This thesis is submitted to the Department of Biochemistry and molecular biology, Shanghai Jiao Tong University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Robust and Efficient Artificial Multiple Enzyme Cascades for Biosynthesis of Bioactive Terpenoid Glycosides

Author: Mohamed Yassin Ali

Student ID: 016080990004

Major: Biology

Research area: Biochemistry & Molecular Biology

Advisor: Prof. Yan Feng

School of Life Sciences and Biotechnology

Shanghai Jiao Tong University

Abstract

Multienzymatic cascade reactions are the most important technology for industrial process development, such as synthesizing pharmaceutical, cosmetic, and nutritional compounds. The widely existing MECs in Nature have inspired researchers to design synthetic analogs to promote the overall catalytic efficiency in *vitro* by co-localizing multiple enzymes to mimic the MECs' unique functionalities. Enzymes complexes are designed using either of three types of routes: fusion proteins, enzyme scaffolds, or immobilization. The development of coimmobilized multi-enzymatic systems is increasingly driven by economic and environmental constraints that provide an impetus to develop alternatives to conventional multistep synthetic methods. As in nature, enzyme-based systems work cooperatively to direct the formation of the desired products within the defined compartmentalization of a cell. In an attempt to mimic biology, coimmobilization is intended to immobilize a number of sequential or cooperating biocatalysts on the same support to impart stability and enhance reaction kinetics by optimizing catalytic turnover. This thesis work has been focusing on establishing multienzymes cascade reactions and developing a novel strategy based on enzyme immobilization to design multienzyme nanodevices for multi-enzyme co-localization to realize kinetics enhancement and strongly control the spatial arrangement of the enzymes.

1. Highly Efficient Biosynthesis of Glycyrrhetinic Acid Glucosides by Coupling of Microbial Glycosyltransferase to Plant Sucrose Synthase

A novel glycosyltransferase, termed as UGT109A3, from *Bacillus subtilis* was identified to catalyze the synthesis of

glycyrrhetinic acid glycoside derivatives from GA. The UGT109A3 was functionally expressed in *Escherichia coli* and purified as the biocatalyst for the GA's glycosylation by Ni-NTA affinity chromatography. As a result, the UGT109A3 can glycosylate both the free C3 hydroxyl and C30 carboxyl groups of GA to yield a unique 3, 30-*O*-*β*-D-diglucoside-GA. To produce valuable GA-glycosides in an efficient and cost-effective way, we constructed an artificial biocatalytic cascade by coupling the microbial UGT109A3 to plant sucrose synthase (SUS). With fed-batch glycosylation, a large scale of GA-glucoside (4.98 g/L, 6.26 mM in 8 hours) could be enzymatically transformed from GA. The obtained GA-diglucoside showed a significant water solubility improvement around 3.4×10^3 fold compared with that of the parent GA (29 μM). Moreover, it also exhibited a dose-dependent cytotoxic towards human colon carcinoma Caco-2 cell line according to MTT assay, having an IC_{50} at 160 μM. This study builds up an efficient platform for producing GA-glucosides and is valuable for further biosynthesis of other complex glycosylated natural products.

2.Ordered Co-Immobilization of Multimeric Enzyme Arrays with Enhanced Biocatalytic Cascade Performance

The immobilization of multi-enzymes on the support materials has been one of the most efficient approaches for increasing the enzymatic activity via substrate channeling and improving the stability and reusability of enzymes. Herein, we explored a novel strategy to rapidly achieve orderly co-immobilizing biocatalysts based on the SpyTag/SpyCatcher system of orthogonally reactive split peptides and polyhistidine (His)-tagged proteins and nitrilotriacetic acid (NTA)-modified surfaces as a general approach for a stable, specific and oriented multiple protein immobilization. The SpyCatcher fragment and its binding partner SpyTag peptide were fused to a UGT51

glycosyltransferase mutant (UGTm, S81A/L82A/V84A/K92A/E96K/S129A/N172D) and the tetrameric sucrose synthase (SUS), respectively. The multimeric fusion proteins were self-assembled into a supramolecular device, forming a covalently linked enzyme cascade that facilitating UDP-glucose regeneration and converting Protopanaxadiol (PPD) into rare ginsenosides Rh2. Next, we immobilized self-assembled MENCs on a Ni^{2+}: NTA-functionalized support via the constituent enzymes' abundant polyhistidine tags. The self-assembled UGTm-SUS MENCs was successfully immobilized onto the microspheres-modified surface.

The morphology and ultrastructure were examined using various electron microscopy techniques to analyze the ordered co-immobilization biomaterials. The morphology of the immobilized MENCs and the results found that particle-like nanolayers cover the supports, and the particles are highly ordered arrayed on the surface square microns in size. More importantly, the enzymatic analysis revealed that enzyme activity of the ordered co-immobilized UGTm-SUS MENCs was 3.8- and 2.3-folds higher conversion rate compared with the random co-immobilization. Moreover, the orderly co-immobilized UGTm-SUS arrays led to a 3-fold decreased loading of the costly UDP than the random co-immobilization.

The ordered co-immobilized UGTm-SUS array significantly enhanced the immobilization rate and catalytic efficiency of the biocatalytic cascade. It displayed significant improvements in UDPG regeneration, storage stability,and reusability compared to those of the random co-immobilized and free MENCs system. This study provides a great promise for fabricating enzyme arrays and highlights the synergistic benefits of nanocomplexes in enhancing biocatalytic cascade performance. Looking forward, the design of sustainable and re-usable multi-enzyme biocatalysts would lead to both scientifically

exciting research as well as economically viable designs for next-generation catalysts and biosensors.

Keywords: Cascade reactions, multimeric enzymes, self-assembly, co-immobilization, glycosyltransferase, sucrose synthase, UDP-glucose regeneration, protopanaxadiol, rare ginsenoside Rh2, glycyrrhetinic acid.