Prompted by this observation, I explored the relationship between the chemical structures of aprotic polyethers and their effects on PHA molecular weight. In the course of this investigation, we unexpectedly discovered that the addition of a cyclic polyether, 18-crown-6 ether (18-crown-6), to the culture of P(3HB)-producing *E. coli* increased the molecular weight of the polymer. To date, there are no reports of exogenous additives capable of increasing the molecular weight of PHAs.

Consequently, the objective of this study was to investigate the effect of 18-crown-6 on the molecular weight of PHAs and to elucidate the underlying mechanism by examining its impact on the expression level and enzymatic activity of PHA synthase.

3.2. Materials and methods

3.2.1 Bacterial strains and plasmids

Escherichia coli JM109 served as the host strain for polymer production. For the biosynthesis of P(3HB), cells were transformed with the plasmid $pBSP_{Re}phaC_{AR}$(opt)pctalkK[12]. This plasmid carries the propionyl-CoA transferase gene (*pct*) from *Megasphaera elsdenii* and the medium-chain-length hydroxyalkanoate-CoA ligase gene (*alkK*) from *Pseudomonas putida*. The $PhaC_{AR}$ gene sequence is partially codon-optimized to enhance expression efficiency in *E. coli*[12]. Furthermore, the mutant plasmid $pBSP_{Re}phaC_{AR}$N149DF314H(opt)pctalkK (NDFH mutant) was employed specifically for the synthesis of P(3HP) and P(3HHx).

For *in vitro* enzymatic assays, the recombinant $PhaC_{AR}$ protein, fused to an N-terminal His6-tag, was expressed using the pQE30-$phaC_{AR}$ plasmid, as described previously[13].

3.2.1. Culture conditions and polymer extraction

Initially, a preculture was established by incubating recombinant *E. coli* cells in 1.5 mL of Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) at 30°C with shaking at 180 rpm for 12 h. One-mL aliquot of this preculture was then used to inoculate 100 mL of fresh LB medium in 500-mL shake flasks. The main culture medium was supplemented with 20 g/L glucose, 100 μg/mL ampicillin, and 2.5 g/L of sodium (*R/S*)-3HB as the monomer precursor. For the production of P(3HHx) and P(3HP), (*R/S*)-3HHx and/or 3HP were used as precursors in place of 3HB. Cyclic polyethers (Figure 1) were added to the medium at final concentrations of 5, 10, and 20 mM.

The main cultures were incubated at 30°C with reciprocal shaking at 120 rpm for 48 h. For time-course analysis, samples were withdrawn at specific time points (9, 12, 15, 18, 24, 36, and 48 h). After cultivation, cells were collected by centrifugation (5,000 × *g*, 4°C, 10 min), rinsed twice with ultrapure water, and lyophilized. To extract the accumulated polymers, the lyophilized biomass was treated with chloroform at 60°C for 48 h. The extracted polymers were subsequently purified by precipitation using an excess amount of ethanol.

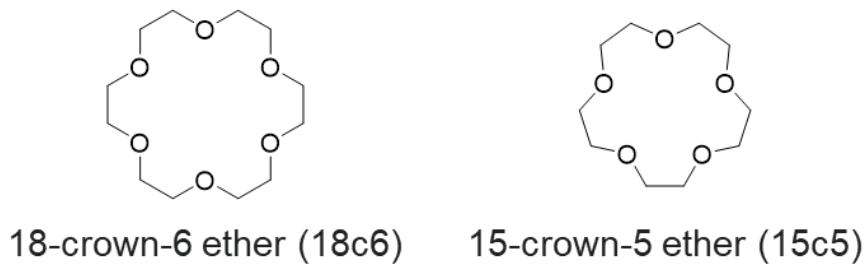


Figure 3-1. Cyclic polyethers used in this study.

3.2.2. ^{1}H NMR analysis

For NMR sample preparation, polymer samples (2–5 mg) were dissolved in 1 mL of deuterated chloroform at 60°C. Upon cooling the solution to room temperature (approximately 25°C), insoluble particulates were removed by filtration through a polytetrafluoroethylene (PTFE) filter with a pore size of 0.2 μm. The NMR spectra of the polymers were acquired using a JNM-ECS400 spectrometer (JEOL, Japan). Chemical shift assignments for each polymer were determined according to previously described methods[14–16].

3.2.3. Measurement of polymer molecular weight

For sample preparation, purified polymer was dissolved in chloroform at 60°C to a final concentration of 1 mg/mL. Before analysis, the solutions were filtered through a polytetrafluoroethylene (PTFE) membrane filter with a pore size of 0.2 μm (Advantec Toyo, Japan) to remove insoluble particulates.

The molecular weight distributions of the polymers were determined via size exclusion chromatography (SEC) using a Gel Permeation Chromatography (GPC) system. The system was equipped with two tandem K-807L columns (Shodex, Japan). The chromatographic analysis was conducted under the following operating conditions: mobile phase, chloroform; flow rate, 0.8 mL/min; column oven temperature, 40°C; and sample injection volume, 100 μL. Molecular weights were calculated based on a calibration curve generated using commercially available polystyrene standards (Shodex) with peak molecular weights (M_p) ranging from 1,180 to 3,210,000 g/mol. All molecular weight values are reported relative to these polystyrene standards.

3.2.4. Measurement of polymer production

Polymer content was determined by converting the intracellular polyesters into ethyl esters via acid ethanolysis. Approximately 10 mg of lyophilized cells were treated with 500 μL of chloroform, followed by the addition of 0.5 mL of a 15% (v/v) sulfuric acid/ethanol solution. The mixture was heated at 100°C for 2 h to facilitate the reaction. After cooling, 4 mL of ultrapure water was added to the mixture and stirred to induce phase separation. The lower organic layer containing the ethyl esters recovered by centrifugation at 1,000 × *g* and 4°C for 1 min and subsequently dehydrated using dried molecular sieves for 30 min.

The resulting 3-hydroxybutyrate (3HB) ethyl esters were analyzed using a Shimadzu GC-2030 gas chromatography (GC) system (Shimadzu, Japan). The system was equipped with a Flame Ionization Detector (FID) and an InertCap1 capillary column (0.25 mm I.D. × 30 m, film thickness 0.25 μm; GL Sciences, Japan).

The GC analysis was conducted under the following temperature program: the column oven temperature was initially held at 45°C, then ramped to 230°C at a rate of 2°C/min, and finally maintained at 230°C for 10 min. Both the injector and detector temperatures were set to 250°C.

3.2.4. *In vitro* assay of PHA synthesis

To evaluate the effect of 18c6 on enzymatic activity, *in vitro* polymerization assays were performed. His_6-tagged $PhaC_{AR}$ was purified using a Ni-NTA column as described previously[13]. The standard reaction mixture contained 5 mM ammonium acetate (pH 7.0), 200 μM (*R*)-3HB-CoA, and varying concentrations of 18c6 (200, 500, or 1,000 μM). The reaction was initiated by the addition of $PhaC_{AR}$ (100 nM) to the mixture (200 μL), which had been pre-warmed to 30°C.

The reaction was incubated at 30°C for 5 min. During this period, 20-μL aliquots were sampled periodically and mixed with an equal volume of 1% trichloroacetic acid to quench the reaction. After filtration (0.2 μm pore size), the samples were subjected to LC-MS analysis using a Shimadzu LCMS-2020 system equipped with a Mastro C18 column and a single quadrupole mass spectrometer with electrospray ionization[13]. Substrate quantification was based on the detection of the deprotonated $[M-H]^-$ ion of 3HB-CoA at m/z 852.2 (retention time: 11.8 min).

3.2.5. Immunoblot analysis

Transformed *E. coli* cells were cultured without precursors (30°C, 12 h) and harvested. The cells were resuspended in lysis buffer (20 mM HEPES, 10% glycerol, 300 mM NaCl; pH 7.5) and subjected to ultrasonication for 10–20 min. After centrifugation (15,000 × *g*, 4°C, 3 min), the supernatant was collected as the crude extract. Protein concentrations were quantified using the Bradford assay (Bio-Rad, USA).

Before electrophoresis, protein samples were normalized to the same concentration, mixed with 4×sanple buffer (200 mM Tris-HCl pH 6.8, 8% SDS, 0.4% bromophenol blue, 40% glycerol, 20% 2-mercaptoethanol), and boiled at 95°C for 5 min. Samples (2 μg total protein) were resolved by SDS-PAGE on a 12% acrylamide gel, with Precision Plus Protein All Blue Prestained Protein Standards (Bio-Rad) used as markers. Proteins were transferred to a PVDF membrane using the iBlot™ system (Invitrogen, USA). Immunoblotting was conducted as described previously[10], utilizing Anti-Rabbit IgG, HRP-linked Whole Ab Donkey (Cytiva, Japan) as the secondary antibody. Signals were detected using ECL Prime reagents (Cytiva). Band intensities were quantified using the lane profiling tool in ImageJ, and the data were normalized against the intensity of the control sample (0 mM).

3.3. Result

3.3.1. Culture results of *E. coli* expressing $PhaC_{AR}$ under crown ether conditions

The effects of crown ether supplementation on polymer synthesis were evaluated, specifically comparing 18c6 and 15c5 to determine the impact of ring structure (**Table 1**). In the presence of 18c6 (5–20 mM), inhibition of cell growth and a reduction in P(3HB) production were recorded. Conversely, the inhibitory effects of 15c5 on cell growth and polymer production were minimal.

A significant difference was observed in the molecular weight profiles. While the molecular weight of P(3HB) slightly decreased with increasing concentrations of 15c5 (Figure 2A), the addition of 18c6 resulted in an unexpected increase in the molecular weight of the obtained P(3HB) (Figure 2B). Based on these observations, 18c6 was selected for further detailed analysis to elucidate its mechanism of action on PHA synthesis.

Table 3-1. P(3HB) production with supplementation of crown ethers in *E. coli* expressing $PhaC_{AR}$

Crown ether	Concentration (mM)	Cell Dry Weight (g/L)	P(3HB) production (g/L)	Molecular weight		
				M_n ($\times 10^5$)	M_w ($\times 10^5$)	M_w/M_n
-	0	4.56±0.32	0.64±0.02	0.98±0.05	1.91±0.03	1.95±0.06
18c6	5	3.26±0.03	0.61±0.04	1.26±0.02	2.54±0.03	2.01±0.01
	10	1.76±0.15	0.23±0.16	2.16±0.10	6.57±0.46	3.04±0.08
	20	0.75±0.08	0.14±0.02	3.98±0.64	19.31±1.24	4.90±0.50
15c5	5	3.65±0.25	0.51±0.05	0.93±0.01	1.69±0.01	1.81±0.03
	10	3.45±0.04	0.50±0.01	0.51±0.07	1.42±0.01	1.88±0.18
	20	3.35±0.17	0.48±0.10	0.67±0.11	1.19±0.03	1.79±0.14

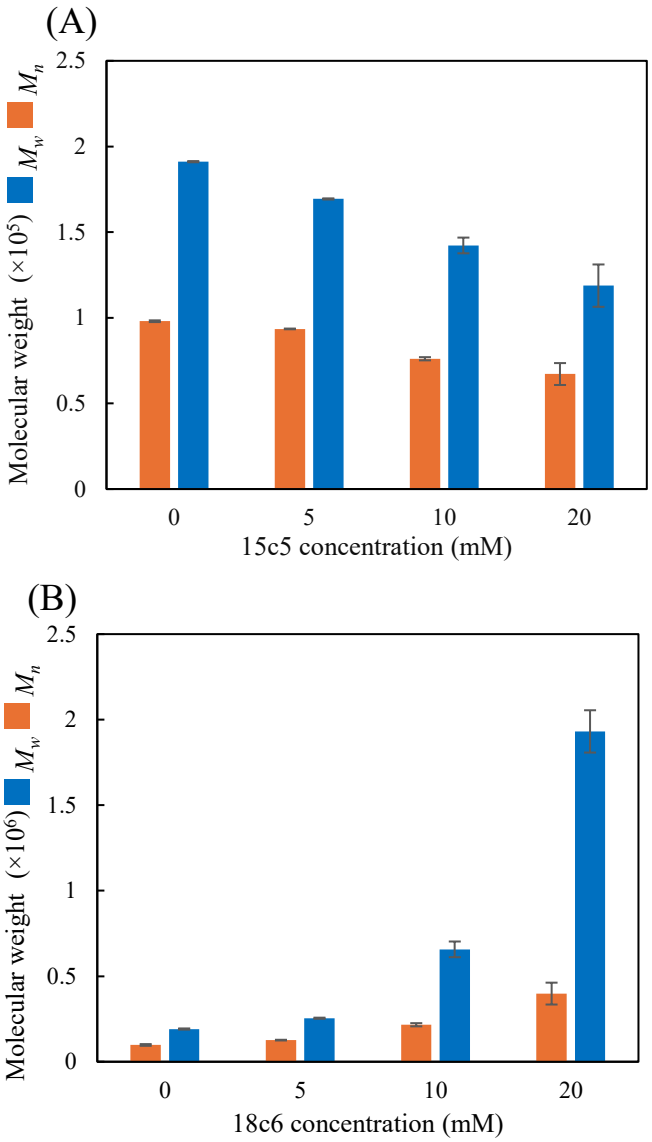


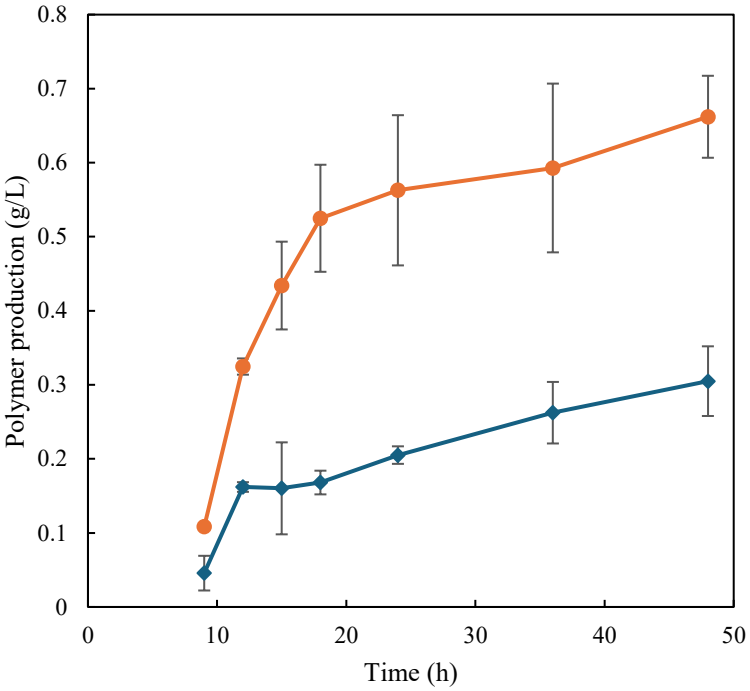
Figure 3-2. Effect of crown ethers on molecular weight of the P(3HB) synthesized in *E. coli*. Varied concentrations of 15c5 (A) and 158c6 (B) were added to the medium. The *orange bars* indicate the number-average molecular weight (M_n), and *blue bars* indicate the weight-average molecular weight (M_w). Data represent the mean ± standard deviation of three independent experiments.

3.3.2. Time-purifiers measurement of P(3HB) molecular weight in the presence of 18-crown-6

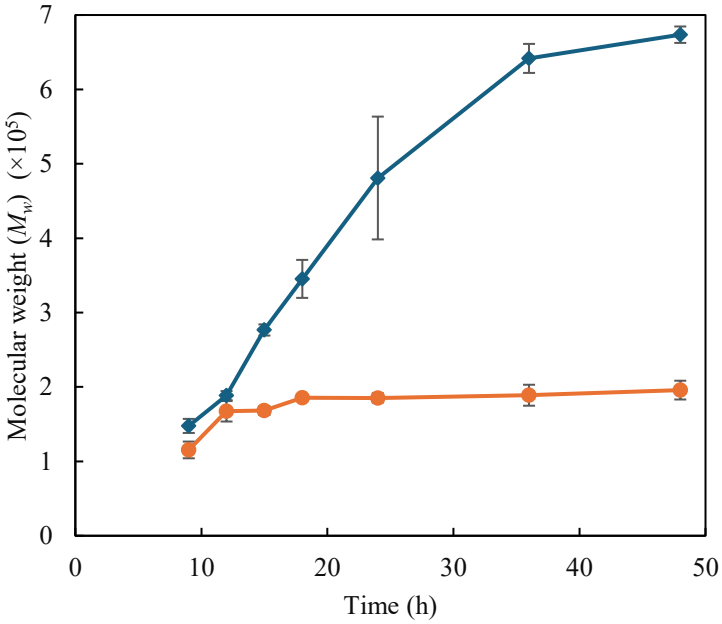
Molecular weight of P(3HB) evolution was evaluated in *E. coli* (Figure 3-3). Rapid polymer accumulation occurred from 9 to 18 h in all cultures, after which the production rate declined (Figure 3-3A). While 18c6 addition reduced the total polymer yield, it induced a unique molecular weight profile. Unlike the control condition, where the M_w plateaued at 18 h, the 18c6-supplemented condition showed a continuous increase in M_w until the end of cultivation (Figure 3-3B). Typically, PHA molecular weight remains constant because chain propagation is extremely rapid relative to the cultivation time[1,17]

To determine if the anomalous increase in M_w under 18c6 supplementation resulted from prolonged chain elongation, the molecular weight distributions were analyzed. If chains were continuously elongating, the low-molecular-weight fraction should diminish over time. However, the SEC traces demonstrated that the low-molecular-weight fraction persisted without significant reduction (Figures 3-3C and 3-3D). Thus, the increase in molecular weight is likely attributed to the synthesis of new, high-molecular-weight polymer chains during the late cultivation stage, rather than the continued growth of pre-existing chains.

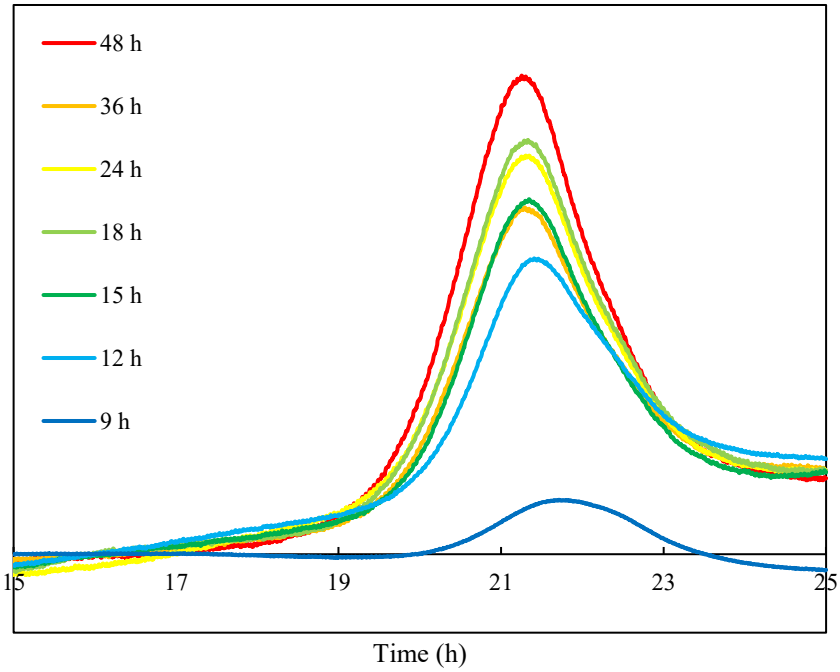
(A)


Polymer production (g/L)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
Time (h)

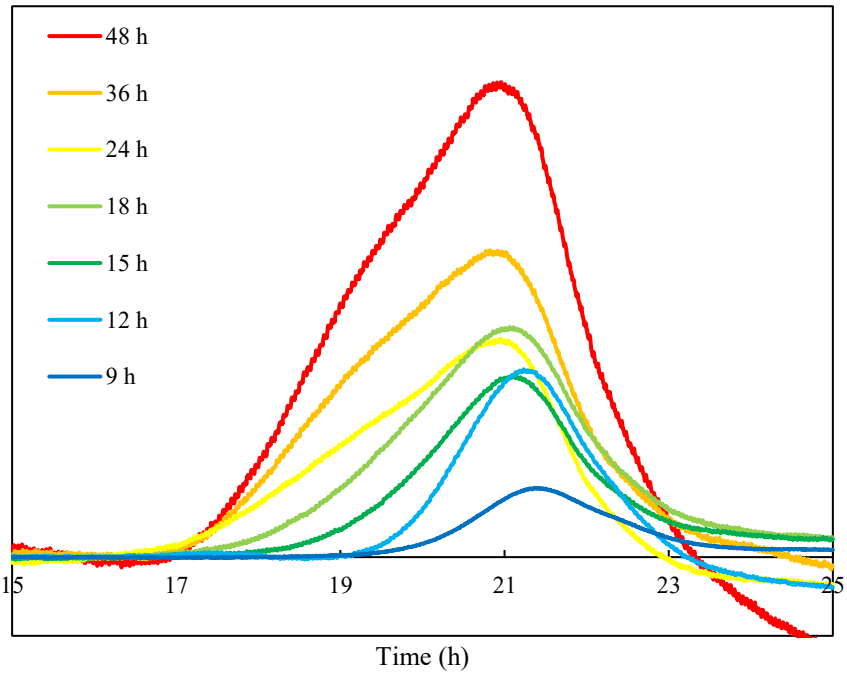
(B)


Molecular weight (M_w) ($\times 10^5$)
7
6
5
4
3
2
1
0
0
10
20
30
40
50
Time (h)

(C)


48 h
36 h
24 h
18 h
15 h
12 h
9 h
15
17
19
21
23
25
Time (h)

(D)


48 h
36 h
24 h
18 h
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12 h
9 h
15
17
19
21
23
25
Time (h)

Figure3-3. Time course analysis of P(3HB) production (A) and their weight-averaged molecular weight (B) with and without 18c6. 18c6 concentrations: *orange circle*, 0 mM; *blue diamond*, 10 mM. Data represent the mean ± standard deviation of three independent experiments. Time-profile of SEC trace of the polymers synthesized without (C) and with (D) supplementation of 18c6.

Ethanol produced endogenously by *E. coli* is known to induce chain termination during PHA polymerization. Consequently, the amount of ethanol released into the culture supernatant was quantified during the cultivation (Figure 2-4A). The results indicated that the ethanol concentration increased in a time-dependent manner throughout the cultivation period. Notably, ethanol accumulation was lower under the 18c6-supplemented conditions compared to the control (no addition). However, when normalized to CDW, the specific ethanol production remained substantially constant, independent of 18c6 supplementation (Figure 2-4B). This suggests that the observed reduction in total ethanol production is attributable to the decrease in cell biomass rather than a specific inhibition of the ethanol biosynthetic pathway.

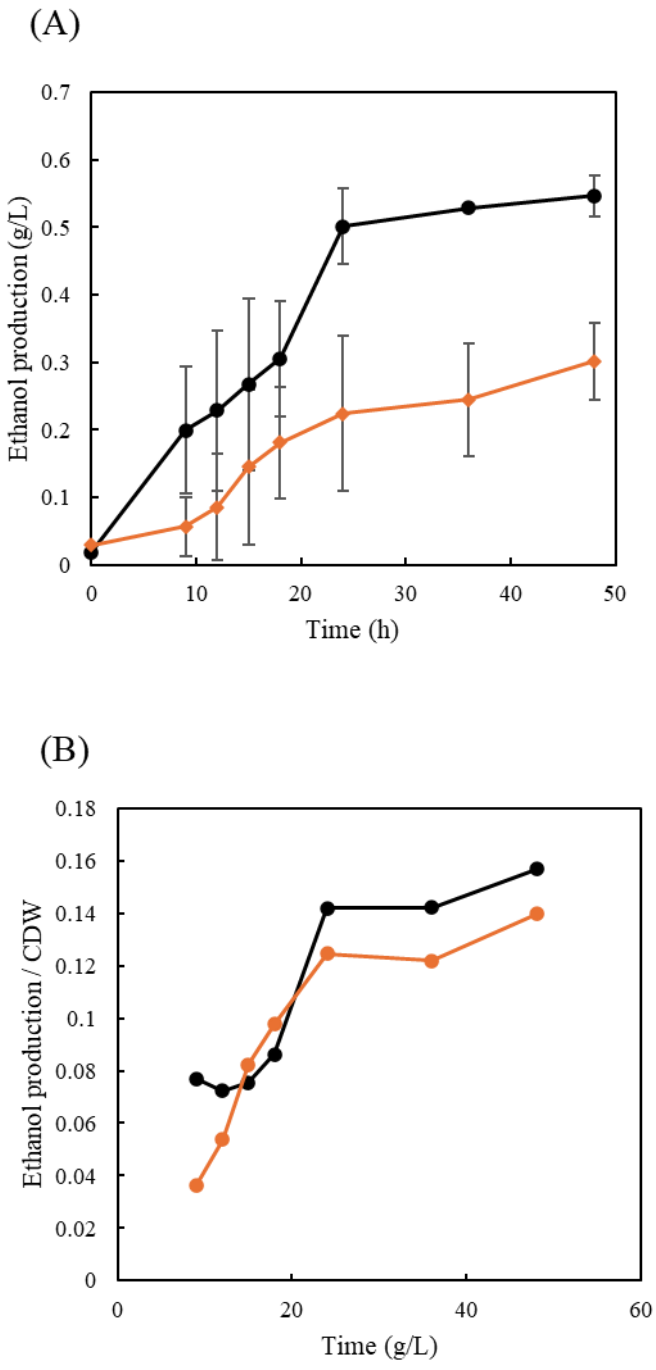

Figure3-4. Ethanol concentration in the culture medium during P(3HB) production by PhaC$_{AR}$-expressing *E. coli* in the presence or absence of 18c6 (A) and ethanol concentration normalized to CDW (B). Data represent the mean ± SD of three independent experiments. *Black circle*, no 18c6; *orange diamond*, 10 mM 18c6. Data represent the mean ± standard deviation of three independent experiments.

3.3.3. Effect of 18-crown-6 on $PhaC_{AR}$ expression levels

Previous studies have proposed that the expression level of PHA synthase significantly influences the molecular weight of the synthesized polymer[7]. To determine whether the observed increase in molecular weight was mediated by changes in enzyme concentration, immunoblot analysis was performed to evaluate the effect of 18c6 on $PhaC_{AR}$ expression (Figure 3-4).

For this analysis, cells were cultured in the absence of substrate precursors. This condition was chosen to prevent the adsorption of PHA synthase onto polymer inclusions, thereby facilitating the recovery of $PhaC_{AR}$ in the soluble fraction.
The results indicated that the expression level of $PhaC_{AR}$ decreased slightly in an 18c6 concentration-dependent manner. However, this marginal reduction in expression was insufficient to account for the substantial increase in P(3HB) molecular weight. Therefore, it is concluded that the molecular weight enhancement induced by 18c6 is unlikely to be caused by the downregulation of PHA synthase expression.

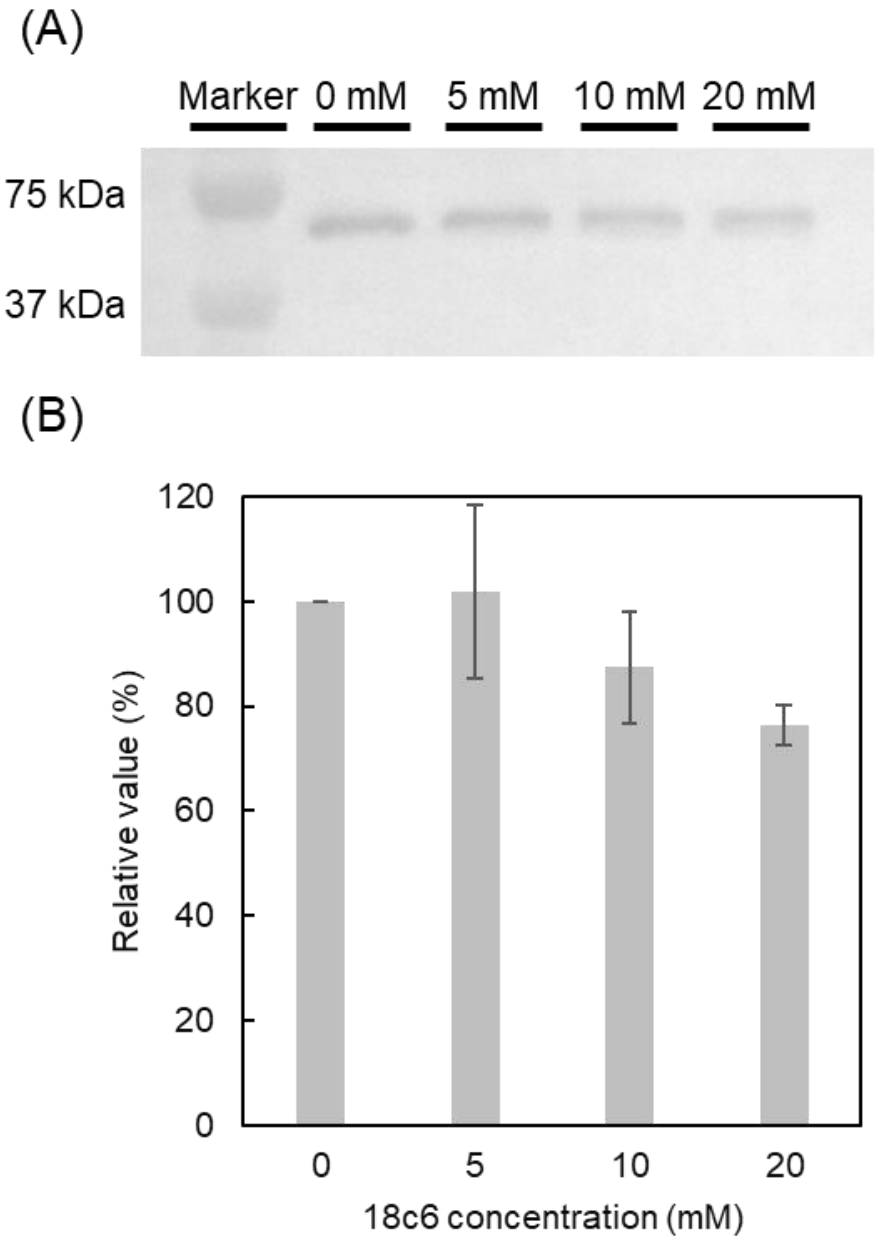

Figure.3-4. Immunoblot analysis of $PhaC_{AR}$ expressed in *E. coli* (A) and quantification of the relative expression levels (B). The molecular weight of $PhaC_{AR}$ is approximately 66 kDa. The values are presented as the mean ± SD of the biological triplicates.

3.3.4. Measurement of $PhaC_{AR}$ activity in the presence of 18-crown-6

To investigate the hypothesis that the observed increase in molecular weight resulted from a direct interaction between 18c6 and PHA synthase, in vitro assays were conducted using purified $PhaC_{AR}$. First, the effect of 18c6 on the kinetic activity of $PhaC_{AR}$ was evaluated. The results demonstrated that the addition of 18c6 did not significantly alter the substrate consumption rate (Figure 2-5A). This suggests that the molecular weight increase is unlikely to be attributable to a modulation of the enzymatic reaction rate by 18c6. Subsequently, the molecular weight of the P(3HB) synthesized in vitro was determined. As shown in Figure 2-5B, no significant difference in molecular weight was observed between the presence and absence of 18c6. It has been proposed that, unlike in vivo synthesis, no chain termination process occurs during in vitro PHA synthesis [24]. Under this premise, the molecular weight of the polymer is determined primarily by the ratio of substrate to the number of PHA synthase molecules actively engaged in polymerization. Given the lack of change in molecular weight in the results, it is suggested that the presence of 18c6 does not reduce the number of active PhaC molecules under the employed in vitro conditions. Therefore, the mechanism driving the molecular weight increase in vivo remains to be fully elucidated and requires further investigation.

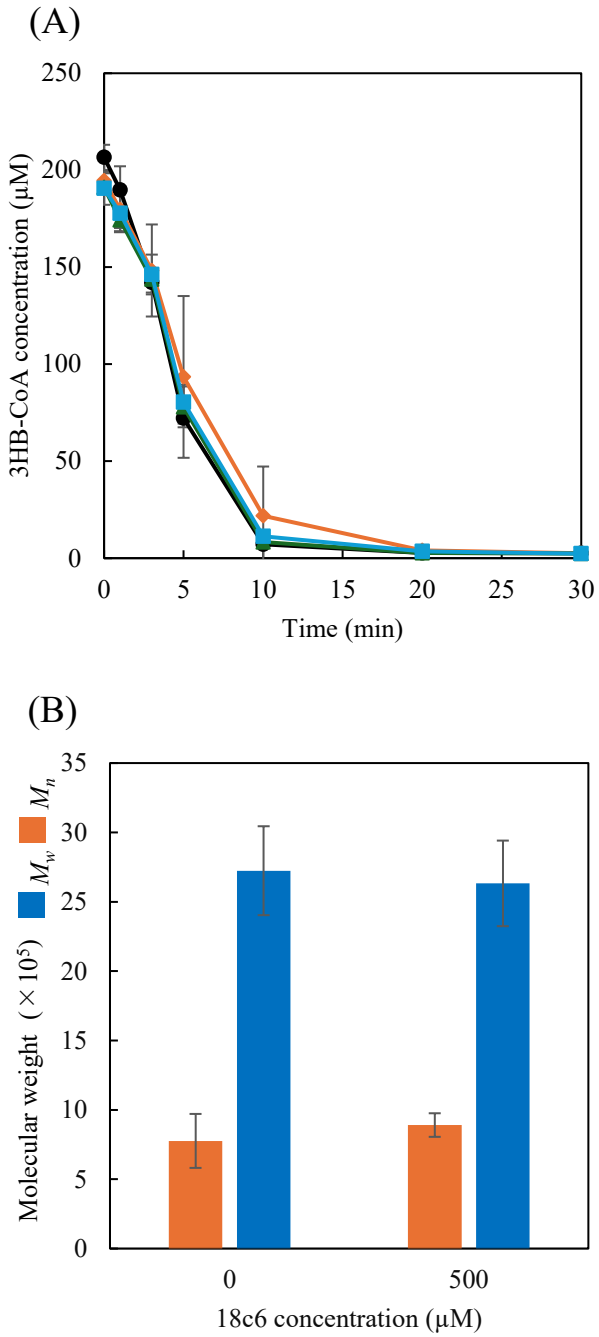

Figure.3-4. *In vitro* assay of PhaC$_{AR}$ with the addition of 18c6. (A) Time course of 3HB-CoA concentration (A) and molecular weight of P(3HB) synthesized using purified PhaC$_{AR}$ (B). 18c6 concentrations: *black*, 0 μM; *orange diamond*, 200 μM; *green triangle*, 500 μM; *blue square*, 1000 μM. Molecular weight: *orange bar*, M_n; *blue bar*, M_w. Data represent the mean ± SD of three independent experiments.

3.3.5. Effect of potassium ion complexation by 18c6

18c6 is known for its ability to specifically encapsulate potassium ion, K^+, within its cavity. Therefore, we investigated whether the complexation of 18c6 with potassium ions influences its ability to enhance molecular weight.

To evaluate the effect of potassium concentration, varying concentrations of KCl (5–100 mM) were added to the culture medium in the presence of 10 mM 18c6. The addition of exogenous KCl alone had a negligible effect on the molecular weight of P(3HB), which remained substantially constant (Figure 3-5A). However, under the conditions supplemented with 18c6, the molecular weight-enhancing effect of 18c6 diminished as the KCl concentration increased (Figure 3-5B). These results suggest that the intracellular ion balance maintained by the host strain may be involved in the polymerization process of PHA biosynthesis.

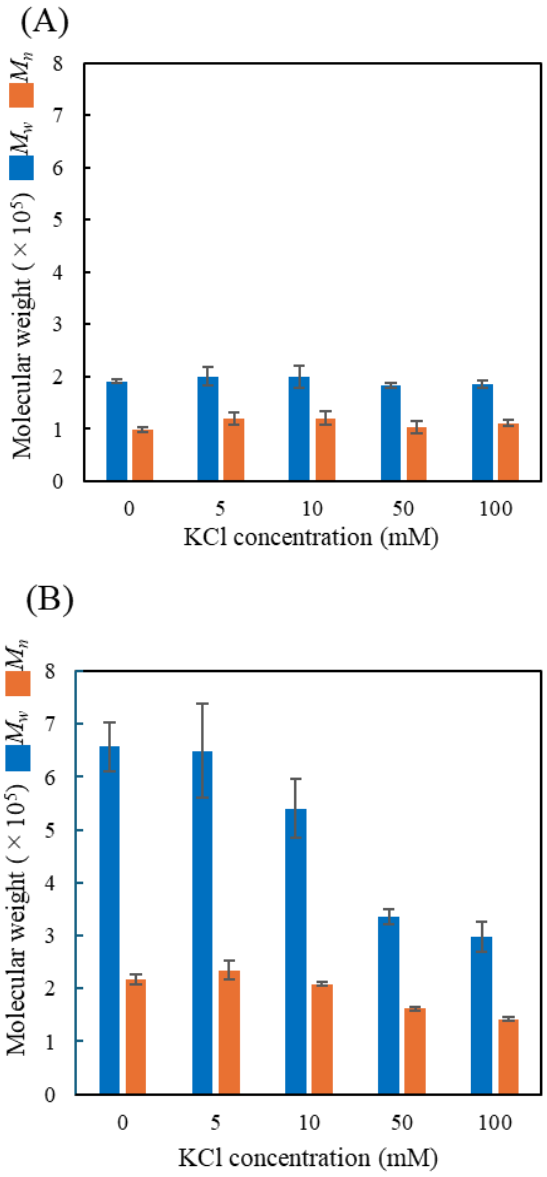

Figure 3-5. Attenuation of 18c6-induced molecular weight enhancement by KCl addition. (A) In the absence of 18c6 and (B) In the presence of 10 mM 18c6. The *blue bars* indicate the weight-average molecular weight (M_w), and *orange bars* indicate the number-average molecular weight (M_n). Data represent the mean ± standard deviation of three independent experiments.

3.3.6. Effect of 18-crown-6 on other PHA homopolymers

I investigated whether the addition of 18c6 increases the molecular weight of polymers other than P(3HB). Specifically, poly(3-hydroxypropionate) [P(3HP)] and poly(3-hydroxyhexanoate) [P(3HHx)] were tested. In this experiment, the mutant synthase $PhaC_{AR}$NDFH was employed for the synthesis of P(3HHx). The synthesis of each homopolymer was confirmed by ^{1}H NMR analysis (Figure 3-6, 3-7, 3-8).
The results indicated that the addition of 18c6 resulted in a slight increase in the M_w of both P(3HB) and P(3HP) (Figure 3-9A, B). However, the magnitude of this increase was lower than the molecular weight enhancement observed for P(3HB) synthesized by $PhaC_{AR}$. In contrast, for P(3HHx), no increase in molecular weight was detected upon the addition of 18c6 (Figure 3-9C). Consequently, these findings demonstrate that the effectiveness of 18c6 varies depending on the specific monomer substrates.

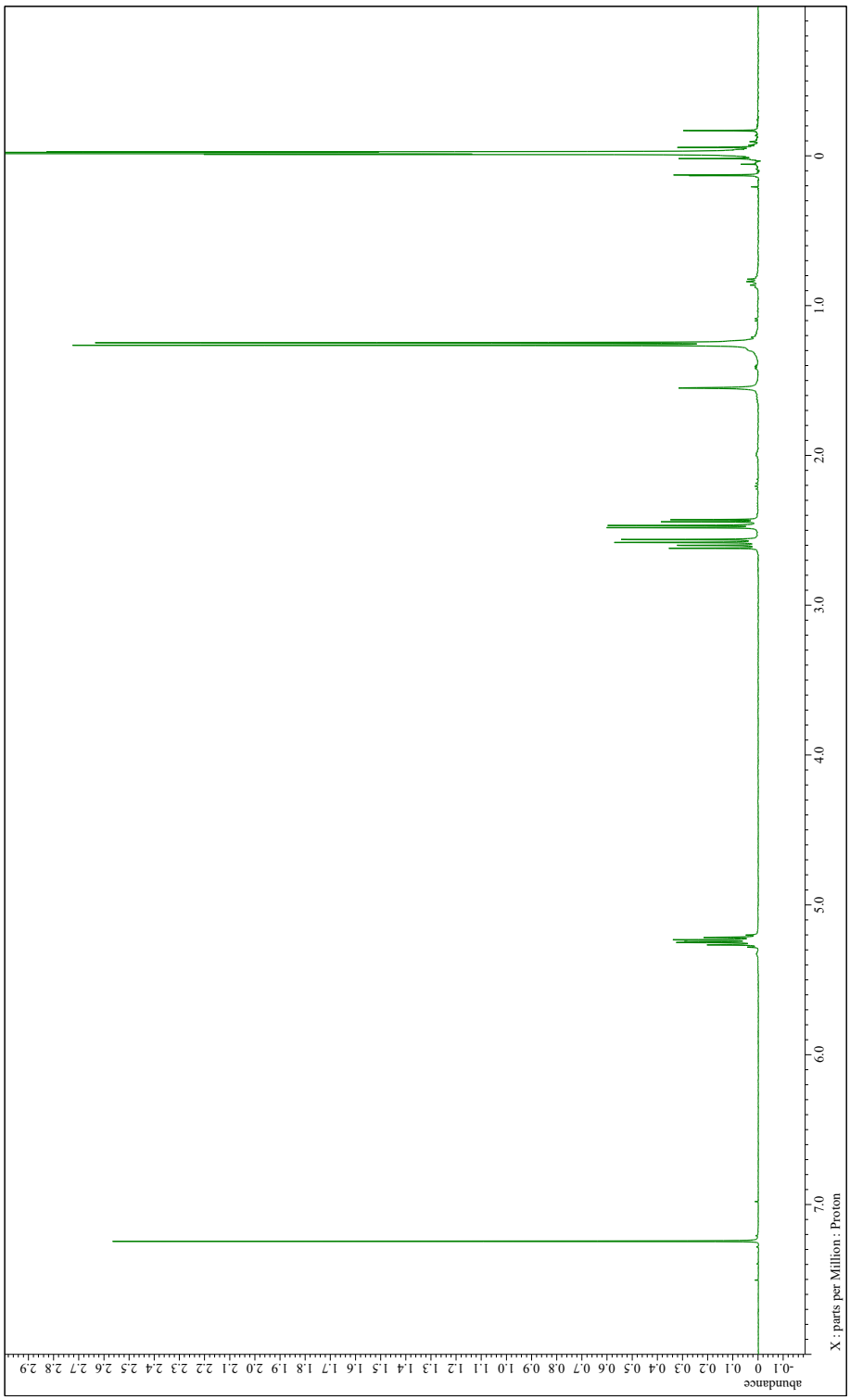

Figure 3-6. ^{1}H NMR of P(3HB) synthesized using PhaC$_{AR}$NDFH.

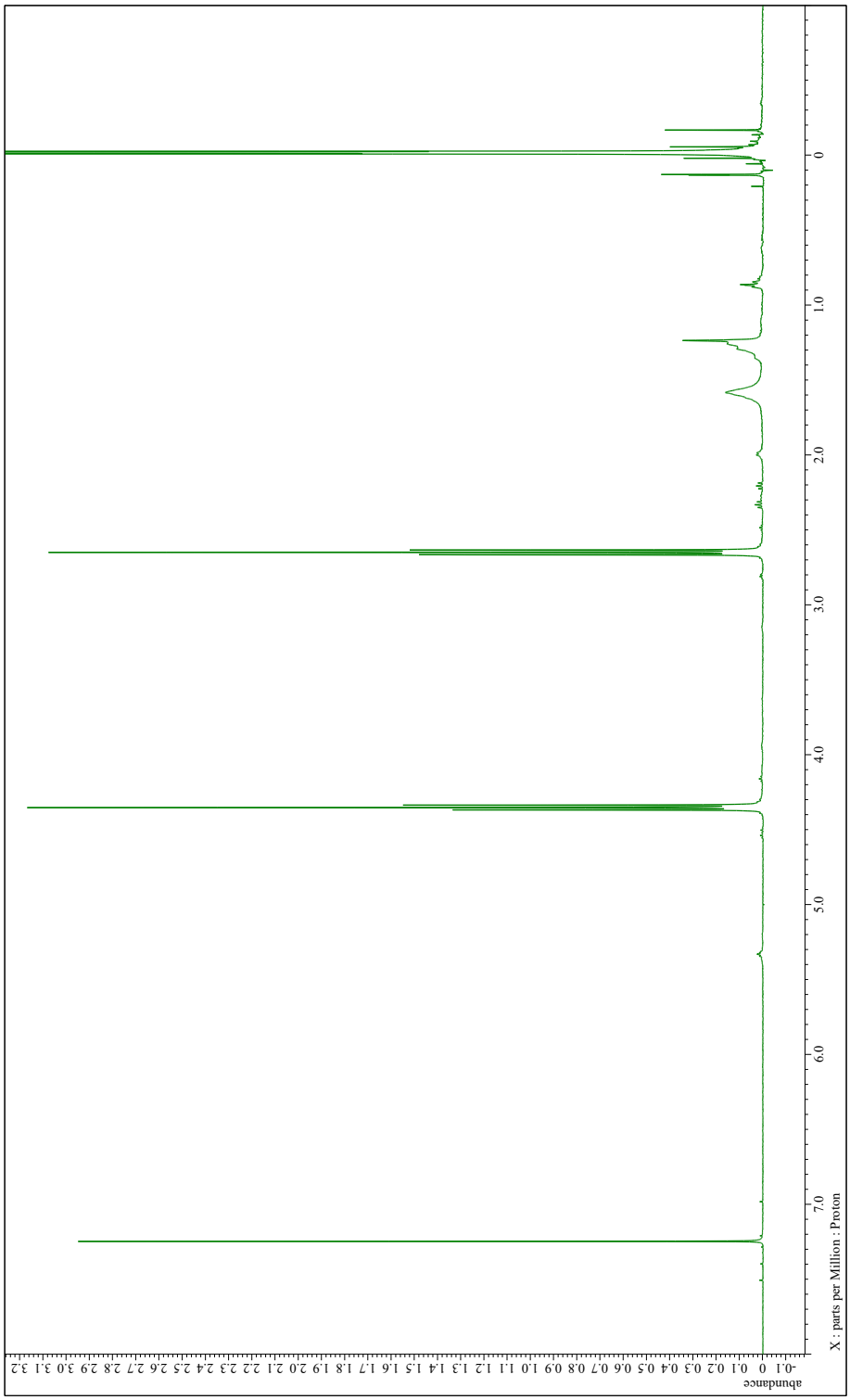

Figure 3-7. ^{1}H NMR of P(3HP) synthesized using PhaC$_{AR}$NDFH

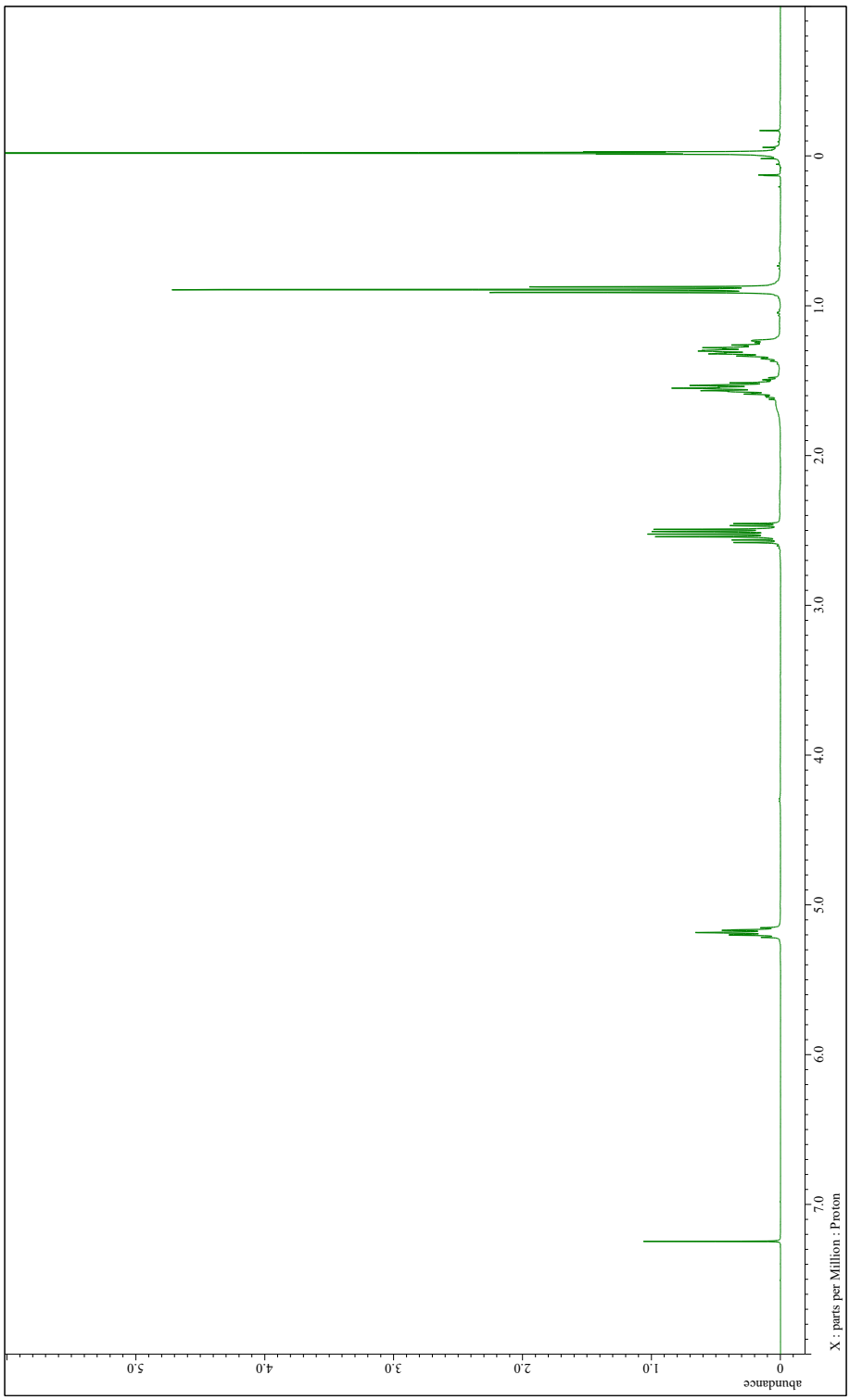

Figure 3-8. ^{1}H NMR of P(3HHx) synthesized using PhaC_{AR}NDFH.

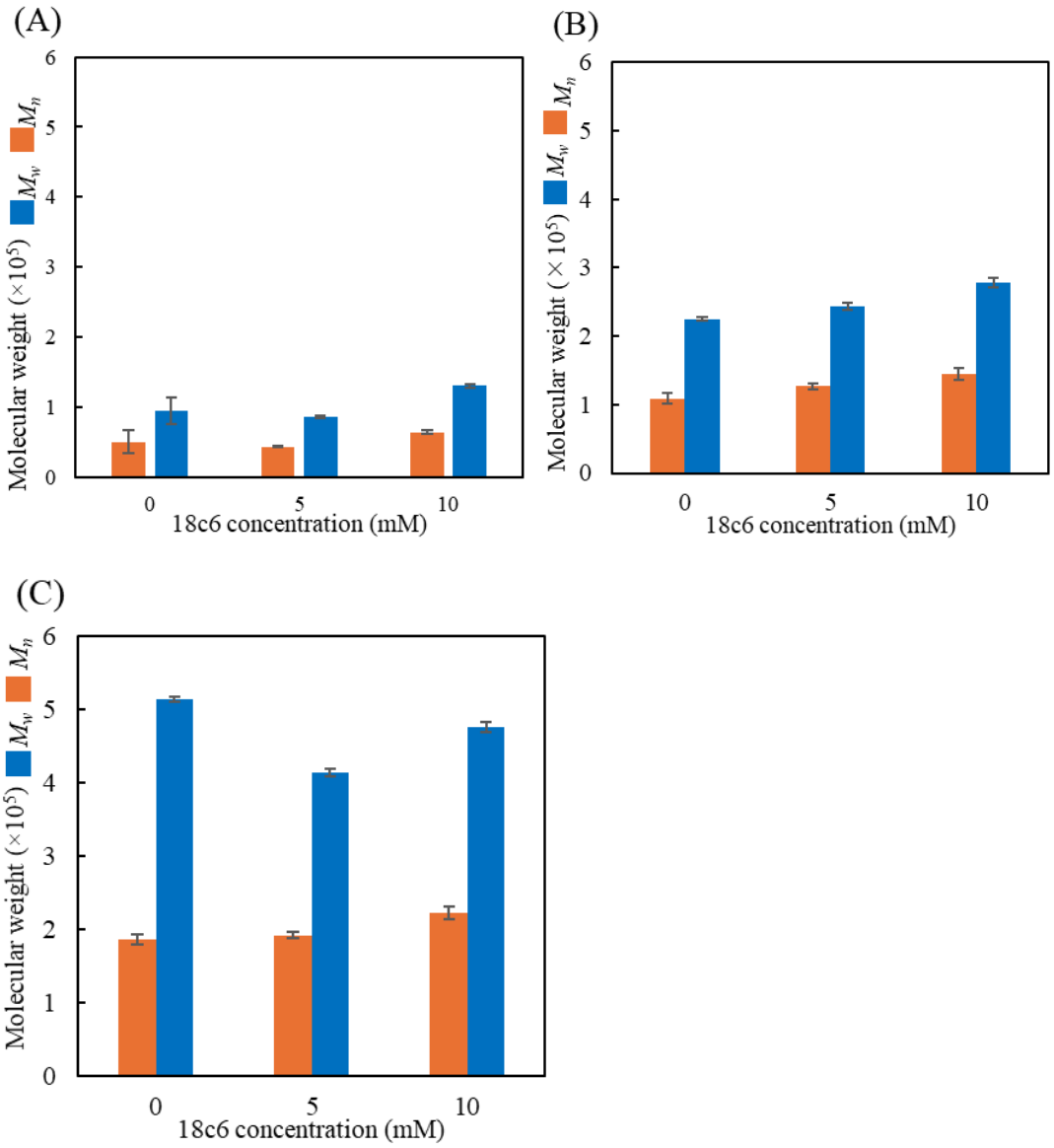

Figure 3-9. Effect of 18c6 on molecular weight of the polymer synthesized in *E. coli* expressing PhaC$_{AR}$NDFH for the synthesis of P(3HB) (A), P(3HP) (B), and P(3HHx) (C). Data represent the mean ± standard deviation of three independent experiments.

3.4. Discussion

In my exploration of the effects of aprotic polyethers on PHA synthesis, we discovered that the addition of 18c6 significantly increases the molecular weight of P(3HB). To the best of my knowledge, this represents the first report of an exogenous culture additive capable of considerably enhancing the molecular weight of PHAs. Consequently, we aimed to elucidate the underlying mechanism of this novel phenomenon.

Previous literature has established that the molecular weight of synthesized P(3HB) is inversely correlated with the expression level of PHA synthase (PhaC). Therefore, a potential mechanism for the M_w increase was that 18c6 might suppress PhaC expression. The analysis confirmed a slight decreasing trend in PhaC expression upon 18c6 addition. However, the observed reduction was marginal (10–20%), which is considered quantitatively insufficient to explain the substantial increase in molecular weight (approximately 10-fold) observed in this study. Thus, the molecular weight enhancement is unlikely to be dependent on the downregulation of PHA synthase expression.

Subsequently, we considered the possibility that 18c6 directly modulates the polymerization activity of PhaC. However, *in vitro* kinetic assays using purified enzyme revealed no significant alterations in activity in the presence of 18c6. Furthermore, 18c6 did not affect the molecular weight of P(3HB) synthesized *in vitro*. These results strongly suggest that the effect of 18c6 is not mediated by a direct action on the PHA synthase molecule itself.

On the other hand, these findings do not preclude the possibility that 18c6 interacts with other enzymes involved in the monomer supply pathway, such as propionyl-CoA

transferase or upstream metabolic enzymes. This remains a potential target for future mechanistic studies.

The versatility of 18c6 was evaluated using different monomers. While 18c6 increased the molecular weight of P(3HB) and exhibited a similar effect on poly(3-hydroxypropionate) [P(3HP)], no such increase was observed for poly(3-hydroxyhexanoate) [P(3HHx)]. These results demonstrate that while 18c6 is applicable to polymers other than P(3HB), its molecular weight-enhancing effect is dependent on the monomer substrate.

The precise reason for this substrate specificity remains to be clarified. Indeed, the fundamental relationship between polymer structure and molecular weight control mechanisms is not yet fully understood. However, as shown in Figure 3-5, the molecular weight-enhancing effect of 18c6 was observed to be attenuated by the encapsulation of potassium ions. This finding suggests that the intracellular ion balance maintained by the host strain may exert an influence on the PHA polymerization process. Further exploration of a broader range of polymer synthetic conditions will be a crucial first step toward the complete elucidation of this phenomenon.

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Chapter 4

Conclusions

This dissertation addresses the development of novel strategies for controlling the molecular weight of polyhydroxyalkanoate (PHA), a class of microbial polyesters, and the elucidation of the underlying mechanisms. The molecular weight of PHA is critical determinants of its physical properties, including tensile strength, processability, and crystallization behavior. Conventionally, molecular weight regulation has relied on methods such as modulating the gene expression levels of PHA synthase (PhaC) or utilizing alcohols as chain transfer (CT) agents. In this study, I demonstrate that the molecular weight of PHA can be controlled broadly and bidirectionally ranging from reduction to enhancement through a facile chemical approach involving the addition of specific compounds to the culture medium. This research provides new insights into the molecular mechanisms governing PHA biosynthesis.

Chapter 2 presents new findings regarding the reduction of PHA molecular weight. Traditionally, it has been believed that compounds possessing hydroxyl groups (such as ethanol and diethylene glycol) are essential for inducing molecular weight reduction via chain transfer reactions. However, I discovered that the addition of aprotic polyethers (e.g., diethylene glycol dimethyl ether), which lack hydroxyl groups, also results in a significant, concentration dependent reduction in the molecular weight of P(3HB). Structural analysis via ^{1}H NMR revealed that these compounds do not bind to the terminal of P(3HB), indicating a mechanism distinct from the classical CT reaction. Furthermore, *in vitro* enzymatic assays and immunoblotting confirmed that these additives affect neither the catalytic activity nor the expression levels of PhaC. Based on these results, I proposed a "decoy molecule" model: aprotic polyethers function as decoy molecules by entering the substrate-binding pocket of the enzyme. By affecting

the behavior or residence time of water molecules near the active center, they indirectly facilitate water-mediated hydrolysis (chain termination). This finding highlights the importance of the steric influence of additives in the PHA polymerization termination reaction.

Chapter 3 explores the mechanism of PHA molecular weight enhancement. During the screening process described in Chapter 2, I found that the addition of 18-crown-6-ether (18c6), a cyclic polyether, resulted in a dramatic increase in the molecular weight of P(3HB). To date, no method has been reported for actively increasing PHA molecular weight using exogenous additives; thus, this represents a notable discovery in the field of PHA biosynthesis research. Since the addition of 18c6 did not significantly affect PhaC expression levels or *in vitro* enzymatic activity, it was inferred that the effect is not due to a direct interaction with the enzyme but rather an indirect effect mediated by the physiological state of the host cell. Specifically, focusing on the ability of 18c6 to specifically encapsulate potassium ions (K^+) within its ring, I conducted experiments with KCl supplementation. The results confirmed that the molecular weight-enhancing effect of 18c6 was negated as the K^+ concentration increased. This suggests that the sequestration of intracellular K^+ or alterations in the ion balance by 18c6 may suppress the frequency of chain termination. This method offers a groundbreaking approach that enables the synthesis of high-molecular-weight PHAs solely by medium supplementation, without the need for genetic engineering.

In summary, this study established two contrasting chemical control strategies: "molecular weight reduction via aprotic polyethers" and "molecular weight enhancement via crown ethers." Furthermore, the insights gained from this research demonstrate that the chain termination and elongation mechanisms in PHA biosynthesis

can be flexibly modulated not only by enzyme-substrate interactions but also by coexisting molecules and the intracellular ionic environment. These findings are expected to serve as new design guidelines for future PHA biosynthetic engineering.

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