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Thesis/dissertation Title

Identification and Functional Analysis of An Exo-Acting PET Hydrolytic Enzyme of the PET-degrading Bacterium, *Ideonella sakaiensis*

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Polyethylene terephthalate (PET) is a petroleum-based thermoplastic extensively used in the production of packaging plastics and polyester fibers. Short service time of PET products and its non-biodegradability properties have triggered serious environmental problems. Recent discovery of *Ideonella sakaiensis* with the capability to grow on the PET has emerged as a promising bioremediation strategy to mitigate the environmental crisis arising from plastic waste accumulation. Two PET-hydrolytic enzymes crucially involved in the PET metabolism, PET hydrolase (PETase) and mono-2-hydroxyethyl terephthalate (MHET) hydrolase (MHETase), have been identified. However, the mechanism underlying efficient PET degradation in *I. sakaiensis* remains unclear, hampering its potential applications.

I found that PETase proteins localize on the PET film surface in *I. sakaiensis* PET-fed culture, indicating that *I. sakaiensis* assembles proteins important for PET degradation on the nutrient. From quantitative proteomics using data-independent acquisition mass spectrometry (DIA-MS), I discovered a novel factor, IS3653, an MHETase homolog exhibiting the highest fold change localization on the surface of PET. This observation suggests that IS3653 holds significant potential in being involved in the PET metabolism and degradation of *I. sakaiensis*, making it the primary focus of this study.

To identify the function of IS3653, I employed *in vitro*, *in silico*, and *in vivo* approaches. IS3653 was expressed as a maltose-binding protein (MBP) fusion protein in *Escherichia coli*. MBP-IS3653 hydrolyzed several PET moieties, such as bis(2-hydroxyethyl) terephthalate (BHET), bis-(benzoyloxyethyl) terephthalate

(BETEB) and 2-hydroxyethyl benzoate (HEB), but not MHET and PET, indicating a unique substrate specificity different from MHETase and PETase. Since IS3653 and PETase share BHET hydrolytic activities, a comparative kinetic study was conducted, revealing their similar k_{cat}/K_m values on BHET hydrolysis. This indicates the significance of IS3653 in the BHET hydrolysis of *I. sakaiensis*, a part of the PET metabolism.

IS3653 structure was successfully modeled using *de novo* modeling with AlphaFold2. The overall structure of IS3653, which resembles that of the tannase/esterase family of enzymes, adopts a bipartite domain architecture: an α/β-hydrolase domain, and a lid domain that partially envelops the active site. The model revealed its catalytic triad consisting of Ser217, His488, and Asp450, where the respective alanine scanning mutations were experimentally confirmed to completely abolish the enzymatic activity of IS3653. Among the protein homologs with known crystal structures, IS3653 showed the highest structural similarity to MHETase. Despite their similar overall structures, the substrate binding pocket of IS3653 consists of more hydrophobic amino acid residues than that of MHETase. Therefore, this abundance of hydrophobic residues, particularly in the inner binding pocket of IS3653 is speculated to stabilize the aliphatic chain of BHET, but destabilize the positioning of the polar carboxylate group of MHET.

Gene disruption of *Is3653* led to a delayed growth of *I. sakaiensis* on PET film and a decreased PET weight loss. In contrast, no notable growth difference was observed with other PET moieties, including BHET, MHET, terephthalic acid (TPA) and EG. These results indicated the direct involvement of IS3653 in promoting PET degradation in *I. sakaiensis*. PETase with a diluted IS3653 led to an improved release of MHET from PET compared to PETase only, suggesting their synergistic effect on PET. Besides, upon partial digestion of PET polymer with PETase to increase the PET termini, the subsequent incubation with IS3653 revealed a notably improved capability of IS3653 to release MHET as the sole product.

Taken together with the structural analysis of IS3653, this study indicated that IS3653 could attack from the EG-terminus of a PET polymer, and cleave the ester linkage with every MHET unit. That is, IS3653 is an exo-PET hydrolytic enzyme that could contribute to efficient PET hydrolysis by the synergistic action with endo-acting PETase, promoting PET degradation and metabolism in *I. sakaiensis*.