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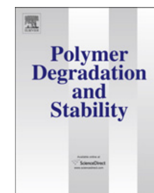

Unusual change in molecular weight of polyhydroxyalkanoate (PHA) during cultivation of PHA-accumulating *Escherichia coli*

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ABSTRACT

This study aimed to investigate the factors affecting molecular weight of poly[(*R*)-3-hydroxybutyrate] [P(3HB)] when polyhydroxyalkanoate (PHA) synthase ($PhaRC_{Bsp}$) from *Bacillus* sp. INT005 was used for P(3HB) synthesis in *Escherichia coli* JM109. It was found that the molecular weight of P(3HB) decreased with time in mid- and late-phase of culture and was strongly affected by culture temperature. At 37 °C culture temperature, the molecular weight of P(3HB) rapidly decreased from 4.4×10^5 to 4.8×10^4 with culture time, whereas it was almost unchanged at 25 °C. Kinetic analysis suggested that the decrease in molecular weight of P(3HB) was due to random scission of the polymer chain. The decrease in molecular weight of P(3HB) was not observed when PHA synthases other than $PhaRC_{Bsp}$ were expressed. This study sheds light on the unique behaviour in molecular weight change of P(3HB) that is synthesized by *E. coli* expressing $PhaRC_{Bsp}$.

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1. Introduction

Polyhydroxyalkanoates (PHAs) are biological polyesters synthesized by a wide range of bacteria, and accumulated as intracellular carbon and energy storage material [1–4]. PHAs continue to attract increasing industrial interest as a renewable, biodegradable and biocompatible thermoplastic that can be used for marine, agricultural, and medical applications [1–4].

A homopolymer of (*R*)-3-hydroxybutyrate [P(3HB)] is the most common type of PHA that bacteria accumulate in nature. P(3HB) is synthesized from acetyl-coenzyme A (acetyl-CoA) by the action of three enzymes as follows: two acetyl-CoA molecules are condensed into acetoacetyl-CoA by 3-ketothiolase (PhaA), and then reduced to (*R*)-3-hydroxybutyryl-CoA (3HB-CoA) by NADPH-dependent acetoacetyl-CoA reductase (PhaB). PHA synthase (PhaC) finally polymerizes the 3HB moiety of 3HB-CoA into P(3HB). The number-average molecular weight (M_n) of P(3HB) produced by native PHA-producing bacteria is usually in the range of $(1–20) \times 10^5$ [1–4].

PHA synthases play a key role in PHA biosynthesis. A number of PHA synthase genes have been isolated from various bacteria so far and grouped into four classes (class I to IV) based on subunit composition and substrate specificity [3,5]. Class I and class II synthases comprise one type of subunit (PhaC), whereas class III and class IV synthases comprise two types of subunits, PhaE and PhaC for PhaEC, and PhaR and PhaC for PhaRC, respectively. Class I, III and IV synthases prefer to polymerize short-chain-length (scl) monomers (C3–C5), whereas class II synthases prefer medium-chain-length (mcl) monomers (C6–C14).

Bacillus sp. INT005 was isolated from gas field soil as a PHA-producing bacterium [6]. This strain possesses class IV synthase genes in the PHA biosynthesis operon consisting of $phaR_{Bsp}$ (480 bp), $phaB_{Bsp}$ (741 bp) and $phaC_{Bsp}$ (1083 bp) [7]. *In vitro* activity assay of the gene product, $PhaRC_{Bsp,}$ revealed a lag time prior to the polymerization reaction [7]. This feature is different from class III synthase (PhaEC), which has been reported to show no lag time. Furthermore, from *in vivo* PHA synthesis experiments by *Bacillus* sp. INT005 at culture temperature of 45 °C, it was suggested that $PhaRC_{Bsp}$ possesses moderate thermal stability.

In our previous study, the molecular weights of P(3HB) synthesized by various class of PHA synthase (class I–IV) in recombinant *Escherichia coli* JM109 were compared under the same experimental conditions [8]. It was suggested that the molecular weights and polydispersities of P(3HB) were strongly dependent on the nature of the PHA synthase employed. Interestingly, an unusual

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molecular weight distribution of P(3HB) was found when $PhaRC_{Bsp}$ was expressed. The strain accumulated P(3HB) to 55 wt% of dried cells after 14 h of cultivation at 37 °C [8]. However, the P(3HB) had relatively low molecular weight ($M_n = 1.7 \times 10^5$). In addition, the molecular weight distribution was bimodal: thus the polydispersity (M_w/M_n) was as high as 9.1 [8].

This study aims to investigate the factors affecting molecular weight of P(3HB) especially when $PhaRC_{Bsp}$ was used for P(3HB) synthesis. First, the time-dependent change of P(3HB) molecular weight in *E. coli* JM109 expressing $PhaRC_{Bsp}$ was investigated at culture temperatures of 37 °C. Furthermore, the effect of culture temperature (30 and 25 °C) and host strains (DH5α and HB101) on molecular weight of P(3HB) were investigated. Kinetic analysis was performed to elucidate the mechanism underlying the decrease in molecular weight of P(3HB). The results shed light on the unique behaviour of molecular weight change of P(3HB) synthesized in *E. coli* expressing $PhaRC_{Bsp}$.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth condition

The bacterial strains and the plasmids used in this study are listed in Table 1. *E. coli* JM109 (Takara Co. Ltd., Kyoto, Japan) was used for production of P(3HB). Inocula of *E. coli* were prepared using Luria Bertani (LB) medium (10 g/L NaCl, 10 g/L tryptone, and 5 g/L yeast extract) at 37 °C. To maintain the plasmids within the cells, 100 μg/ml of ampicillin was added into the medium.

2.2. Biosynthesis and analysis of P(3HB)

For P(3HB) synthesis, the cells were grown using a reciprocal shaker (130 strokes/min) in a 500-mL flask with 100 mL of LB medium supplemented with 20 g/L of glucose. The P(3HB) content in the cells was determined by gas chromatography (GC) after methanolysis of lyophilized cells in the presence of 15% (v/v) sulfuric acid, as described previously [9].

P(3HB) was extracted from cells by stirring with chloroform for 72 h at room temperature, and then cells were removed by filtration. The extracted P(3HB) was purified by precipitation with methanol twice. Molecular weight data were obtained by gel permeation chromatography (GPC) at 40 °C, using a Shimadzu 10A GPC system and a 10A refractive index detector with two joint columns of Shodex K-806M and K-802. Chloroform was used as an eluent at a flow rate of 0.8 mL/min, and the sample was applied with a concentration of 1 mg/mL. Polystyrene standards ($M_p = 1.3 \times 10^3$–7.5×10^6) with a low polydispersity were used to make a molecular weight calibration curve.

3. Results

3.1. Change in molecular weight of P(3HB) at 37 °C

Initially, to investigate the time-dependent change of molecular weight of P(3HB) synthesized by $PhaRC_{Bsp}$, recombinant *E. coli* JM109 harbouring pGEM-$phaRC_{Bs}$AB was cultured at 37 °C in LB medium containing glucose (20 g/L) for various culture times (12, 14, 34, 50, and 60 h).

The results are shown in Fig. 1(A). The cell concentration increased until 14 h of cultivation, and then remained almost unchanged with further culture time. This means that the cells entered a stationary phase-like growth state after 14 h. The P(3HB) accumulation in the cells was in the range of 46–61 wt% of dried cells during 12–60 h of cultivation. Also, P(3HB) content increased until 14 h, but further cultivation did not enhance accumulation of P(3HB) in the cells.

The M_n of P(3HB) showed the highest value of 4.4×10^5 at 12 h, and then decreased with time to 4.8×10^4 at 60 h. The polydispersity (M_w/M_n) of P(3HB) reached 9.1 at 14 h, then turned down and decreased to 6.4 (60 h) with culture time. Fig. 2(A) shows the molecular weight distributions of P(3HB) synthesized at 37 °C for various cultivation times. The narrowest polydispersity of 3.6 was observed at 12 h, where the molecular weight had an almost unimodal distribution, like the case for other PHA synthases. However, from 14 to 60 h, the molecular weight changed to a bimodal distribution, resulting in the increased polydispersity. Subsequently, the molecular weight distribution returned to unimodal, with the decreased molecular weight.

To examine whether decreasing P(3HB) molecular weight is observed for other synthases, two PHA synthases from *Ralstonia eutropha* ($PhaC_{Re}$) and *Delftia acidovorans* ($PhaC_{Da}$) were expressed in *E. coli* JM109 under the same conditions as $PhaRC_{Bsp}$. The time courses of P(3HB) molecular weight are shown in Fig. 3. In the cases of $PhaC_{Re}$ and $PhaC_{Da}$, unlike $PhaRC_{Bsp}$, the molecular weights did not change with culture time, and showed unimodal distribution. Therefore, this pattern of decreasing molecular weight is an unusual phenomenon for P(3HB)-accumulating *E. coli*.

3.2. Change in molecular weight of P(3HB) at 30 °C

Because the interesting phenomenon was found at a culture temperature of 37 °C, other cultivations were performed at 30 °C for

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference or source
Strain		
E. coli JM109	*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR*17($r_K^- m_K^+$), *e*14$^-$ (*mcrA*$^-$), *supE44*, *relA1*, Δ (*lac-proAB*)/F′ [*traD*36, *proAB*$^+$, *lac*I^q, *lacZ* Δ M15]	Takara Co. Ltd.
E. coli DH5α	F$^-$, ϕ80d*lacZ* Δ M15, Δ (*lacZYA-argF*) U169, *deoR*, *recA1*, *endA1*, *hsdR17*($r_K^- m_K^+$), *phoA*, *supE44*, λ$^-$, thi-1, *gyrA*96, *relA*1	Takara Co. Ltd.
E. coli HB101	*supE44*, Δ (*mcrC-mrr*), *recA13*, *ara-14*, *proA2*, *lacY*1, *galK*2, *rpsL*20, *xyl*-5, *mtl-1*, *leuB6*, *thi*-1	Takara Co. Ltd.
Plasmid		
pGEM-$phaC_{Re}$AB	pGEM-T derivative carrying pha_{Re} promoter and $phaCAB_{Re}$ (“Re” represents *R. eutropha*); This plasmid is identical with pGEM’$phaCAB_{Re}$.	[17]
pGEM”ABex	pGEM- $phaC_{Re}$AB derivative carrying pha_{Re} promoter-$phaAB_{Re}$.	[8]
pGEM-$phaRC_{Bs}$AB	pGEM”ABex derivative carrying pha_{Re} promoter-$phaRC_{Bsp}$-$phaAB_{Re}$ (“Bsp” and “Bs” represent *Bacillus* sp. INT005).	[8]
pGEM-$phaC_{Da}$AB	pGEM”ABex derivative carrying pha_{Re} promoter-$phaC_{Da}$-$phaAB_{Re}$ (“Da” represents *D. acidovorans*); This plasmid is identical with pGEM”ABexCaC.	[18]

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