学 位 論 文 要 旨

Relation of 3-Hydroxybutyrate Oligomer Hydrolase and 3-Hydroxybutyrate Dehydrogenase to Polyhydroxyalkanoate Degradation in *Paracoccus denitrificans*

Paracoccus denitrificans における３－ヒドロキシ酪酸オリゴマーヒドロラーゼと３－ヒドロキシ酪酸デヒドロゲナーゼのポリヒドロキシアルカン酸分解への関与

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Microbial polyesters are high-molecular-weight compounds with degradability in natural habitat. They are attractive in industrial or commercial fields. Polyhydroxyalkanoates (PHAs) are examples of such polymers. It has well been known that PHAs are intracellularly synthesized and degraded by many bacteria because they are carbon and energy reserve materials. *Paracoccus denitrificans* used in this study is a methylotrophic bacterium which synthesizes different type of PHAs from different alcohols. Also, the strain was previously used for analyzing PHA synthesis in genetic and biotechnological studies. Intracellular PHA depolymerase gene (*phaZ*) of *P. denitrificans* was previously isolated and analyzed only for poly(3-hydroxybutyrate) (PHB) degradation. Although there are many studies on PHA synthesis, many things on the intracellular PHA degradation system has still remained to be cleared.

In this study, genes encoding 3-hydroxybutyrate oligomer hydrolase (3HBOH or PhaZc) and 3-hydroxybutyrate dehydrogenase (Hbd) were isolated from *P. denitrificans*. *P. denitrificans* NBRC13301 (*P. denitrificans* PD01) was used. *P. denitrificans* Pd1222 genome sequence is available in the NCBI data base. By referring to its nucleotide sequence, PCR was performed with the *P. denitrificans* PD01 DNA. PCR products obtained were cloned in *E.coli* cells and sequenced, and the ORFs were named *phaZc* and *hbd*. When the *phaZc* and *hbd* cloned in pUC19 or pTAC-1 were expressed, two proteins with predicted molecular masses were produced. 3HBOH activity was detected with 3-hydroxybutyrate (3HB) oligomers as a substrate. Also, Hbd activity was detected with 3HB as a substrate. It was demonstrated that the gene chosen as one of “hydrolases” was for 3HBOH. pQE30-phaZc and pQE30-hbd were then constructed in *E.coli* M15 to purify the enzymes as His-tagged proteins, His-PhaZc and His-Hbd.

For purifying His-PhaZc and His-Hbd, IPTG concentrations were optimized to avoid inclusion body formation. IPTG was used at 10 μM and 0.1 mM for expressing His-PhaZc and His-Hbd, respectively.

His-PhaZc and His-Hbd were purified via two-step chromatography using affinity and gel filtration columns to a single band on SDS-PAGE. These purified proteins had molecular masses of 31 kDa and 120 kDa (a tetramer of 29-kDa subunits), respectively. Enzymatic characteristics (substrates specificity, pH, temperature, and inhibitors) of His-PhaZc were determined and were almost the same as the enzyme previously purified as 3HBOH from *P. denitrificans*. Especially, the purified His-PhaZc catalyzed the hydrolysis of 3-hydroxyvalerate (3HV) oligomers in addition to 3HB oligomers. His-Hbd catalyzed the dehydrogenation of 3-hydroxyvalerate as well as 3-hydroxybutyrate at similar rate. When both enzymes were included in the same enzymatic reaction system with 3HV dimer, sequential reaction occurred, suggesting PhaZc and Hbd play an important role in the intracellular degradation of poly(3-hydroxyvalerate) (PHV) homopolyester and PHB-PHV copolyester.

The *phaZc* gene of *P. denitrificans* PD01 was disrupted in order to know the function of PhaZc in PHA degradation. Kanamycin resistant (Km^r) gene was introduced inside the *phaZc* region of *P. denitrificans* PD01 genome by a traditionally and commonly used procedure of homologous recombination. Colonies displaying Km^r were obtained on selective agar plates. One of them was chosen and named *P. denitrificans* PD01Km. Introduction of the Km^r gene into the *phaZc* region was confirmed by Southern blotting and PCR. A 1.2-kb *Sal*I fragment (Km^r gene) from pUC4K was used as a probe for hybridization. The probe hybridized with the mutant DNA, but did not hybridize with the wide type DNA. When PCR was performed, the size of PCR product amplified with the mutant was approximately 1.3 kb larger than that with the wild type. PhaZc activity was not detected in *P. denitrificans* PD01Km. When the *phaZc*-disrupted *P. denitrificans* mutant was complemented with the *phaZc* gene, PhaZc activity was restored. These results suggest that *P. denitrificans* carries a single *phaZc* gene. Effect of the *phaZc* disruption on intracellular PHA degradation in *P. denitrificans* PD01 was examined. PHA contents in *P. denitrificans* PD01 and PD01Km were measured in both PHA synthesis and degradation stages. *P. denitrificans* PD01 and PD01Km were cultivated for analyzing cellular PHA contents. The strains were first grown to late exponential growth phase. The second cultivation stage was under carbon-sufficient and nitrogen-deficient. The third cultivation stage was under carbon-depleted conditions. In the second stage, the wild and mutant strains produced PHA in a similar way. In the third stage, especially, in the first 5 h of this stage, PHA content in the wild strain decreased by 40.5% (w/w), whereas that in the mutant strain decreased by 26.3% (w/w). PHA degradation in the mutant strain proceeded more slowly than in the wild type strain. It was demonstrated that PhaZc activity affects PHA degradation rate in *P. denitrificans* PD01.