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Enhancing Enantioselectivity of an Esterase from *P. fluorescens* by Focusing Mutations into the Substrate-Binding Site

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Enzymes are highly active and selective toward natural substrates. Researchers have successfully used these characteristics of enzymes in many research fields, such as industrial, especially pharmaceutical and agrochemical, and academic research. [1] However, when researchers deal with unnatural substrates, they may need to modify reaction system or enzyme itself because enzymes often do not show sufficient activity or selectivity. Among the approaches to improve activity or selectivity of enzymes, the most effective one is protein engineering, such as rational design by site directed mutagenesis based on crystal structure or directed evolution by error prone PCR, mutator strain, or DNA shuffling.

Rational protein design requires detailed structural and mechanistic understanding to choose the mutations. [2] The rational approach can avoid the need for huge screening, which is required in directed evolution. However, the result is not always predictable. For example, Hult and coworkers reported a rational approach to increase enantioselectivity of lipase B from *Candida antarctica* towards bromo- or chlorohydrin.[3] On the basis of a crystal structure and molecular modeling, the alcohol-binding region was identified as was the possible binding pocket for the bromo or chloro group of the fast-reacting enantiomer. In this binding pocket, there are four hydrophilic amino acids, Thr40, Ser47, Thr42, and Trp104. They proposed that those hydrophilic residues would make repulsive interaction because of partial negative charge on bromo or chloro group. One of mutants, Ser47Ala, showed doubly increased enantioselectivity towards 1-bromo-2-octanol or 1-chloro-2-octanol. But a double mutant, Ser47Ala/Thr42Val did not show high enantioselectivity as that of the single one.

Although directed evolution (recursive generation and screening of mutants) requires no structural and mechanistic information, it requires extensive screening of mutants. For example, if the enzyme contains 300 amino acids, one amino acid substitution creates 5700 mutants. [4] Reetz's group reported the first example of increased enantioselectivity by directed evolution. [5] The wild type of a lipase from *Pseudomonas aeruginosa* (PAL) has $E = 1.1$ towards hydrolysis of *p*-nitrophenyl 2-methyldecanoate. They improved enantioselectivity to 11.3 after four rounds of random mutagenesis using the error prone PCR and screening 1000-2400 clones per each round (total 5600 clones).

In this study, we show an efficient approach to produce high enantioselective mutants of *Pseudomonas*

fluorescens esterase (PFE) using random mutagenesis within the substrate-binding site at Trp28, Val121, Phe198, and Val225 for hydrolysis of MBMP (methyl 3-bromo-2-methylpropionate). Instead of a detailed mechanistic study based on a crystal structure, we have used a homology model of PFE to select the substrate-binding sites. For each site, we screened about 192 clones that account >99.8% of the 32 possible codons (assuming equal incorporation) [6]. Five of the catalytically active mutants showed better enantioselectivity (up to 5-fold; $E = 61$ for Val121Ser, $E = 58$ for Trp28Leu) than wild-type PFE ($E = 12$). This result was more efficient than that from directed evolution ($E = 19$ for Thr229Ile) [7]. The enantioselectivity was more improved ($E = 61$ vs. 19) and the fraction of mutants with increased enantioselectivity is higher either (13% vs. <1%). In the further mutagenesis based on one of the best mutant (Trp29Leu), we found an even higher enantioselective mutant (Trp28Leu/Val225Cys, $E = 80$).

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