Functional Analysis of a Gene Cluster of Two Aliphatic-Aromatic Polyester Type Plastic Degrading Enzymes from *Roseateles depolymerans* strain TB-87

July 2019

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Abstract

Plastic waste pollution is one of the major environmental concerns in recent years caused by the rapid growth of plastic utilizing industries. To overcome many problems associated with plastics waste, the development of biodegradable plastics for use especially in packaging have been gaining attention since these plastics can be degraded into low molecular weight monomer generating carbon dioxide, methane, and water by suitable microorganisms. However, in recent years, concerns regarding the fate of biodegradable plastics have arisen; the production of micro- and nanoplastics as a result of the breakdown of the plastics. The development of biodegradable plastics comprise of recyclable monomers is, therefore, more economical and environmentally benign approach to the problems. To achieve this final aim, the biodegradation mechanism by microorganisms first needs to be clarified.

Previously in this laboratory, the isolation and characterization of polyester-degrading enzymes, designated as Est-H and Est-L from freshwater bacterium, *Roseateles depolymerans* strain TB-87, capable to degrade various polyester-based biodegradable plastics such as poly(butylene succinate) (PBS), poly(butylene succinate-*co*-adipate) (PBSA) and poly(Ɛ-caprolactone) (PCL), have been reported. Although both Est-H and Est-L have similar substrate specificity and degradation activity as well as nearly identical molecular masses, studies suggested that both proteins are of different proteins. While many other similar studies reported on the single enzyme responsible for the degradation, strain TB-87 possesses two identical enzymes with similar functions. This study aims to elucidate the functions of the genes encoding for the enzymes.

Two open reading frames (ORFs) consisting of 1083 bp and 870 bp nucleotides, corresponding to *est-H* and *est-L*, encoding enzymes of 360 and 289 amino acids, respectively, were predicted. In addition, another ORF consisting of 735 bp encoding a chaperone-like protein (Est-Ch) of 244 amino acids was identified in the intergenic region of *est-H* and *est-L*. The presence of a promoter region upstream of *est-H* and the absence of a terminator region downstream of the ORF and vice versa for *est-Ch*, suggests that *est-H* and *est-Ch* are polycistronically expressed. Est-H and Est-L showed homology with plastic degrading enzymes, such as esterase and cutinase while Est-Ch showed homology with bacterial lipase chaperone. The presence of consensus lipase sequences (-Gly-His-Ser-Met-Gly-) was observed in these enzymes, thus Est-H and Est-L were hypothesized to be hydrolases with serine (Ser) in their active center.

Following gene annotation, Est-H and Est-L were overexpressed in *E. coli* BL21 (DE3) to obtain purified protein for further biochemical characterization studies. Results of overexpression in *E. coli* BL21 (DE3) suggested possible gene toxicity, therefore overexpression in another strain, *E. coli* Lemo21 (DE3) was attempted. Analysis by sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) revealed the insoluble expression of both proteins. The refolding of both proteins was performed *in vitro* using immobilized metal affinity column chromatography (IMAC). The proteins could not be retrieved even after changing many buffers and refolding conditions, suggesting aggregation of the proteins in the column during simultaneous refolding and purification.

The entire gene cluster was disrupted using a kanamycin antibiotic cassette to elucidate the functions of esterase-encoding genes previously annotated. The mutant strain lost the ability to form a clear zone on PBSA emulsion-overlaid NB agar plates, suggesting that the annotated esterase-encoding genes are indeed the genes responsible for the PBSA degradation ability of *R. depolymerans* strain TB-87. However, the exact mechanism of PBSA-degradation at the molecular level remains unclear. Further, gene annotation data showed the presence of a chaperon-encoding gene in the intergenic region of *est-H* and *est-L*, which functions remains unclear, therefore, gene disruption was performed to elucidate the functions of the gene. The deletion of only *est-Ch* produced mutants unable to form halo zone on PBSA emulsion-nutrient agar plate, similar to that observed after the deletion of the entire esterase gene cluster. Unpublished preliminary results obtained suggested that the *est-Ch* is possibly existed as a subunit or as a part of a protein complex with Est-H and Est-L.

Understanding of function of genes encoding for plastic-degrading enzymes could lead to the elucidation of the decomposition activity and substrate specificity exclusive to the biodegradable plastic enzymes. This will allow manipulation and modification of the genes to obtain enzymes having more specific properties particularly for the development of enzyme-based monomer recycling system in the future.