S 2-3

Genetic and Catalytic Trajectory of Tyrosine Phenol-lyase in Evolutionary Engineering

Eugene Rha, Su-Lim Choi, Jae Jun Song, Seung-Pyo Hong[1], Moon-Hee Sung[1,2], Seung-Goo Lee*

Lab. of Microbial Function, KRIBB, Daejeon 305-333, Korea. [1]BioLeaders Co., Daejeon 305-500, Korea. [2]Dept. Bio & Nanochemistry, Kookmin University, Seoul 136-702, Korea.

Evolutionary protein engineering is opening a new stage of biotechnology where an enzymatic function is engineered precisely by a screening-based molecular engineering without intensive biochemical or structural studies. Improvement of catalytic rate, thermal stability, and the specificity in molecular interaction has been the major subject of the evolutionary engineering for the last decade, and several of which are on the way to industrial applications.

Tyrosine phenol-lyase (TPL) catalyzes the synthesis of tyrosine analogs from phenolics, pyruvate, and ammonia. The phenolics include hydroxylated or halogenated derivatives of phenol or catechol. Two representative products, L-tyrosine and L-dopa, have been widely used as drug materials for Basedow's disease and Parkinson's disease, respectively. Though the reaction has been one of successful enzymatic processes, it is faulted by the low catalytic rate and the destabilization of the enzyme by phenolic substrates. Recently authors reported that the TPL of thermophilic *Symbiobacterium toebii* represents high stability at elevated temperatures, and also at high concentrations of phenolic materials. However, because pyruvate and phenolic analogs are deteriorated by heat or oxygen, the enzymatic process should be operated at suboptimal temperatures where the enzyme shows a low activity.

In this context the TPL from *Symbiobacterium toebii* was subjected to evolutionary engineering to improve the catalytic rate and thermal stability. Genetic and catalytic trajectory of the evolutionary engineering was analyzed to understand the structure-function relations of TPL. Genetic library of the enzyme was prepared by an error-prone PCR, cloned in a constitutive expression vector pHCEIIb, and subjected to a wellplate-based high throughput screening. After an extensive screening, three genetic variants superior in the activity and four variants improved in thermal stability were obtained. Sequence analysis showed selected variants bears one or two changes in amino acid sequences.

Evolutionary protein engineering takes the advantage of accumulating beneficial mutations and simultaneous removing deleterious mutations by combinatorial recombination of DNA fragments. This process greatly accelerates the move of protein function to the targeted direction. DNA shuffling, the first combinatorial recombination tool was enormously effective for this purpose, but a drawback at least was high frequency of mutations during the re-ligation PCR. The unexpected mutations ruined the synergistic combination of beneficial mutations. Conceptually similar but handier recombination protocol is the

staggered extension PCR (StEP) by F. A. Arnold and coworkers. Vent polymerase was very useful for a high accuracy StEP in order to recombine beneficial mutations, resulting in the improved activity and stability.

Sequence analyses showed certain mutations came up more frequently through the screening of shuffling-libraries: for example, T129I in the activity-screening and A13V in the stability-screening. The occurring frequency of the mutations was dependent on the screening conditions: for example, the more A13V-mutations on the higher stability-screening.

Structure alignment, based on the 3D-structure of *Citrobacter freundii* TPL, has assigned the A13V mutation at the N-terminal arm, which interacts with the symmetric arm of the other catalytic dimer and it forms H-bonds with the hydrophobic core of the tetrameric protein (Fig. 1). T129I locates at the interface between two catalytic dimers, in van der waals distance to T124, which is known to bind to the hydroxyl-group of the substrate.

Based on the additive increase in the activity and stability of TPL, it is likely individual characteristics assigned to certain structural units could be combined by a 'Lego-like' principle, in which different components are put together to get the designed output. Several mutants of this work acquired the activities comparable to the highly active enzymes from mesophilic bacteria, and at the same time they were stable up to 65℃, where the wild-type TPL is inactivated within 30 min. [This project was supported by Cleaner Production Program 10007946 of NCPC]

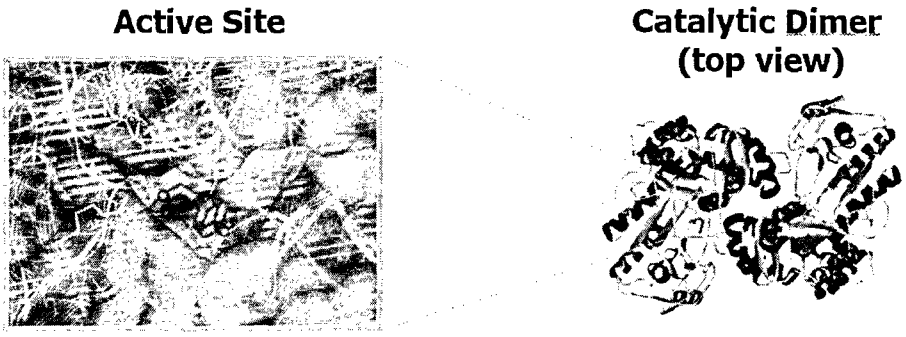

Fig. 1 Magnified view of the catalytic site (left) and structure of a catalytic dimer unit (right).