INVESTIGATION OF KEY ENZYMES PROPERTY IN NARINGENIN BIOSYTHESIS

by

XINYU GONG

(Under the Direction of YAJUN YAN)

ABSTRACT

Flavonoids, such as naringenin, are natural products with high bioactivities and pharmaceutical value, whose titer, however, was limited in microbial biosynthesis. Microbes are enabling platforms to acquire naringenin via introduced artificial pathways. The metabolic pathway derived from L-tyrosine for naringenin biosynthesis requires four enzymes, including tyrosine ammonium lyase (TAL), 4-courmarate:CoA ligase (4CL), chalcone synthesis (CHS), and chalcone isomerase (CHI). Investigating enzyme property, the key elements of the pathway, shows insight for future advances. In this thesis, we systemically characterized $K_M$ and $k_{cat}$ of RgTAL, Pc4CL, and MsCHI for the first time with sufficient energy and cofactors supply. Meanwhile, the enzyme performance of PhCHS was evaluated by product conversion rate and identified as a rate-limiting enzyme here, with a 3% naringenin conversion. These results implied directions to pathway engineering. The high-efficiency naringenin microbial factory will boost its potential industrial production, and further make great contributions to human health and nutrition.

INDEX WORDS: in vitro enzyme assay, naringenin, flavonoid, cell-free, biocatalysis

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XINYU GONG

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| | |
|---|---|
| Major Professor: | Yajun Yan |
| Committee: | James N Warnock |
| | Cheryl Gomillion |

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
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# CHAPTER 1

## INTRODUCTION

Flavonoid is a large family of secondary metabolites from plants, especially citrus fruits, which have high pharmaceutical activity (1). As a member of flavonoid, naringenin is a high-value bioproduct, mainly existing in grapefruit naturally, with antioxidant (2), anti-bacterial (3), anti-tumor (4), and anti-inflammatory (5) properties. It can also be extracted from grapefruit, orange, and lemon (6), however, its natural sources are very limited due to its low content in plants, the long growth cycle of plants, and complicated chemical extraction process (7, 8). Therefore, the microbial factory, as a promising strategy, is widely used in the biosynthesis of valuable compounds. A comprehensive naringenin pathway was constructed in *Escherichia coli* (*E. coli*) for years (9-11), derived from endogenous aromatic synthesis pathway with four heterogenous enzymes (Figure 1). This pathway starts at L-tyrosine and ends with naringenin. Initially, with the deamination reaction catalyzed by tyrosine ammonia lyase from *Rhodotorula glutinis* (RgTAL) (10), tyrosine converts into p-coumaric acid substituting the amino group to a double bond. Next, p-coumaric acid and coenzyme A could form its CoA ester, p-coumaroyl CoA, with the help of 4-coumarate:CoA ligase from *Petroselinum crispus* (Pc4CL) (10). After that, the cycloaddition of malonyl-CoA and naringenin chalcone generation are triggered by chalcone synthase from *Petunia hybrida* (PhCHS) (10). Although, naringenin chalcone is unstable and is easy to form naringenin spontaneously by a slight change of PH or temperature in the reaction system, finally, chalcone isomerase from *Medicago sativa* (MsCHI) (10) will accelerate the isomerization process to produce naringenin.

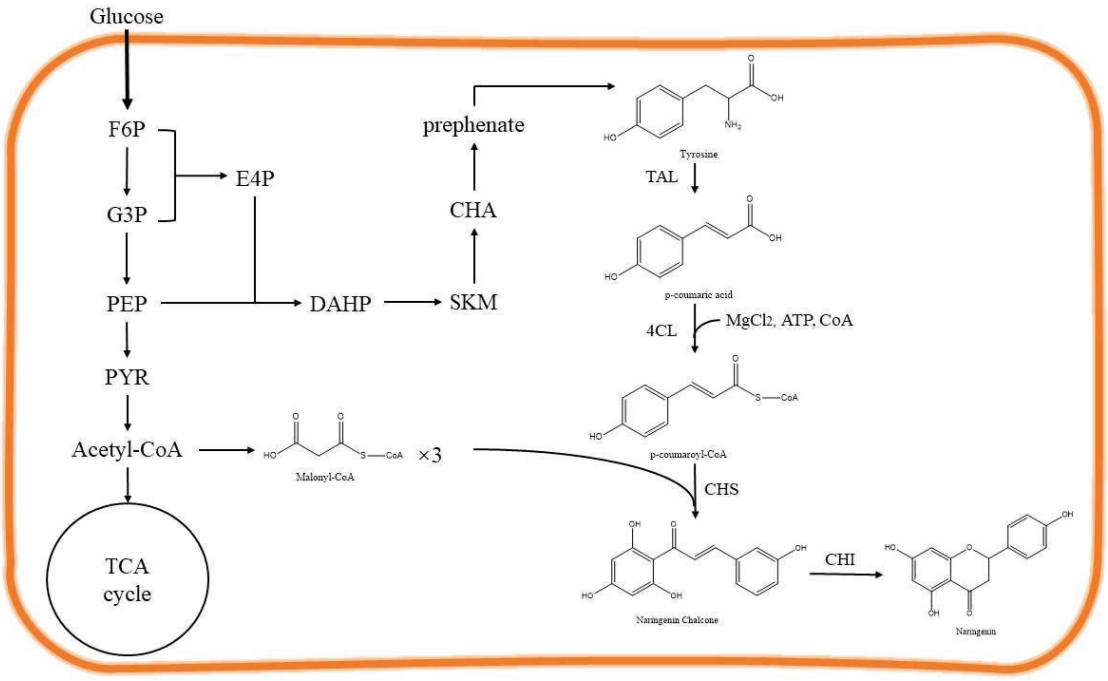


Figure 1: First naringenin pathway constructed in *Escherichia coli* (9). This pathway was derived from endogenous aromatic synthesis pathway with four heterogenous enzymes, TAL, 4CL, CHS, and CHI, respectively.

The naringenin biosynthesis pathway had been successfully constructed in *E. coli* since 2003, while the low titer of naringenin in microbial factories remained the biggest challenge and impeded its industrial application (9). A lot of effort was applied to engineer the host cell, such as adjustment of the carbon flux and optimization gene expression of this pathway, including different enzyme sources comparison (12), the different expression level of the gene clusters (11), inhibition of bypass by anti-sense RNA or CRISPRi (13-15), quorum-sensing circuit for metabolic flux control (16), etc. Nevertheless, the highest production of naringenin in *E. coli* so far is 421.6 mg/L from tyrosine achieved by Wu et al. (14). The previous study came up with the obstacles existing in this pathway as follows. Firstly, p-coumaroyl CoA would abolish the enzyme activity

of TAL and was addressed as a limiting precursor for naringenin biosynthesis (10). Secondly, malonyl-CoA was defined as a limiting cofactor during naringenin production due to the distinctly positive effect of malonyl-CoA supply improvement (12, 13). Lastly, nearly all research put their eyesight on pathway engineering and ignored the importance of pathway enzyme. It is noticeable that the enzyme candidates are very limited and there are not so many candidates showing a prominent enzyme property. As the result, enzyme mining and screening are particularly important and tough tasks in naringenin biosynthesis.

The cell-free system eliminates the dependence on the host cell for biosynthesis through the synthetic biology approach, which allows direct monitoring and easy manipulation of the reaction system. The aim of such in vitro system focuses on accelerating the design-build-test (DBT) process, verifying the enzyme feasibility, and shortening the experiment cycle (17). To achieve this goal, Karim et. al demonstrated a platform for in vitro prototyping and rapid optimization of biosynthesis enzymes (18). The superiority of this method was embodied in the controllable and flexible reaction process. The content, condition, and time course of the reaction were all under control. The results even can be predictable by building up the mathematic model (19). Zang et. al investigated a three-step enzyme reaction from p-coumaric acid to naringenin biosynthesis in vitro (12). Through comparison of the two to three enzyme candidates from different sources for each step reaction, they searched the best in-vitro reaction condition and combination of the enzymes by evaluating naringenin yield. However, the significance of the enzyme was rarely demonstrated, and the basic enzyme properties had not been systematically studied so far in vitro. One of the parameters used to describe the enzyme property is Michaelis constant ($K_M$) (20). It is numerically equal to the substrate concentration at which the reaction rate is half of Vmax. The other one is turnover number, called $k_{cat}$ (21), representing the maximum

number of substrate molecules converted to products activity site per unit time. And the ratio of $k_{cat}$ and $K_M$ ($k_{cat}/K_M$) is defined as catalytic efficiency (21), which can be used to quantitate the specificity of the enzyme for a substrate.

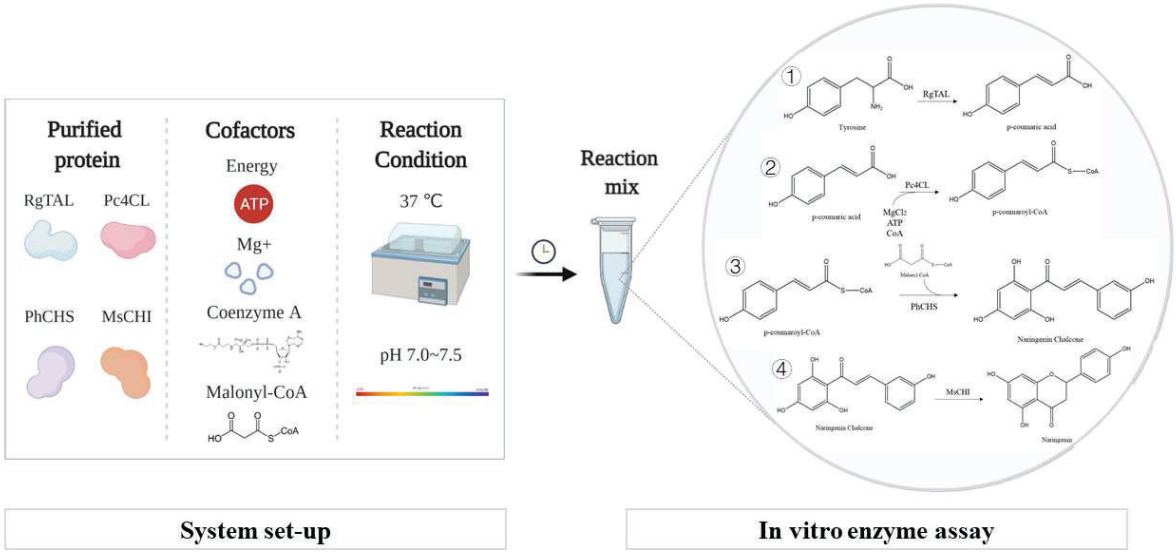


Figure 2: In vitro enzyme assay system. The cell-free system contained purified protein and necessary cofactors with suitable reaction conditions (constant temperature: 37℃, pH: 7.0~7.5).

In this thesis, the enzyme property of the RgTAL, Pc4CL, PhCHS, and MsCHI was investigated by in vitro reaction system that mimicked the fermentation condition in *E. coli* with pH 7~7.5 and constant temperature 37℃ (Figure 2). Based on the stoichiometric experiment design, we successfully obtained the basal parameters for each enzyme except CHS. The catalytic efficiency (kcat/KM) of RgTAL, Pc4CL, and MsCHI were 1.490×10-3, 2.798×10-3, and 52.472, respectively. Unfortunately, the enzyme property of PhCHS was not able to gain directly. This was because of the quite low conversion of its product, caused by low enzyme activity. Therefore, the enzyme performance of PhCHS was showed as the conversion rate from p-coumaric acid to

naringenin. The highest conversion here was 3% at 1000 μM p-coumaric acid. Meanwhile, this step was also recognized as rate-limiting here. This enzymatic information would give great support to the naringenin pathway optimization in vivo in further research.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Market value and global demand of naringenin

Flavonoids are aromatic and colorless glycosides ubiquitously existed in plants (22). Naringenin, as one type of flavonoid, has outstanding biological activities in the medical area. Not only can naringenin play an important role in nutraceuticals, but also it has remarkable performance in disease treatments (23). The preclinic pharmacological activities of naringenin in several disease models, such as anti- Alzheimer (24), anticancer (25), and antidiabetic (26), were proved by many research groups. With the rising health consciousness and increasing awareness of the benefits of consuming naringenin, the demand for naringenin will continue improving. According to a marketing report from MARKET RESEARCH FUTURE in 2021, the global flavonoids market is expected to register a growth rate of 3% during the forecast period of 2019 to 2024 to reach USD 140 million by the end of 2024 (34). However, insufficient bioavailability is hampering its market growth.

The current naringenin synthesis approaches mainly divides into three parts, which are extracted from plants, chemical synthesis, and biological synthesis. (8, 27). The chemical synthesis was also not recommended, because strict reaction conditions are needed to keep the high selectivity (8). Instead, the microbial factory was a well-developed strategy with low-cost, environment-friendly, and time-saving properties, relying on microorganism's metabolism (28). The plant-derived pathways and enzymes gave a hand to the biological synthesis of naringenin in

microbial hosts through metabolic engineering approaches (29). In this way, the biosynthesis has potential to fill the gap in the naringenin production global market and boost its market size.

2.2 Naringenin biosynthesis in *E. coli*

The naringin biosynthesis of naringenin in the model organism, *E. coli*, was successfully construct for years (Figure 1). Hwang et al. firstly reported the microbial synthesis of flavonoids in *E. coli* containing an artificial gene cluster in 2003; a variety of flavonoids biosynthetic pathways have been flourished in microorganisms from then on (9). In 2011, Nicole et al. optimized this heterogenous pathway for naringenin production from glucose with a titer of 84 mg/l with the addition of cerulenin (10). The optimization included the fatty acid synthesis bypass inhibition with cerulenin addition and the improvement of malonyl-CoA supply by utilizing a recombinant malonate assimilation pathway from *Rhizobium trifolii*. They also demonstrated that one intermediate here, p-coumaryl-CoA, inhibited TAL activity, which influenced the production yield of naringenin. Based on this statement, Dinh et. al developed an autonomous and bifunctional quorum-sensing circuit for metabolic flux control in 2019 (16). In this study, they dynamically down-regulated endogenous gene expression for malonyl-CoA accumulation to improve naringenin titers. The final titer was 125.9mg/L in modified MOPS minimal medium containing 5 g/L D-glucose and 500 mg/L tyrosine.

Static pathway regulation was also applied to this pathway. Wu et al. believed that modular static optimization of heterologous pathways by changing the expression level of the enzymes would benefit the naringenin de novo synthesis (11). Here in this research, total modular expression was regulated using different promoter strength and plasmid copy numbers; and the optimum strain could produce 100.64 mg/L naringenin directly from glucose in *E. coli*. With the development of asRNA and CRISPR interference systems, the naringenin production strategies

were further updated by applying these biotechnologies. The asRNA was used to silence genes related to fatty acid synthesis in order to fine-tune the fatty acid pathway for malonyl-CoA accumulation by Wu et al. in 2014 (13). The naringenin production from L-tyrosine with appropriate inhibitory efficiency of the anti-fabB/fabF asRNA improved the production titer to 391 mg/L. Later, in 2015, systematically tuning the central metabolic pathways based on a CRISPRi system in *E. coli* for naringenin production was established by the same research group (14). This system was able to efficiently channel carbon flux toward malonyl-CoA. By coupling the genetic modifications to cell growth, the combined effects of these genetic perturbations increased the final naringenin titer to 421.6 mg/L.

From the previous study, it was evident that most of the studies put their effort into cofactor accumulation by host cell engineering to improve production. However, little effort was put into pathway enzyme investigation. Only by fully understanding the characteristics of the key enzymes in the metabolic pathway and troubleshooting the limiting factors can we better engineer and optimize this pathway.

2.3 Biosynthesis within cell-free system

The cell-free based metabolic engineering was a hotspot research area in recent years. Cellular metabolic engineering exhibited several restrictions like suitable pH (7.0~7.5), temperature (37℃), and culture medium for cell production (30). Meanwhile, cell viability is easily influenced by involving intermediates or products that are toxic to the cell. The complexity regulation of cellular metabolism is another barrier for efficient target compound production (31). Because of these characteristics, most natural products do not have efficient host cells to support high-yield production to meet the demands of current natural product markets. The most

straightforward solution to the limitations is to use purified enzymes to build cell-free metabolic pathways (32, 33).

Cell-free metabolic engineering has great enzyme screening capability. In 2020, Karim et al. published a cell-free system called iPROBE, namely, in vitro prototyping and rapid optimization of biosynthetic enzymes (18). This system allowed simple and fast screening for the enzyme candidates for pathway design with their TREE score metric. One application of iPROBE was in vitro prototyping of limonene biosynthesis using cell-free protein synthesis (19). They tested 580 unique pathways with different cell-free protein combinations, and the highest limonene titer was 610 mg/L.

For cell-free naringenin synthesis, Zang et al. constructed a three-step naringenin pathway starting from p-coumaric acid in vitro with two to three enzyme candidates for each step (12). After analysis of different pathway enzyme ratios, substrate concentration, and cofactors concentration, 4CL and CHS were found to be crucial to the reaction and malonyl-CoA was identified as the limiting factor. The results indicated that the best loading ratio of 4CL/CHS/CHI was 10:10:1, which was the first attempt for in vitro naringenin synthesis. Cell-free metabolic engineering is expected to provide an alternative method for the biological pathway design, optimization, and build for those valuable compounds with low in vivo production.

# CHAPTER 3

# METHODOLOGY

## 3.1 Media, strains, and chemicals

Luria-Bertani (LB) medium was used for inoculants preparation, cell cultivation, and protein expression. When needed, ampicillin was added to the medium to the final concentrations of 100 μg/ml. *E. coli* XL1-Blue was used as the host strain for plasmid construction and propagation. *E. coli* BL21 Star (DE3) was used for protein expression and purification. Plasmid pETDuet-1 was employed for protein expression and purification. The details of the strains and plasmids used in this study are depicted in Table 1.

**Table 1. Strains and plasmids used in this study.**

| **Strain** | **Genotype** | **Source** |
|---|---|---|
| **XL1-Blue** | recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacIqZΔM15 Tn10 (Tetr)] | Stratagene |
| **E. coli BL21* (DE3)** | F- ompT hsdSB ($r_B^- m_B^-$) gal dcm rnel31 | Invitrogen |
| | | |
| **Plasmids** | **Description** | **Reference** |
| **pETDuet-1** | pT7, PBR322 ori, Ampr | Novagen |
| **pETDuet-RgTAL** | pETDuet-1 containing RgTAL from Rhodotorula glutinis | This study |
| **pETDuet-Pc4CL** | pETDuet-1 containing Pc4CL from Petroselinum crispus | This study |
| **pETDuet-PhCHS** | pETDuet-1 containing PhCHS from Petunia hybrida | This study |
| **pETDuet-MsCHI** | pETDuet-1 containing MsCHI from Medicago sativa | This study |

All chemicals were purchased from Sigma-Aldrich unless otherwise noted. Phusion High-Fidelity DNA polymerase, restriction endonucleases and Quick Ligation Kit were purchased from

New England Biolabs (Beverly, MA, USA). Plasmid Miniprep Kit, Gel Recovery Kit, DNA Cleanup kit and His-Spin protein miniprep kit were purchased from Zymo Research (Irvine, CA, USA).

3.2 DNA manipulation

The codon-optimized gene clusters RgTAL, Pc4CL, PhCHS and MsCHI are invited for previous published study(10). Plasmid pETDuet-RgTAL was constructed by cloning RgTAL sequence into pETDuet-1 using BamHI and SalI. The same DNA manipulation process was applied to the construction of other plasmids, including pETDuet-Pc4CL, pETDuet-PhCHS, and pETDuet-MsCHI.

3.3 Expression and extraction of enzymes

The TAL gene from *R. glutinis* (RgTAL), 4CL genes from *P. crispus* (Pc4CL), the CHS genes from *P. hybrid* (PhCHS), and the CHI genes from *M. sativa* (MsCHI) were expressed in *E. coli* BL21 Star (DE3) for protein expression. The recombinant *E. coli* strains were grown in 250 ml shake flask with 50ml LB medium containing ampicillin (100 μg/ml) at 37 °C and 270 rpm until the optical density at 600 nm ($OD_{600}$) reached to 0.6−0.8. After that, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to 0.5 mM, and the further cultivation was grown at 30 °C overnight. Cells were then harvested and lysed by French Press. The recombinant protein with an N-terminal multi-histidine tag was purified using His-Spin protein miniprep kit (ZYMO RESEARCH). The enzyme concentration was measured using BCA kit (Pierce Chemicals).

3.4 In vitro enzyme activity assay of RgTAL and Pc4CL

The standard enzyme assay of RgTAL was performed by making an 100ml assay mixture containing 50mM Tris-HCL buffer (PH=7~7.5), 7.58μg purified enzyme, and 0 to 200 μM L-tyrosine as the substrate. The reaction system was kept at 37 °C for 30 min and stopped by boiling.

The reaction rates were represented by measuring the formation of p-coumaric acid via HPLC. The kinetic parameters were calculated with Lineweaver-Burk plot. Each data point was the average of duplicate or triplicate experiments. Error bars indicate standard deviations of the presented results.

To assess the activity of Pc4CL from different plant sources, p-coumaric acid was used as a substrate to produce coumaroyl-CoA. The reaction containing p-coumaric acid ranging from 0 to 100 μM, 5 mM MgCl2, 5 mM ATP, and 0.5 mM CoA was initiated by adding 8.1μg purified 4CL, and then using Tris-HCL buffer (PH=7~7.5) to adjust the final volume to 100 μL. The enzyme reaction was terminated by boiling. The reaction rates were presented by measuring the consumption of p-coumaric acid via HPLC. The kinetic parameters were calculated with Lineweaver-Burk plot. Each data point was the average of duplicate or triplicate experiments. Error bars indicate standard deviations of the presented results.

3.5 In vitro enzyme activity assay of MsCHI

The spectrophotometric assays of MsCHI to detect the formation of naringenin were performed at 390 nm at room temperature for 30 s. The 100 μL reaction system contains Tris–HCl (50 mM, pH=7.0~7.5), purified enzyme (8.465×10-3μg/100μL MsCHI) and different amount of naringenin chalcone. The substrate concentrations varied from 0 to 100 μM. The kinetic parameters were calculated with Lineweaver-Burk plot. Each data point was the average of duplicate or triplicate experiments. Error bars indicate standard deviations of the presented results.

3.6 Synergistic enzymatic assay of Pc4CL, PhCHS, and MsCHI

The enzyme assay of PhCHS was conducted with the combination of Pc4CL and MsCHI. The synergistic enzymatic activity of these enzymes was monitored based on the yield of the product naringenin. The p-coumaric acid, as the substrate, concentrations varied from 0 to 1000

μM. The 100 μL reaction system consisted of all the cofactors needed for 4CL and the three enzymes. Analysis of the enzymatic products was performed using high-performance liquid chromatography (HPLC). The ideal naringenin production showed 100% substrate convert to naringenin. The calculation of conversion rate was followed according to the formula below:

$$conversion\ rate = \frac{real\ naringenin\ production}{ideal\ naringenin\ production} \times 100\%$$

All results were obtained with three biological replicates. Error bars indicate standard deviations of the presented results.

3.7 HPLC analysis

For quantification of compounds in vitro enzyme assay, HPLC system 1260 infinity II (Agilent technologies) equipped with a reverse phase ZORBAX SB-C18 column and a 1260 infinity II Diode Array Detector WR was used. The oven and column temperature were both set at 25°C. The mobile phase at a flow rate of 1 mL per min consisted of solvent A was trifluoroacetic acid (TFA, 0.1% v/v) and solvent B was 100% methanol. The flowing gradient was as follows: 5–80% solvent B for 15 min, 80–5% solvent B for 1min and 5% solvent B for an additional 4 min. Quantification was based on the peak areas referring to the commercial standards at the wavelength of 280nm for L-tyrosine and naringenin, 308nm for p-coumaric acid, respectively. Titer analysis was conducted by comparing the sample reads with the standard curve ($R2 > 0.999$). All results were obtained with three biological replicates. Error bars indicate standard deviations of the presented results.

# CHAPTER 4

# RESULTS

## 4.1 RgTAL, Pc4CL, PhCHS and MsCHI protein purification

Enzymes are the foundation of pathway construction and the enzyme properties determine the performance of the designed pathway to a large extend. Some plants are rich in naringenin; therefore, plants are the main enzyme resources for its synthesis. To investigate the enzyme properties for the currently reported naringenin biosynthesis pathway, TAL, 4CL, CHS, and CHI genes were invited from *Rhodotorula glutinis*, *Petroselinum crispus*, *Petunia hybrida*, and *Medicago sativa*. These genes were expressed in *E. coli* BL21 and purified by attached with a histidine tag. The molecular weight and protein purity were verified by SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) (Figure 3). The molecular weight of RgTAL, Pc4CL, PhCHS, and MsCHI were 74.7kDa, 59.65kDa, 43.98kDa, and 25.31kDa, respectively.

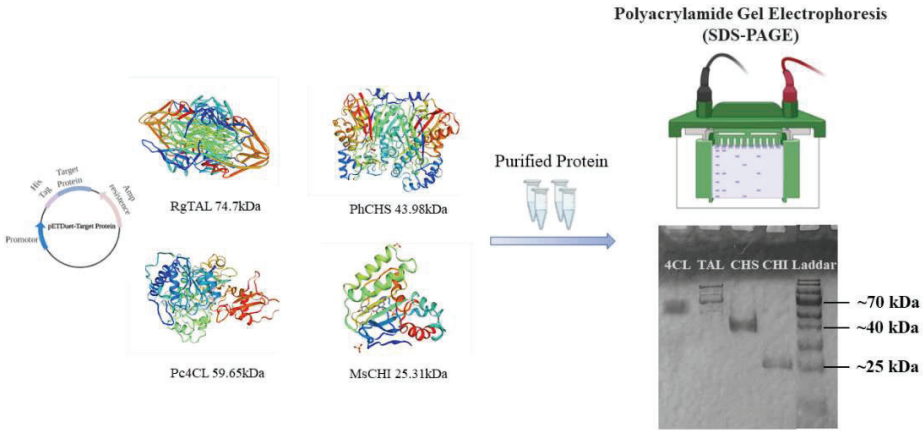


Figure 3: SDS-PAGE for purified enzyme. The left side of figure showed the construction of protein expression plasmid and the protein structures and their molecular weight. The right side of figure showed the results from SDS-PAGE.

The results showed that each enzyme had a very high degree of purity and the molecular weight was in an appropriate range with the calculation from ApE software (A plasmid editor). Furthermore, the purified enzymes were used in subsequent studies.

4.2 Enzyme property of RgTAL and Pc4CL

To investigate the enzyme property of RgTAL, the reaction was under constant temperature 37℃ with constant pH=7.0. In the pretest, it took an hour to convert L-tyrosine to p-coumaric acid in a 100 μL reaction system. However, no substrate was detected by HPLC after such a long-time reaction time. Michaelis-Menten kinetics requires that the substrate needs to be excessive during the reaction so that the enzyme can fully interact with the substrate and perform its function. Consequently, the control variates method was applied to find a suitable reaction time. Finally, the reaction was conducted for 30 min and we successfully measured the Michaelis constant ($K_M$), and maximum rate achieved by the system ($V_{max}$) by using the Lineweaver-Burk plot method; and we further calculated the turnover number ($k_{cat}$) and catalytic efficiency ($k_{cat}/K_M$) (Figure 4). The turnover number of RgTAL was $1.606\times10^{-4}$ ($s^{-1}$); and its catalytic efficiency was $1.490\times10^{-3}$ ($s^{-1}\cdot mM^{-1}$) (Table 2).

Table 2: RgTAL enzyme properties.

| | |
|---|---|
| $V_{max}$ (μM/s) | 0.0163 |
| $K_M$ (μM) | 107.807 |
| $k_{cat}$ (/s) | $1.606\times10^{-4}$ |
| $k_{cat}/K_M$ ($s^{-1}\cdot mM^{-1}$) | $1.490\times10^{-3}$ |

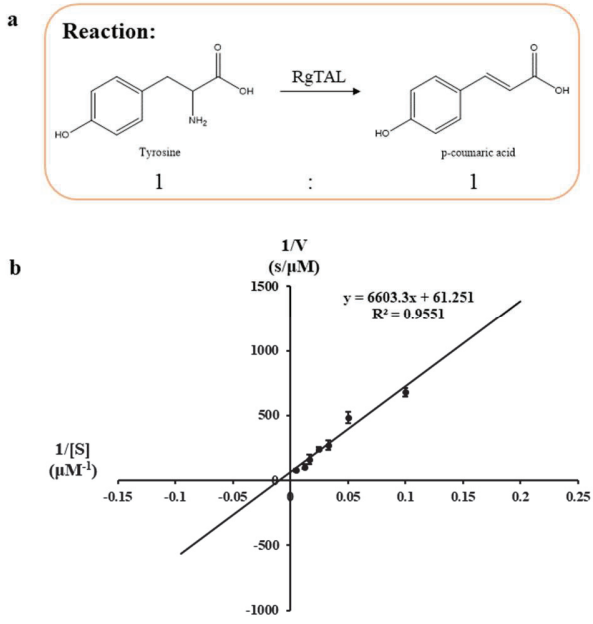


Figure 4: RgTAL enzyme assay. (a) Reaction for RgTAL enzyme assay. (b) Data analysis by Lineweaver-Burk plot method.

The conversion from p-coumaric acid to p-coumaroyl CoA involves cofactors, namely ATP, $MgCl_2$, and coenzyme A. These cofactors naturally exist in cell-matrix or can be generated by cell metabolism. However, the cell-free system cannot provide these cofactors without addition. Thus, it is necessary to consider adding these cofactors for in vitro enzyme assay for Pc4CL. After pretesting with sufficient cofactors supply, we found that p-coumaroyl CoA production was unable to be directly detected by HPLC. Therefore, we hypothesis that the consuming rate of p-coumaric acid was equal to p-coumaroyl CoA generating rate. Herein, the consumption of p-coumaric acid was borrowed for enzyme kinetics calculation, within a 100 μL constant temperature (37℃) and pH (7.0~7.5) system (Figure 5a, 5b). The turnover number of Pc4CL was $1.171\times10^{-4}$ ($s^{-1}$); and its catalytic efficiency was $2.798\times10^{-3}$ ($s^{-1}\cdot mM^{-1}$) (Table 3). Compared with RgTAL, the turnover

number ($k_{cat}$) and catalytic efficiency ($k_{cat}/K_M$) were at the same level, indicating that these two enzymes had very similar enzyme activity.

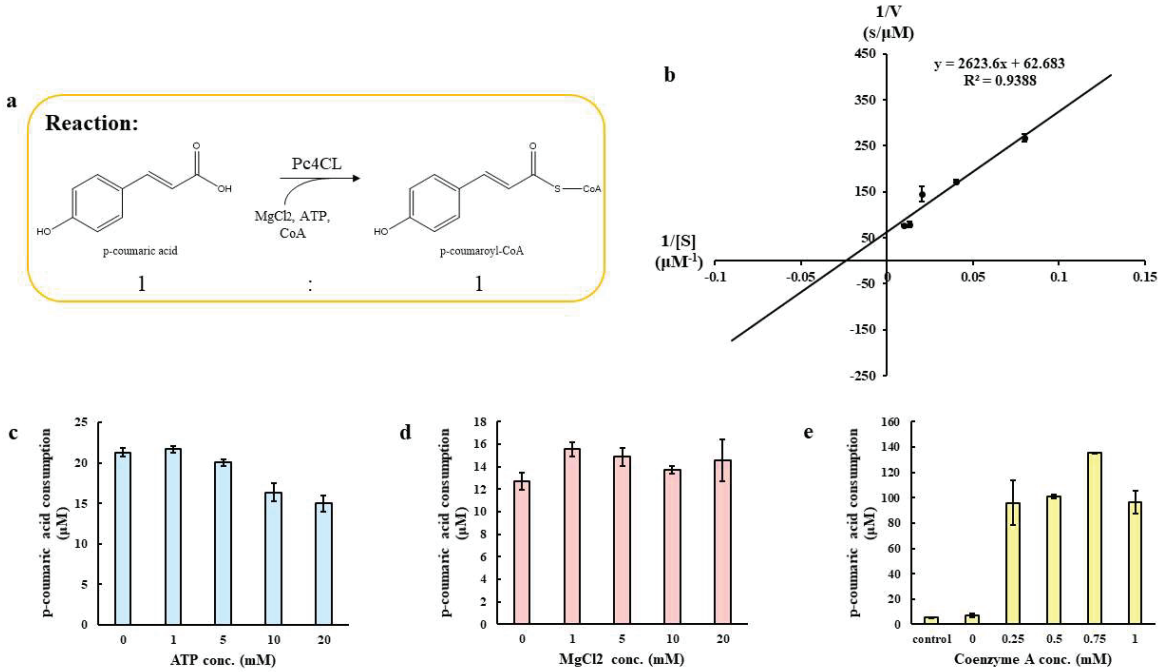


Figure 5: Pc4CL enzyme assay and its cofactors effect. (a) Reaction for Pc4CL enzyme assay. (b) Data analysis by Lineweaver-Burk plot method. (c) Effect of ATP. (d)Effect of $MgCl_2$. (e) Effect of Coenzyme A.

Table 3: Pc4CL enzyme properties.

| | |
|---|---|
| $V_{max}$ (μM/s) | 0.0159 |
| $K_M$ (μM) | 41.855 |
| $k_{cat}$ (/s) | $1.171\times10^{-4}$ |
| $k_{cat}/K_M$ ($s^{-1}$ • $mM^{-1}$) | $2.798\times10^{-3}$ |

Moreover, considering the content of the cofactors might be important for the reaction, the effect of cofactors was worth exploring. To figure out the role of each cofactor, the control variates method was applied by changing the concentration of ATP, $MgCl_2$, and coenzyme A, respectively (Figure 5d, 5e, 5f). The effect of ATP showed a negative effect on this reaction, which means too much energy supply would inhibit the consumption of p-coumaric acid. It was interesting that even without the ATP supply, the reaction can operate normally. We speculated that there were amounts of ATP existing in the purified protein solution because the enzyme was extracted from *E. coli* cell. As for the effect of $MgCl_2$, it had no dosage effect on the reaction. Instead, the effect of coenzyme A showed a positive effect on this reaction, which means the increasing amount of CoA would attach a positive driving force here.

4.3 Enzyme property of MsCHI

The analytically pure naringenin chalcone solution was yellow. MsCHI was able to catalyze the isomerization of naringenin chalcone to naringenin. The difference was that the analytically pure naringenin solution was colorless and transparent. The same reaction condition and system were invited from RgTAL enzyme assay with the different substrate (naringenin chalcone) and the enzyme (MsCHI). In the beginning, the reaction mix was analyzed by HPLC, but no naringenin chalcone could be observed even in several minutes' reaction. Therefore, we supposed that the reaction was super-fast and could be finished in several seconds. Next, we used a UV spectrophotometer to time-monitor this enzyme reaction. The flaw of this strategy is that the reaction temperature was no longer controllable. By processing the data obtained from a UV spectrophotometer, the reaction velocity was easily acquired (Figure 6). The enzyme properties of MsCHI were also calculated through the Lineweaver-Burk plot method. The turnover number of MsCHI was 2.383 ($s^{-1}$); and its catalytic efficiency was 52.472 ($s^{-1}\cdot mM^{-1}$) (Table 4). It is obvious

that the enzymatic activity of MsCHI exceeds that of RgTAL and Pc4CL by several orders of magnitude, showing its outstanding enzyme performance.

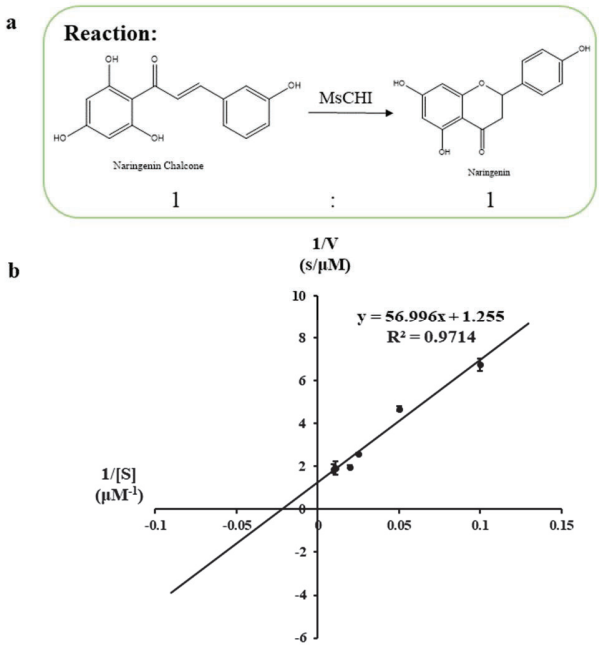


Figure 6: MsCHI enzyme assay. (a) Reaction for MsCHI enzyme assay. (b) Data analysis by Lineweaver-Burk plot method.

Table 4: MsCHI enzyme properties.

| | |
|---|---|
| $V_{max}$ (μM/s) | 0.797 |
| $K_M$ (μM) | 45.415 |
| $k_{cat}$ (/s) | 2.383 |
| $k_{cat}/K_M$ ($s^{-1}$ • $mM^{-1}$) | 52.472 |

4.4 The synergistic enzymatic assay of Pc4CL, PhCHS, and MsCHI

The enzyme assay of PhCHS was first conducted with Pc4CL in a two-step reaction. This was because it was difficult to get the analytical reagent p-coumaroyl CoA from a commercial company. Therefore, we would like to prepare p-coumaroyl CoA from p-coumaric acid through the catalysis of Pc4CL. However, it was hard to detect the naringenin chalcone peak after HPLC analysis. According to MsCHI enzyme assay before, naringenin was known can be easily detected by HPLC. As a result, we decided to test the synergistic enzymatic activity of Pc4CL, PhCHS, and MsCHI by considering the three-step reaction as a whole. In the reaction system with the addition of MsCHI, naringenin was successfully detected with a conversion rate of only 3% (Figure 7). The previous test of Pc4CL and MsCHI exhibit a good enzyme efficiency to their substrates. Hence, the results indicated that PhCHS had a very low enzyme activity by showing a low naringenin conversion. Meanwhile, the reaction from p-coumaroyl CoA to naringenin chalcone was defined as the limiting-step in the naringenin synthesis pathway here.

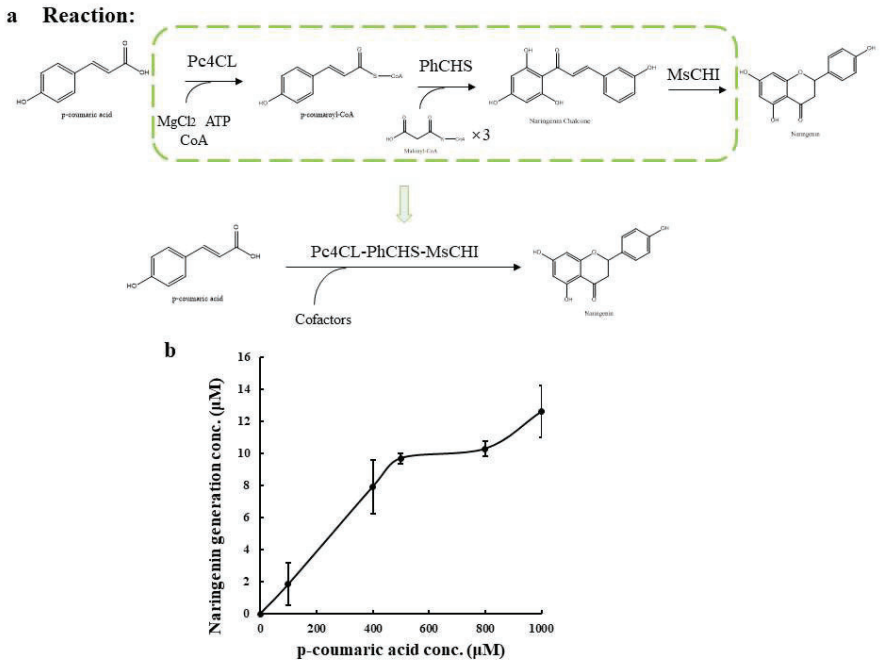


Figure 7: Synergistic enzymatic assay of Pc4CL, PhCHS, and MsCHI. (a) Reaction for synergistic enzyme assay. (b) Naringenin generation with increased p-coumaric acid concentration.

# CHAPTER 5

# CONCLUSION & DISCISSION

Enzymes play a decisive role in the performance of metabolic pathways. The naringenin biosynthesis pathway derived from L-tyrosine with four heterogenous enzymes containing tyrosine ammonium lyase (TAL), 4-courmarate:CoA ligase (4CL), chalcone synthesis (CHS), and chalcone isomerase (CHI) showed a low production yield in *Escherichia coli* in previous research. Characterization of the enzymes by in vitro enzyme assay will help figure out the drawback of the pathway and help pathway regulation.

In the previous study, a lot of strategies were applied to the naringenin biosynthesis pathway, including both dynamic and static regulation. The fatty acid synthesis pathway downstream of malonyl-CoA was recognized as a completing pathway by many research groups (10, 14). The researchers put much effort into balancing the carbon flux between the fatty acid pathway and the naringenin pathway. It was obvious that the research focus lay on host cell engineering by driving the central metabolic carbon flux to precursors or cofactors accumulation. Interestingly, the shortcoming of the naringenin synthesis pathway was rarely studied. We speculated that the unbalanced carbon flux might also exist in the naringenin biosynthesis pathway. Therefore, we proposed that the systematic investigation of enzymes was one potential direction that was worth studying. The enzyme properties would provide informative hints for us to have a better understanding of naringenin synthesis.

In this thesis, we tested the enzyme properties of a four-step naringenin pathway with RgTAL, Pc4CL, PhCHS, and MsCHI. The catalytic efficiency (kcat/KM) of RgTAL, Pc4CL, and

MsCHI were 1.490×10-3, 2.798×10-3, and 52.472, respectively. It was obvious that the catalytic efficiency of RgTAL and Pc4CL were at the same level, but the catalytic efficiency of MsCHI was higher than the other two enzymes. Also, the highest conversion in the synergistic enzymatic assay of Pc4CL, PhCHS, and MsCHI here was only 3% at 1000 μM p-coumaric acid. Therefore, PhCHS was claimed as a rate-limiting enzyme through its bad performance in the synergistic enzymatic activity test. The cell-free system did a favor to the in vitro enzyme. This simple and clear system, without disturbing the complicated cell intracellular environment, benefited the straightforward enzyme properties analysis. Furthermore, the investigation of these enzyme properties would provide helpful information for in vivo pathway regulation by adjustment of enzyme expression level and propel the production of microbial synthesis of naringenin. In conclusion, the four enzyme properties were successfully demonstrated here. By adopting a cell-free biocatalytic system and mimicking in-vivo reaction parameters in vitro, the naringenin bioproduction pathway can be analyzed in detail and the issues associated with traditional microbial fermentation can be easily solved to achieve highly efficient bioconversion.

According to the results, there are some potential future works that we can dig out. Firstly, based on the current in vitro reaction system, we can adjust the ratio between these four enzymes to enhance the conversion rate of naringenin. This is to change the expression level of each enzyme in the cell-free system. According to the enzyme properties we got, the expression level of RgTAL and Pc4CL should be the same. As for the rate-limiting enzyme, PhCHS, it requires a much higher expression level than those two. The turnover number of MsCHI is several magnitudes larger than those three enzymes, so only a little bit MsCHI is needed here. Another direction is based on the current enzyme candidates to do protein engineering. We can mutate the current four enzymes and further modify their substrate-binding site or enzyme active site and screen the mutations with

higher enzyme activity by in vitro enzyme assay. The last direction is to do the pathway enzyme mining. Through this strategy, we might BLAST the new enzyme candidates in the protein database and next test their enzyme properties, and then compare them with the former enzyme candidates. In the end, we hopefully are able to get a new enzyme combination with higher efficiency to gain a higher naringenin titer.

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