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Regulation of Molecular Weight of Biopolyesters Produced by Bacteria

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Research aims

A variety of bacteria can synthesize biopolyester, polyhydroxyalkanoate (PHA), as carbon and energy storage materials in the cells. Unlike petroleum-based polymers, PHA can be produced from renewable resources such as biomass and carbon dioxide and be used as biodegradable thermoplastic material. Natural PHA-producing bacteria produce PHA with the weight-average molecular weight ranging from 500,000 to 1,000,000. Molecular weight of polymer is one of the important factors for material properties and the molecular weight-regulated polymers are practically useful. However, as for PHA, regulation of the molecular weight in biosynthesis method has not been established. This study aims to develop a method for regulating molecular weight of PHA by use of PHA synthase mutants in recombinant bacteria.

Methods

PHA synthase gene (*phaC*Ac) from *Aeromonas caviae* was used in this study. The *A. caviae* PHA synthase mutants, substituting an alanine at position 505 (A505) with the other 19 amino acids, were generated by site-specific mutagenesis. As a host for PHA accumulation, PHA negative mutant of *Ralstonia eutropha* H16, strain PHB^{-}4, was used. Recombinant plasmids for functional expression of $phaC_{Ac}$ genes in *R. eutropha* PHB^{-}4 were constructed using a broad-host-range vector and introduced into the host cells by a transconjugation method.

Recombinant strains of *R. eutropha* PHB^{-}4 were cultivated on a reciprocal shaker in 500-ml flasks containing 100 ml mineral salt (MS) medium supplemented with 2% (wt./vol.) fructose at 30°C for 72 h. After cultivation, collected cells were washed with water to remove the remaining carbon source, and then lyophilized.

The PHA content in dried cells was determined by gas chromatography (GC) after methanolysis of lyophilized cells. The polymers accumulated in the cells were extracted with chloroform at room temperature and purified by reprecipitation with methanol. Molecular weight data were obtained by gel permeation chromatography (GPC).

Results

Sequence alignment analysis of PHA synthases

At present, approximately 60 different PHA synthases have been found and characterized. Based on their substrate specificity and subunit composition, PHA synthases have been classified into four types, type I to IV. PHA synthase from *Ralstonia eutropha* is the well-known enzyme belonging to type I group. Previously, we reported that an amino acid substitution at position 510 (A510) in *R. eutropha* enzyme, a highly conserved position in type I enzymes and located two residues downstream from active-site histidine (H508), is beneficial to change the molecular weight of PHA[1]. Also, similar effect was observed for type II mutant enzyme, in which the corresponding amino acid residue (Q481) was substituted with other amino acids[2].

PHA synthase from *Aeromonas caviae* belongs to type I group; this enzyme shows broader substrate specificity than typical type I enzymes. Thus, it has been recognized as an important tool for biosynthesis of high performance PHA. Based on the sequence alignment analysis of PHA synthases, we identified that alanine at position 505 (A505) in *A. caviae* enzyme is the effective residue to change in molecular weight of PHA by introducing amino acid substitution.

PHA synthesis by use of mutant enzymes

R. eutropha PHB^{-}4 expressing PHA synthase mutants A505X from *A. caviae* were cultivated and all strains showed comparable growth (2.8–4.1 g/l of dry cell concentration). To determine the monomer composition of PHA and the content in the cells, the cells were subjected to GC analysis. The results showed that all strains produced homopolymer of (*R*)-3-hydroxybutyrate, P(3HB). As for PHA accumulation, high content was observed for the strain expressing the wild-type enzyme (79 wt%), followed by A505M (78 wt%) and A505T (77 wt%). The low PHA content was observed for three mutants of A505P, A505R and A505K (66 wt%). All mutant enzymes, however, produced relatively large amounts of PHA in the cells, suggesting that amino acid substitution at position 505 does not affect primary polymerization function of the enzyme.

Molecular weight analysis

All PHA samples isolated from each strain were subjected to GPC analysis. The weight-average molecular weight (Mw) of PHA synthesized by the wild-type enzyme was 650,000, whereas those by mutant enzymes were in the range of 280,000–1,070,000. These mutants did not synthesize PHA with significantly higher molecular weight than the wild-type. However, A505M (Mw=280,000) and A505I (Mw=400,000) synthesized low-molecular weight PHA with high reproducibility. The polydispersity index of

PHA synthesized by the wild-type enzyme, A505M and A505I were 2.6, 2.5 and 3.3, respectively. Taking PHA content and polydispersity index into consideration, A505M mutant is a possible candidate for synthesis of PHA with the molecular weight regulated to half of that synthesized by the wild-type enzyme.

Conclusion

The molecular weights of PHA synthesized by mutants of PHA synthase from *A. caviae* were characterized. Amino acid substitution at position 505 was found to be effective for changing molecular weight of PHA without reducing polyester productivity. Particularly, A505M mutant is a possible candidate for synthesis of low-molecular weight PHA. This study demonstrates the feasibility of synthesizing PHA with regulated-molecular weight by use of PHA synthase mutants from *A. caviae*.

References

1) Tsuge, T., Saito, Y., Narike, M., Muneta, K., Yahaya, N. M., Kikkawa, Y., Hiraishi, T., Doi, Y.: Mutation effects of a conserved alanine (Ala510) in type I polyhydroxyalkanoate synthase from *Ralstonia eutropha* on polyester biosynthesis; *Macromol. Biosci*., **4**, 963–970 (2004)
2) Tsuge, T., Yano, K., Imazu, S., Numata, K., Kikkawa, Y., Abe, H., Taguchi, S., Doi, Y.: Biosynthesis of polyhydroxyalkanoate (PHA) copolymer from fructose using wild-type and laboratory-evolved PHA synthases; *Macromol. Biosci*., **5**, 112–117 (2005)