Creation of *Rhizopus oryzae* lipase with unique substrate specificity by using molecular display and combinatorial mutagenesis methods

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Lipase catalyzes the hydrolysis of lipids, the ester synthesis, and ester transfer reactions with high substrate specificity in organic solvents. *Rhizopus oryzae* lipase (ROL) shows broad substrate specificity toward middle-chain fatty acids and its active site consists of Ser, His, and Asp, and it has a flexible a-helical lid domain (the open and closed forms), which contributes to the substrate-recognition and covers the active site.

In this study, to create the modified ROL with shifted substrate specificity, a combinatorial mutation was introduced into 6 amino acid residues composed of the lid domain. Activity of mutants obtained was evaluated by halo assay and hydrolysis against fluorescent substrates by displaying modified ROLs with the mutated lid domains on the cell surface of yeast, *Saccharomyces cerevisiae* using the molecular display system, which is a powerful tool for high-throughput screening and for comprehensive investigations of relationship between structure and function of proteins. By the combinatorial mutations of Phe88, Ala91, and Ile92 in the lid domain, it revealed that the hydrophobic interaction between the substrate and amino acid residues in the open form of the lid domain is critical for ROL activity. Next, Thr93 and Asp94 were further combinatorially mutated to obtain a lager hydrophobic region in the lid open form. Among the combinatorial libraries, two clones, having (Thr93Thr and Asp94Ser) and (Thr93Ser and Asp94Ser), exhibited the substrate specificity toward short-chain fatty acids. It was suggested that unique oxyanion holes were formed and the hydrophobic patch became to be narrow with the analysis by computer modeling. Thus, a combination of combinatorial mutagenesis and the yeast cell surface display of ROL lead to the creation of modified ROLs with the unique substrate specificity toward short-chain fatty acids. Such unique ROLs would make important contributions to liquid biofuel production and food, oil, and pharmaceutical industries.