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Redesigning the enzymatic synthetic pathway of adrenaline to produce non-natural compound, Phenylephrine

(非天然型アドレナリン作動薬フェニレフリンの酵素による合成経路の構築)

Phenylephrine is an α 1-adrenoceptor agonist and primarily used as a decongestant to treat the common cold. Because phenylephrine is less likely to cause side effects like central nervous system stimulation, insomnia, anxiety, phenylephrine has become more widely use now. Phenylephrine is a non-natural compound and it is produced by organic synthesis. Although effective synthesis method has been developed, the synthesis processes usually require high pressure, high temperature, and organic solvents, which are unfriendly to the environment. On the other hand, recently, along with the developing of life science, using biosynthesis way to produce functional non-natural compound is becoming more and more viable. Hence, we consider employing biosynthesis in *E. coli* instead of organic synthesis for producing phenylephrine.

Based on the structural similarity between phenylephrine (m-OH on the benzene-ring) and adrenaline (p-OH and m-OH on the benzene-ring). The biosynthesis pathway of adrenaline could be treated as a candidate to produce phenylephrine by modifying substrate-recognition of enzymes. In nature, adrenaline is synthesized from Dopa in chromaffin cells of adrenal medulla by three crucial enzymes: 3,4-dihydroxyphenyl-L-alanine (Dopa) decarboxylase (DopaDC) which catalyze Dopa to produce dopamine, Dopamine β -monooxygenase (DBH) which catalyze dopamine to noradrenaline, and Phenylethanolamine N-methyltransferase (PNMT) which catalyze noradrenaline to adrenaline. We purposed to design a new biosynthesis pathway, though mimic the adrenaline biosynthesis of 3 steps reaction catalyzed by DDC, DBH and PNMT but using one nature exist compound, m-tyrosine as starting substrate to produce phenylephrine.

For the first step, understanding the substrate recognition mechanism of aromatic amino acid decarboxylases (AADCs) which including DopaDC and Plant tyrosine decarboxylase (TyrDC) will assist us to create one new decarboxylase that catalyzes the m-tyrosine to m-tyramine. TyrDC is a group II pyridoxal 5'-phosphate (PLP) -dependent decarboxylase that mainly catalyzes the decarboxylation of p-tyrosine to tyramine. In this study, we determined crystal structures of plant TyrDCII (type II) form *Papaver somniferum*, in apo form (*PsTyrDCII*), PLP-binding form (*PsTyrDCII*-PLP), PLP-inhibitor-binding binding (*PsTyrDCII*-PLP-CarbiDopa), and PLP-substrate-binding form of mutant H203F (*PsTyrDCII*H203F-PLP-Tyr). Like other DopaDCs and TyrDCs, *PsTyrDCII* forms a homodimer with two

active sites each contributed to by two monomers, and three homodimers (molAB, molCD, and molEF) exist in an asymmetric unit. By structural comparison with DopaDC and some other AADCs, PLP-binding and the substrate specificity of TyrDC and DopaDC have been cleared. The structures of *PsTyrDC* and *PsTyrDC*-PLP showed that, unlike mammalian DopaDC, the binding of PLP does not induce distinct conformational changes in plant TryDC regarding the overall structure, but the PLP binding pocket displays conformational changes at Phe124 of the other monomer (Phe124^{MolB}), His203, and Thr262. The center (Ala368^{MolB} – Ser370^{MolB}) of a loop (Trp365^{MolB} – Arg374^{MolB}) that interacts with the phosphate group of PLP to anchor PLP, did not show conformational changes between *PsTyrDC* and *PsTyrDC*-PLP but differed from the structures of DopaDC. The enzymatic activity assay experiments of TyrDC and DopaDC with dopa, p-tyrosine, m-tyrosine show that TyrDC reacts with dopa and p-tyrosine in similar level but it has rarely activity to m-tyrosine, while DopaDC is mainly react with Dopa and also has activity for m-tyrosine but no activity for p-tyrosine. By superimposing the structures of *PsTyrDCII*-PLP-carbiDopa with DopaDC-PLP-carbiDopa, we found that the substrate specificity is related to Ser103/Thr82 for p-OH and Ser370^{MolB}/Gly354^{MolB} and also the center (Ala368^{MolB}/Pro352^{MolB} – Ser370^{MolB}/Gly354^{MolB}) of the loop for m-OH, in TyrDC/DopaDC, respectively. The crystal structure of *PsTyrDCII*-PLP shows that His203 is important for stabilize the PLP binding by forming the pi-pi stacking with PLP pyridine ring. We also found that mutants of H203F and H203A lost the almost activity. The crystal of *PsTyrDCII*H203F-PLP-Tyr shows H203 not only important for PLP binding but also play key role for binding substrate in right position to start the reaction.

For the second step reaction from m-tyramine to producing norphenylephrine, we tried several enzymes. DBHs from *Homo sapiens* and *Rattus* were tried to overexpress in *E. coli* and yeast expression system. Unfortunately, the most proteins were expressed in inclusion body. Now, we are trying to a promise cytochrome P450 which could change its substrate specificity by adding decoy molecules may help us finish this step reaction.

For the last step reaction from norphenylephrine to phenylephrine, PNMT from *Homo sapiens* was overexpressed and purified using *E. coli* expression system successfully. The enzymatic activity assay shows PNMT could react with noradrenaline and norphenylephrine as similar level. Based on the structure of PNMT, we found Val53, Met258, Val269 and Val272 is closed to the benzene ring of substrate, and mutated of these residues to change the substrate specificity. However, the results were not show the changes as we expected. The structural simulation showed that the space around these residues allow the enzyme reacts varies substrate in a same level.

In this study, we tried to build one possible biosynthesis pathway to produce non-natural compound, phenylephrine. The study provided a structural basis for engineering and application of AADC, DBH, PNMT in the biocatalytic synthesis of phenylephrine.