Summary of Dissertation

Tittle: Development of activity-based ultra-high-throughput screening system of peroxidase by using bead display

Name: 朱　博　(ZHU Bo)

The C1a isoenzyme of horseradish peroxidase (HRP) is an industrially important heme-containing enzyme that utilizes hydrogen peroxide to oxidize a wide variety of inorganic and organic compounds for practical applications, including synthesis of fine chemicals, medical diagnostics, and bioremediation. Bead display is an *in vitro* biomolecular display technology established in our laboratory by combining the emulsion PCR and cell-free protein synthesis (CFPS) system, which can be used for making the linkage between the genotype and phenotype of an individual mutant of target proteins. The purpose of this study is to develop a ultra-high-throughput screening system for peroxidase (Figure 1), such as HRP, by using the optimized bead display technology.

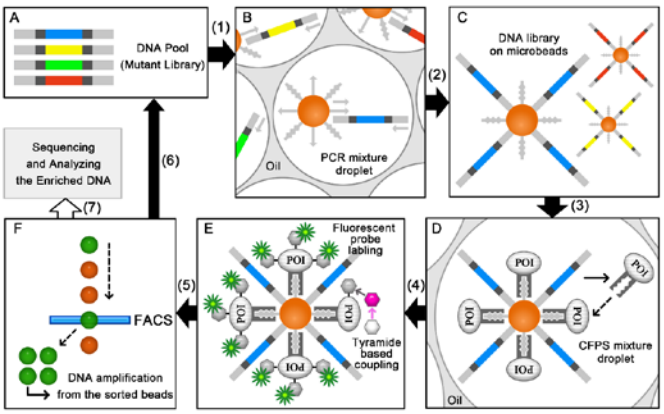

Figure 1. Scheme showing the principle of the bead display-based uHTS platform for peroxidase.

HRP is difficult to be expressed in *Escherichia coli* since it contains four non-consecutive disulfide bonds and hemin prosthetic group. In the Chapter 2, we established the *E. coli*-based CFPS synthesis system for producing the soluble and active holo-HRP. The sequence of mature HRP with hemagglutinin-tag and polyhistidine-tag was first optimized for *E. coli* expression by removing rare codons, adjusting GC content and avoiding the formation of secondary structure of its messenger RNA. T7 promoter was used to start the expression. Then, this sequence was synthesized and utilized as the template in CFPS. The soluble and active expression was successful by adding disulfide bond

isomerase DsbC and optimizing the calcium ion concentration, hemin concentration, redox potential and temperature of CFPS reaction. The soluble HRP was purified and its specific activity was 76% of that of the native enzyme commercially available.

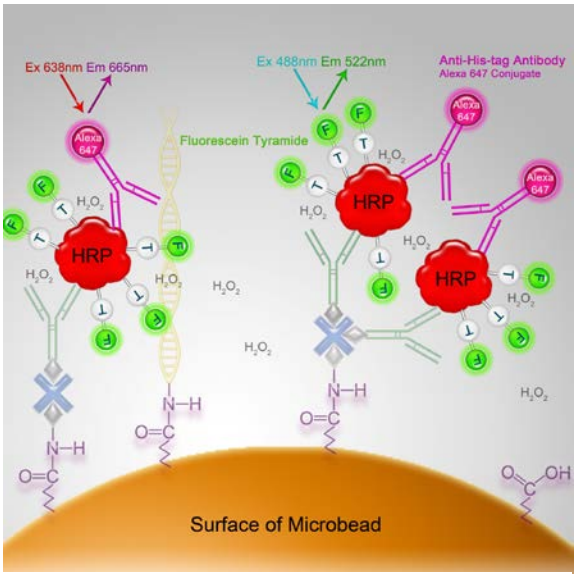

Figure 2. Fluorescein-tyramide-based fluorogenic assay for HRP immobilized on microbeads.

In the Chapter 3, the immobilization method of HRP on microbeads by antibody and the fluorogenic assay of HRP for ultra-high-throughput activity detection were established (Figure 2). Together with H_2O_2, HRP can catalyze tyramides to short-lived tyramide radicals, which can form a covalent bond with nearby tyrosine or tryptophan residues of HRP or antibody. The HRP immobilized beads were subjected to fluorescein-tyramide assay, then the microbeads were analyzed by flow cytometer. The significantly higher fluorescent signal of HRP immobilized beads was observed comparing with the result of microbeads without HRP.

In the Chapter 4, the biosynthesized HRP was fused with a DNA-binding tag at its N-terminal and C-terminal sites for improving its immobilization efficiency, and a model screening by using the improved display method was demonstrated. we have generated a DNA-binding tag, scCro-tag, developed from a single-chain derivative of the bacteriophage lambda Cro repressor. It has relatively low molecular weight, and it binds O_R consensus DNA (ORC) as a 1:1 complex with high affinity. The addition of the scCro-tag at both ends increased the solubility of HRP. The HRP fusion proteins were displayed on microbeads attached with double-stranded ORC DNA. The activities of the immobilized HRP fusion proteins were detected with a biotin-tyramide/streptavidin-Cy5-based fluorogenic assay using flow cytometry (Figure 3). Moreover, a model microbead library containing wild type *hrp* (WT) and inactive mutant (MUT) genes was screened

using fluorescence-activated cell-sorting, thus efficiently enriching the WT gene from the 1:100 (WT:MUT) library.

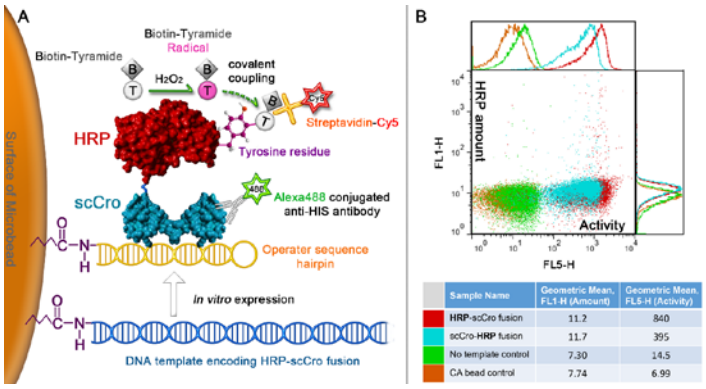

Figure 3. A tyramide-based fluorogenic assay for the HRP displayed on microbeads. (A) Scheme corresponding to the fluorogenic assay. (B) Dot-plot results of the flow cytometric analysis.

In the Chapter 5, the biochemical reactions for bead display were optimized, including emulsion PCR and emulsion CFPS. For the DNA template longer than 450 bp, such as *hrp-sccro* (aprox. 1700 bp), the amplifying efficiency is much lower than the shorter DNA in emulsion PCR. By examining the different DNA polymerases, additives and cycling programs, the amplifying efficiency of emulsion PCR was significantly improved. Next, the emulsion competent used for CFPS was optimized, because the choice of surfactant and continuous phase for emulsion is critical for the activity and stability of the enzymes synthesized in emulsion droplet. An *in situ* assay (Figures 4) was established to detect the expression level of the active HRP in different kinds of emulsions, by using the substrate delivery method. The HRP synthesized in emulsion with 3% SunSoft No. 818SK showed the highest activity among tested conditions. The post-translational hemin integrating method for HRP synthesized and immobilized inside emulsion droplets had also been established. It makes the survey of non-natural prosthetic groups for HRP possible during the screening of the desired HRP mutants.

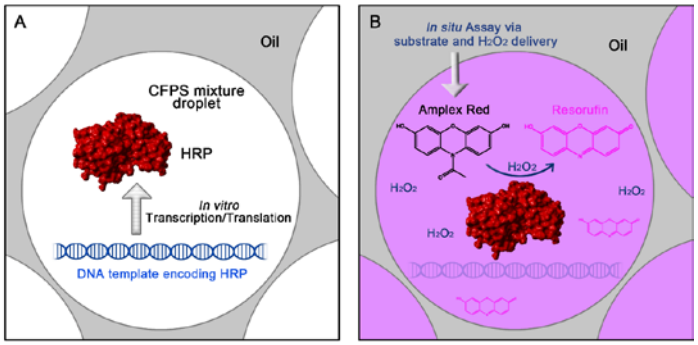

Figure 4. Scheme of the *in situ* activity assay for optimization of emulsion conditions for CFPS of HRP. (A) CFPS of HRP inside emulsion droplets. (B) Substrate delivery for detecting the activity of HRP.

In the Chapter 6, a method of stabilizing the linearized DNA template in CPFS reaction by using the scCro had been discussed. The expression level of superfolder green fluorescent protein

was 4 times increased by the protection of the linearized DNA templates from degradation using scCro.

The techniques described here could serve as a novel platform for the ultra-high-throughput discovery of more useful HRP mutants and other heme-containing peroxidases.