Engineering of PET Hydrolase for Plastic Recycling and Environmental Remediation, and Engineering of Plastic Binding Domains for Detection and Quantification of Particles

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Plastic is an indispensable material in our daily lives. It is used in a wide variety of products because it is low-cost, mass-producible, and easy to mold and process. However, due to its convenience, the amounts of plastic used and disposed are increasing every year, raising concerns about its environmental impact and sustainability. Therefore, there is a need to develop efficient and environmentally friendly recycling methods, as well as simple and rapid methods to detect plastics that have leaked into the environment.

We are attempting to obtain more active mutants by exhaustively mutating amino acid residues on the enzyme surface and screening their activity using a dispensing robot. We are also attempting to create plastic adsorption domains by modifying the amino acids constituting the adsorption surface of the carbohydrate binding domain and using a phage display method.

These studies will promote the recycling of used plastics by enzymatic degradation and promote more sustainable use of plastics. In addition, by creating a protein that detects and stains plastics, we will contribute to the protection of the natural environment by monitoring the small plastics runoff into the environment (Figure 1).

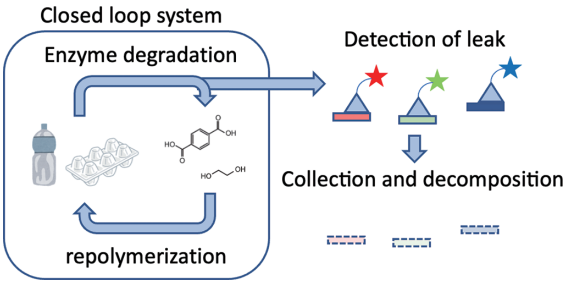

Figure 1. Scheme of plastic recycling and detection system.

Selected Publications

- A. Nakamura, N. Kobayashi, N. Koga and R. Iino, "Positive Charge Introduction on the Surface of Thermostabilized PET Hydrolase Facilitates PET Binding and Degradation," *ACS Catal.* **11**, 8550–8564 (2021).
- A. Nakamura, D. Ishiwata, A. Visootsat, T. Uchiyama, K. Mizutani, S. Kaneko, T. Murata, K. Igarashi and R. Iino, "Domain Architecture Divergence Leads to Functional Divergence in Binding and Catalytic Domains of Bacterial and Fungal Cellobiohydrolases," *J. Biol. Chem.* **295**, 14606–14617 (2020).
- A. Nakamura, K. Okazaki, T. Furuta, M. Sakurai and R. Iino, "Processive Chitinase Is Brownian Monorail Operated by Fast Catalysis after Peeling Rail from Crystalline Chitin," *Nat. Commun.* **9**, 3814 (2018).
- A. Nakamura, T. Ishida, K. Kusaka, T. Yamada, S. Fushinobu, I. Tanaka, S. Kaneko, K. Ohta, H. Tanaka, K. Inaka, Y. Higuchi, N. Niimura, M. Samejima and K. Igarashi, "'Newton's Cradle' Proton Relay with Amide-Imidic Acid Tautomerization in Inverting Cellulase Visualized by Neutron Crystallography," *Sci. Adv.* **1**, e1500263 (2015).

1. High Throughput Screening Method of PET Hydrolase Activity

Mutations were introduced into PET hydrolytic enzyme PET2 derived from a metagenomic library to create a mutant with improved thermostability and PET degrading activity.[1)] In this study, we tried to further improve the activity of PET2 with a combination of saturation mutations. For a combination of saturation mutation at two positions, we need to test 399 mutants. Since it is difficult to purify and measure the activity of each mutant individually, a screening method was created to evaluate the activity without purification of the enzyme and without the use of HPLC (Figure 2).[1)]

We prepared PET particulate suspensions by dissolving PET in Hexafluoro-2-propanol and regenerating it in water. Since the concentration of the suspension correlates with the intensity of light scattering at 595 nm, we attempted to measure PET degradation activity using turbidity as an indicator.

The correlation between the reduction of turbidity and concentration of soluble product was analyzed by HPLC. At the first stage of degradation, the two parameters showed linear correlation. Therefore, we created a library in which saturation mutations were introduced at two substrate recognition sites of the PET2 mutant and performed selection. colonies were cultured in 96-well plates, and after one night of enzyme induction, the culture medium was collected and mixed with the PET suspension. The rate of decrease in turbidity was analyzed in the culture medium of 720 colonies, and 13 enzymes were found to be more than twice as active as the PET2 mutant that used as the template.

To further verify thermostability, activity measurements were performed using heat-treated enzymes. The use of 384 wells for activity measurement enabled analysis of many enzymes at one time and efficient screening. The best H229T-F233M mutant showed 3.4 times higher degradation rate against PET film than the template enzyme at the initial time. The molecular dynamics simulation implied that F233M mutation make the space for making alpha helix and H229T mutation resolved steric hindrance with Trp199. These mutations were speculated to change the angle of the Trp199 side chain of PET2 to an angle similar to that of the Trp185 of IsPETase, making it suitable for PET binding to the active center. Screening of activity using PET suspensions is compatible with robotic automation and is expected to be useful for validating computationally predicted mutations.

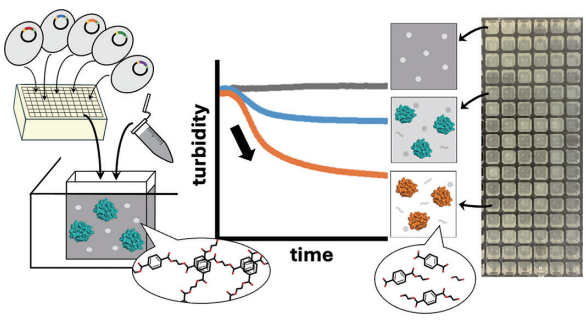

Figure 2. Scheme of PET degrading enzyme activity measurement using turbidity as an indicator.

2. Artificial Evolution of Carbohydrate Binding Domain to PET Binding Domain

To develop a method for detecting plastics using plastic binding proteins, we first developed a protein targeting PET. The template enzyme was a thermostable domain that binds on chitin, which has hydrophobic planes similar to PET.

Saturation mutations were introduced at four amino acid residues oriented on the binding surface and involved in chitin recognition. Since the number of candidates of mutants is about 160,000 and it is difficult to verify them one by one, we created a library of phage vectors with binding domain mutant genes inserted into, which were then used to create a library of M13 phage presenting adsorption domain mutants on their surface. The M13 phage library was incubated with PET film, and unbound phage were washed away. The phage bound on PET were recovered by trypsin digestion and re-amplified by infection with *E. coli*. The concentrated library of phage was further screened with more strong condition of washing.

The 3 cycles of screening of the first-generation phage library resulted in the 29 colonies of PET binding domain candidates. The 27 colonies were the PfCBD-K270H-N272P-E279V-D281G mutant (PfCBD-4M) and showed higher binding affinity to PET than WT. But the binding affinities to chitin and cellulose were remained. The other two mutant were rejected because the RFP fusion proteins were insoluble.

To improve the binding specificity, three mutations were further added to PfCBD-4M. After the 3 cycles of screening, the 425 colonies were obtained. The colonies were mixed, and the CBD genes were amplified by PCR. The CBD genes were ligated with RFP gene and the fusion proteins were produced in small scale. The solubilities of proteins were analyzed based on the RFP fluorescence signal in the solution to remove the insoluble proteins. The gens of the top 10 proteins were sequenced, and the 6 candidates were obtained. The binding amount of 2nd generation of 6 mutants, 4M and PfCBD-WT against chitin, cellulose and low and high concentrations of PET were measured. The all of 6 mutants showed almost no binding to chitin and cellulose but higher binding amount than 4M and WT (Figure 3). Therefore, we succeeded to change the binding specificity of the domain from chitin/cellulose to PET. Now we tried to make a fusion enzyme with PET hydrolase to increase the affinity to PET.

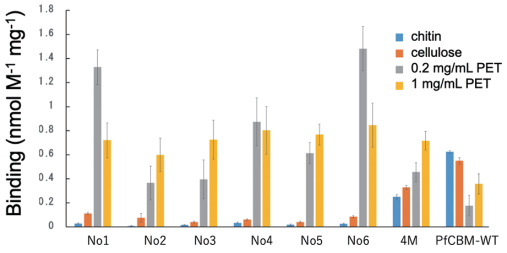

Figure 3. Binding measurement of 2nd generation of mutants against chitin, cellulose and PET.

Reference

1) Y. Ogura, Y. Hashino and A Nakamura, *ACS Omega* (2024), in press.