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 runoff into the environment (Figure 1).

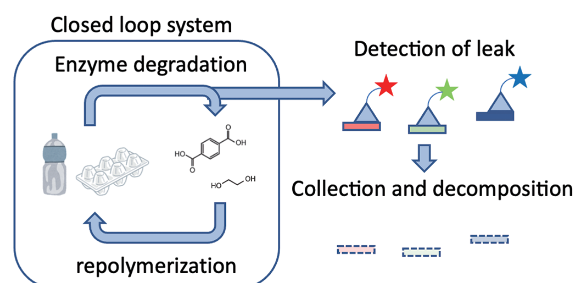

Figure 1. Scheme of plastic recycling and detection system.

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, the improvement was achieved with a combination of single mutations, but a saturation mutation, for example, in which a combination of all amino acids is tested in two locations, we need to test a library of 399 candidate mutants. Since it is difficult to purify and measure the activity of each mutant individually, a method was created to evaluate the activity without purification of the enzyme and without the use of HPLC.

We artificially prepared PET particulate suspensions by dissolving PET in Hexafluoro-2-propanol (HFIP) 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.

When the temporal changes in degradation rate and turbidity were analyzed by changing the concentration of PET degrading enzyme, it was observed that the turbidity decreased over time, and it was found that the high degradation activity could be evaluated from the rate of decrease in turbidity (Figure 2). 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 approximately 800 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 three mutants were produced and purified at large scare cultivation and the activities against PET film are under comparison

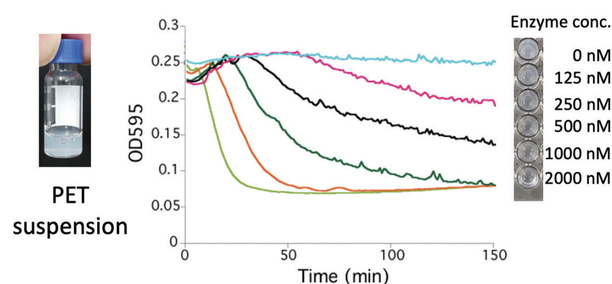

Figure 2. Results of PET degrading enzyme activity measurement using turbidity as an indicator.

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

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

Saturation mutations were introduced at four amino acid residues oriented on the adsorption surface and involved in chitin recognition. Since the number of candidate mutants is about 160,000 and it is difficult to verify them one by one, we created a library of phage vectors with adsorption 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, unbound phage were washed away, and phage bound on PET were recovered by trypsin digestion and reamplified by infection with *E. coli*. The reproduced first-generation phage library was again mixed with PET and the number of washes was increased to 10 before PET adsorbed phage were collected and infected with *E. coli*. After the second-generation phage library was produced, the phage vectors were purified from *E. coli*, and the mutated portions were analyzed using a next-generation sequencer. In the pre-screening library, the percentage of amino acids that appeared for the four mutations was almost the same, but in the second-generation library, a large difference was observed in the probability of occurrence of different amino acids depending on the position of the mutation. This was thought to be the result of selection of amino acids structurally important for PET recognition. In order to select mutants with strong binding and high thermal stability, a third-generation screening was conducted with binding and washing temperatures set at 60 °C. As a result, 29 colonies were obtained, 27 of which were the same mutant. The mutant enzyme was conjugated with RFP, and when mixed with PET powder, the mutant complex stained the PET powder red, whereas the template enzyme did not (Figure 3). Currently, we are introducing further mutations into the mutant to improve the specificity of binding on PET.

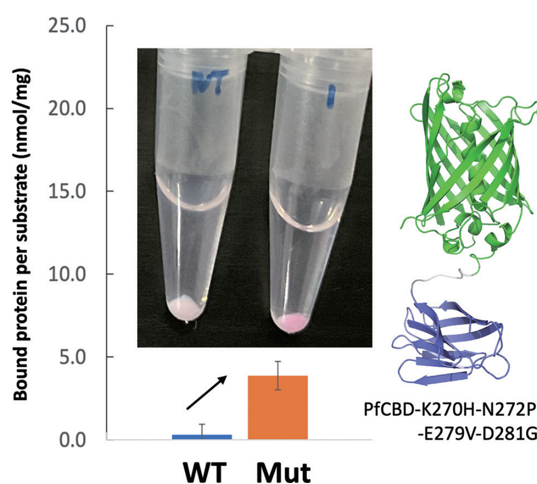

Figure 3. Binding measurement results using PET binding domain and RFP complex.

Reference

1) A. Nakamura, N. Kobayashi, N. Koga and R. Iino, *ACS Catal.* **11**, 8550–8564 (2021).