Microbial Production of Diosmetin from Hesperetin in Engineered *Escherichia coli* with Flavone Synthase and Flavonol Synthase

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Abstract

In this study, diosmetin was synthesized with recombinant *Escherichia coli* expressing flavone synthase (FNS) or flavonol synthase (FLS). Forty-four FNS/FLS were selected from 40 different plants and their bioinformatic data, such as isoelectric point, instability index, grand average of hydropathicity, transmembrane structure, secondary structure, and conservative domain were analyzed with computer tools or software. Nine recombinant *E. coli* strains expressing FNS/FLS were constructed for diosmetin synthesis, and the products were detected through UPLC, LC-MS, and SDS-PAGE. Results showed that FNS/FLS from different sources were different in transmembrane structures, instability coefficients, and conservative regions. Among the nine recombinant *E. coli* strains, six recombinant *E. coli* strains were observed expected bands by SDS-PAGE, four recombinant *E. coli* strains were detected to have diosmetin with a molecular weight of 300.06 confirmed by LC-MS in broth, and the diosmetin concentration of a DE3/pAnFNS fermentation broth was the highest (39.6 mg/L). The enzyme expression and catalytic reaction were accordance with the results of bioinformatics analysis. In addition, the ratio of predicted intermediate product (4'-O-Methyl taxifolin) and final product (diosmetin) was significant different among FNS/FLS from different sources, although they are similar in physicochemical properties and structures. Therefore, the hypothesis that FNS/FLS catalyzed the synthesis of diosmetin from hesperetin by hydroxylation at the C-2 and C-3 positions respectively, 2-hydroxyhesperetin undergoes an elimination reaction and is converted to diosmetin, 3-hydroxyhesperetin (4'-O-Methyl taxifolin) was retained as a byproduct has been proposed.

Key points:

1) A possible biosynthesis pathway of diosmetin was proposed.

2) Diosmetin was biosynthesized by expressing FNS and FLS in recombinant Escherichia coli strains.

3) Enzyme selection in biosynthesis was guided with bioinformatic analysis.

4) The speculation that simultaneous hydroxylation of the reaction occurs at both C-2 and C-3 positions of flavanones has been proposed.

Introduction

Diosmetin as a secondary product of metabolism in plants (Poór et al. 2014) has been used to treat Alzheimer's disease (Sawmiller et al. 2016), protect retina (Shen et al. 2015), prevent acute liver injury (Yang et al. 2017), and inhibit melanin formation (Ge et al. 2016) and other physiological activities. In comparison with other flavonoids, such as hesperetin, whose molecular structure is similar to diosmetin, the physiological activity of diosmetin is more positive, and diosmetin can achieve the same effect of lower doses for the same physiological function. For example, diosmetin and hesperetin can affect serine/threonine-protein kinase 1 (PLK-1) through the same binding site to inhibit tumor cell division. The binding ability of diosmetin to PLK-1 (Binding energy of 48.88 kcal/mol) is more potent than that of hesperetin (Binding energy of 46.50 kcal/mol) (Alajmi et al. 2018). Similarly, diosmetin and hesperetin can improve the efficacy or reduce the side effects of anticancer drugs, such as paclitaxel, by inhibiting the activity of CYP2C8. The effect of diosmetin is 16-fold that of hesperetin (mean IC_{50} of 4.25 and 68.5 μM for diosmetin and hesperetin, respectively) (Quintieri et al. 2011). In addition, diosmetin is more positive than hesperetin in inducing tumor cell autophagy (Lascala et al. 2018) and regulating intestinal absorption (Sanchez-Bridge et al. 2015). However, only a small amount of dissociated diosmetin is found in plant species (16.86 μg/g in *Herba Desmodii Styracifolii* or 616 μg/g in *Citrus limonum*) (Ding et al. 2017; Guo et al. 2015). Directly obtaining a large amount of diosmetin from plants is difficult. Therefore, the effective synthesis methods of diosmetin should be studied.

Diosmetin and its glycoside (diosmin, a cardiovascular protective drug) can be synthesized through chemical methods. However, during this synthesis, a large amount of reagents, such as pyridine and iodine, are used as catalysts and solvents (Eric and Herve 2000), which can cause environmental and safety issue with reagent residues and excessive organic solvents. Biosynthesis, as a safe method for natural product synthesis, has broad research and application prospects to construct a heterologous protein expression system in microorganisms to achieve the catalysis of target substances (Li et al. 2019). Rare and valuable substances, such as phenylpropanoid derivatives (Wang et al. 2015), glycoside derivatives (Kim al. 2015), phenolic acid derivatives (Bai et al. 2016), special amino acids (Zhang et al. 2015), and natural pigments (Carbon et al. 2015), have achieved environmentally friendly, safe, and efficient production through biosynthesis. Therefore, constructing of engineered microbial to produce diosmetin can be a desirable alternative for it eco-friendly mass production.

The biogenic synthesis of diosmetin in plants begins with the conversion of glucose to form phenylalanine. Phenylalanine is catalyzed with phenylalanine ammonia lyase (PAL), cinnamic acid-4-hydroxylase (C4H), 4-acyl CoA ligase (4CL), and chalcone synthase (CHS) to *p*-coumarin CoA. Subsequently, naringenin chalcone is synthesized from *p*-coumaroyl CoA and malonyl CoA (produced through the malonyl pathway). Naringenin chalcone forms hesperetin under the catalysis of chalcone isomerase (CHI), flavonoid 3□-monooxygenase (F3□H), and flavonoid *O*-methyltransferase (Pandey et al. 2016). Thereafter, hesperetin is converted into diosmetin by a specific enzyme; however, the enzyme catalyzing diosmetin synthesis is not yet proven. Cheng et al. (2014) and Zhou et al. (2017) reported that flavone compounds, such as apigenin and kaempferol, can be synthesized by heterologously expressed flavone synthase (FNS) and flavonol synthase (FLS). Diosmetin shows similar molecular structures to apigenin and kaempferol. Therefore, FNS/FLS that functions in the catalytic conversion of flavanones to flavones can play a key role in diosmetin biosynthesis. FNS/FLS exhibited the ability of catalyzing the conversion of flavanone to flavone. Therefore, this study suggested that FNS/FLS showed potential for application in synthesizing diosmetin (Fig. 1A). FNS includes FNS□ (dioxygenase dependent on α-ketoglutaric acid) and FNS□ (monooxygenase-dependent on NADPH) (Martens and Mithöfer 2005). FLS was highly similar to FNS□ in terms of structure and function. Unlike FNS□, which can catalyze 2S-flavanone, FLS can catalyze 2S-flavanone and-2R flavanone and the production of flavonol or flavonol (Marten et al. 2003). The activity of FNS□ is related to the microsomal membrane of eukaryotes (Davydov et al. 2015). Then, diosmetin is synthesized via the reaction of ferryl compounds with hesperetin under the action of enzymes through electron transfer, hydroxylation, and elimination (Akashi et al. 1999; Welford et al. 2001). Previous studies also demonstrated that ferryl compounds can directly synthesize diosmetin from hesperetin without C-ring

hydroxylation (Martens et al. 2001). However, insufficient reports are available about directly changing single carbon bonds into double carbon bonds without intermediate reactions or cleavage reactions. Based on these findings, our hypothesis was that FNS/FLS could biosynthesize diosmetin via electron transfer, hydroxylation, and elimination (Fig. 1B).

Enzymes involved in biosynthesis can be heterologously expressed by constructing the required microbial expression systems. *Escherichia coli* (Watts et al. 2004), *Saccharomyces cerevisiae* (Koopman et al. 2012), and *Streptomyces venezuelae* (Park et al. 2011) are commonly used for flavonoid biosynthesis because of the advantage of suitability for cultivation and clear genetic background. Among them, *E. coli* shows a great advantage in research on diosmetin biosynthesis because of its ease of genetic modification, rapid growth, high titer, cost-effectiveness, and efficient heterologous expression (pET prokaryotic expression system) (Song et al. 2014). In addition, Zhang et al. (2013) revealed that the mechanism of diosmetin synthesis from hesperetin catalyzed by enzymes from multiple sources may be different. Therefore, isozymes with different structures and physicochemical properties should be screened. However, a simple and rapid screening method for enzymes has not been reported. Bioinformatic analysis and molecular docking technology based on computer technology can be utilized to conveniently predict the physical and chemical properties, structural characteristics, and catalytic mechanism of proteins (Fakhar et al. 2016) (Ding et al. 2011). By analyzing information such as protein isoelectric point, instability index, conserved domains and active sites, some of the enzymes that are not stably expressed, do not function independently, or have protein properties that are not suitable for the reaction conditions can be indicated. Therefore, bioinformatics analysis can be used to determine the accuracy of enzyme screening in biosynthesis research and reduce research workload.

Materials And Methods

Genes, bacterial strains, genetic manipulation, and chemicals

All the genes collected in this study are listed in Table 1. All the bacterial strains and plasmids used in this study are listed in Table 2. The FNS/FLS genes were synthesized from Sangon Biotech (Shanghai, China). Restriction nuclease was obtained from Sangon Biotech (Shanghai, China). Hesperetin was procured from Macklin (Shanghai, China). The diosmetin reference material was purchased from Sigma-Aldrich (St. Louis, MO, USA). Tris-HCl, sodium dodecyl sulfate, polyacrylamide, tetramethyl ethylenediamine, tris-glycine running buffer, isopropyl-β-D-thiogalactoside (IPTG), Coomassie brilliant blue G-250, Luria-Bertani broth (LB), Kanamycin, BCA Protein Assay Kit, and ammonium persulfate were bought from Solabio (Beijing, China).

Bioinformatic analysis

ProtParam tool was used to predict the physicochemical properties of FNS/FLS (https://web.expasy. org/protparam). TMHMM 2.0 online service was utilized to predict the transmembrane structure of FNS/FLS (http://www.cbs.dtu.dk/servi-ces/TMHMM). SPOMA online service was applied to predict the secondary structure of FNS/FLS (https://npsa-prabi.ibcp.fr/NPSA/npsa_sopma.html). The CDD database of NCBI was used to analyze the conservative domain of FNS/FLS (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Modeller 9.18 software (Singular Inversions Inc., Canada) was used to model the FNS/FLS homology. AutoDock Vina 1.1.2 software (Olson Lab., Scripps Research, USA) was used to simulate docking between FNS/FLS and hesperetin (Trott O and Olson 2010). The docking results of FNS/FLS and hesperetin were visualized with PyMol 1.7 software (Schrödinger Inc., USA).

Construction of expression plasmids and recombinant *E. coli* strains

The plasmids pAnFNS, pCaFLS, pCrFLS, pInFLS, pLoFLS, pPoFNS, pPoFNS-GST, pViFLS, and ScFNS were constructed to produce flavone compounds through the restriction enzyme digestion of Nco□ and Xho□. The as-obtained plasmid was transferred into *E. coli* TOP10 for amplification. After extraction, the as-obtained plasmid and pET-28a(+) plasmid without the FNS/FLS gene fragment was transferred into *E. coli* BL21(DE3), leading to the formation of recombinant strains, namely, DE3/pET-28a(+), DE3/pAnFNS, DE3/pCaFLS, DE3/pCrFLS, DE3/pInFLS, DE3/pLoFLS, DE3/pPoFNS, DE3 pPoFNS-GST, DE3/pViFLS, and DE3/pScFNS, respectively. The effects of protein stability and water solubility on the activity of FNS were analyzed by comparing PoFNS and PoFNS-GST. The sequence of GST tag was ATGAGCCCGATCCTGGGTTACTGGAAGATTAAAGGCCTGGTTCAGCCGACCCGCCTGCT

GCTGGAATACCTGGAAGAGAAATACGAAGAGCACCTGTATGAGCGTGACGAGGGCGACAAATGGCGCAACAAAAAATTCGAACTGGGCCTGGAGTTCCCGAACCTGCCG

Production of diosmetin by using recombinant *E. coli* strains

The recombinant *E. coli* strains were grown 24 h in 50 mL of the LB medium with 50 mg/L Kanamycin at 37 °C. Accurate 5 mL of each recombinant bacterial liquid was inoculated into 50 mL of a fresh LB medium at an initial optical density at 600 nm of 0.2 and cultivated until a value of 0.8–1.0 was reached. Thereafter, IPTG was added to the culture broths until the final concentrations became 0.2 mM to induce gene expression and produce FNS/FLS, and the recombinant *E. coli* strains were cultured at 23 °C for another 24 h. The final concentrations of hesperetin (100 mg/L), α-ketoglutaric acid (100 mg/L), and ferrous sulfate (1 g/L) were added. The recombinant *E. coli* strains were cultured at 23 °C for 24 h.

Analysis of heterologous protein expression in recombinant *E. coli* strains by SDS-PAGE

Accurate 5 mL of the recombinant *E. coli* strain broth was centrifuged with 10,000 ×g at 4°C for 15 min. After centrifugation, 5 ml phosphate buffer was added. The bacteria were resuspended and broken by ultrasound. The samples were centrifuged again, and BCA Protein Assay Kit was used to detect the protein concentration in the supernatant of different samples. Accurate 4 mL of supernatant was added to the loading buffer containing DL-dithiothreitol. The samples of the expressed protein were prepared by boiling at 100 °C for 5 min. The expressed protein samples were separated through SDS-PAGE. The amount of protein in each lane was 10 mg. The gel was dyed and decolorized with the Coomassie brilliant blue method.

Identification and detection of products from fermentation broth of recombinant *E. coli* strains

Accurate 5 mL of the recombinant *E. coli* strain broth was added with an equal volume of ethyl acetate to extract the synthesized diosmetin. After centrifugation with 4,000 ×g at 4°C for 10 min, 4 mL of ethyl acetate containing diosmetin in the upper layer was taken. Ethyl acetate was evaporated with nitrogen, and the dry matter was redissolved by adding 1 mL of methanol. After the specimen was passed through a 0.22 μm filter membrane. Liquid chromatography-mass spectrometry (LC-MS) was used to identify diosmetin synthesized with recombinant *E. coli* strain broth. The instrument consisted of Agilent 1290 UPLC and Agilent Q-TOF 6550 MS. UPLC was equipped with an Agilent Extend C18 column (2.1 × 50 mm, 1.7 μm), and the mobile phase was 10% acetonitrile water. The scanning range of MS was 50–400 m/z, the gas temperature was 150 °C, the dying gas was 15 L/min, the shear gas temperature was 350 °C, the shear gas flow was 12 L/min, the ESI mode voltage was 4000 V, and the fragment voltage was 175 V. The content of diosmetin synthesized by different recombinant *E. coli* strain broth was detected through ultra-performance liquid chromatography (UPLC). An Acquity UPLC system was equipped with a BEH C18 column (2.1 mm × 50 mm, 1.7 μm). The mobile phases were water and methanol. The content of hesperetin, diosmetin and 4'-O-Methyl taxifolin was detected by UPLC. Hesperetin and diosmetin was detected and quantified at a wavelength of 254 nm, and 4'-O-Methyl taxifolin was detected and quantified at a wavelength of 330 nm to avoid interference from other substances in the medium.

Statistical analysis

The obtained data were calculated with Statistical Product and Service Solutions 25.0 (IBM, USA).They were analyzed and plotted with GraghPad Prism 8 (GraphPad Software, USA). SDS-PAGE results were processed and examined with Image Pro Plus 6.0 (Media Cybernetics, USA).

Results

Results of bioinformatics analysis of FNS / FLS

A total of forty-four kinds of FNS or FLS amino acid sequences were collected for bioinformatic analysis to infer their physicochemical properties in this study (Table 3). The results revealed that the isoelectric points of FNS/FLS from nine kinds of sources (CeFNS, DaFNS, EpFNS, EriFNS, GlFNS, MeFNS, PiFNS, PlFNS, and ScFNS) were approach the pH of the medium (pH = 7.5). The instability index of FNS/FLS from seventeen kinds of sources (ArFNS, ArFLS, CamFLS, CameFLS, CaFNS1, CaFNS2, CaFLS, CarFLS, ChFLS, EpFLS, ErFLS, FaFLS, InFLS, LoFLS, PoFNS, MoFLS, and NeFNS) was above 40 been considered unstable proteins. The remaining twenty-seven kinds of FNS/FLS (AnFNS, ApFNS, CalFNS, CeFNS, ChFNS, CrFLS, DaFNS, DauFNS, EpFNS, EriFNS, ErFNS, FrFLS, GlFNS, LiFLS, LoFLS, MaFLS, MeFNS, NiFLS, PaFNS, PiFNS, PlFNS, SaFNS, SauFNS, ScFNS, SoFLS, VaFLS, ViFLS) instability index was below 40. With the exception of SaFNS, all FNS/FLS grand average of hydropathicity were less than 0, showing hydrophilicity.

Table 3
Bioinformatic information

Proteins	Molecular weight, kU	Isoelectric point	Instability index	GRAVY
AnFNS	40.9	5.55	38.56	−0.455
ApFNS	29.2	5.16	38.03	−0.380
ArFNS	37.2	5.14	45.27	−0.487
ArFLS	38.3	5.63	43.04	−0.513
CaIFNS	58.4	6.75	30.25	−0.062
CamFLS	37.4	5.59	43.32	−0.404
CameFLS	38.4	5.33	41.23	−0.446
CaFNS1	60.5	6.66	42.21	−0.051
CaFNS2	60.5	6.51	42.41	−0.051
CaFLS	37.5	5.62	40.83	−0.397
CarFLS	40.8	5.55	43.35	−0.357
CeFNS	52.9	7.09	34.86	−0.199
ChFNS	58.6	6.78	36.56	−0.122
ChFLS	38.0	5.41	43.12	−0.381
CrFLS	37.6	5.55	37.90	−0.365
DaFNS	58.3	8.95	30.21	−0.081
DauFNS	67.4	5.51	36.86	−0.303
EpFNS	59.3	7.67	35.63	−0.067
EpFLS	11.1	4.27	46.59	−0.195
EriFNS	59.0	7.64	34.25	−0.085
ErFNS	57.1	6.58	35.75	−0.035
ErFLS	38.3	5.70	46.88	−0.394
FaFLS	38.1	5.70	45.06	−0.430
FrFLS	38.2	5.41	38.33	−0.513
GlFNS	59.6	8.29	29.61	−0.112
InFLS	37.2	6.08	44.36	−0.401
LiFLS	37.9	5.41	36.51	−0.483
LoFLS	37.7	5.49	42.33	−0.359
LoFNS	36.0	8.68	38.77	−0.052
MaFLS	38.1	5.48	38.94	−0.506
MeFNS	59.2	7.17	36.20	−0.128
MoFLS	38.0	5.55	42.57	−0.435
NeFNS	59.4	6.62	45.00	−0.106
NiFLS	39.2	6.43	35.15	−0.382
PaFNS	59.3	6.74	36.89	−0.088
PiFNS	52.6	8.01	31.74	−0.108
PlFNS	57.5	8.49	23.74	−0.053
PoFNS	39.2	5.14	59.54	−0.330
SaFNS	57.3	8.59	38.59	0.015
SauFNS	58.8	6.67	39.57	−0.162
ScFNS	56.7	5.96	37.42	−0.068

Proteins	Molecular weight, kU	Isoelectric point	Instability index	GRAVY
SoFLS	40.0	5.60	39.37	−0.432
VaFLS	37.7	5.59	35.97	−0.364
ViFLS	37.0	5.16	25.84	−0.321

The secondary structure, conservative domain characteristics, and transmembrane structures of FNS/FLS are shown in Fig. 2. Twenty-three kinds of FNS/FLS had heme-independent monooxygenase (DIOX) and α-ketoglutaric acid-dependent dioxygenase (2OG-Fe(II)_Oxy). Nineteen kinds of FNS had NADPH-dependent cytochrome P450 monooxygenases. The DIOX conservative domain was identified in EpFLS and ViFLS, whereas the 2OG-Fe(II)_Oxy conservative domain was not found in them. The results of transmembrane structures revealed that all FLS did not contain transmembrane structures. In FNS from multiple sources, seven FNS (AnFNS, ApFNS, ArFNS, CeFNS, DauFNS, PoFNS, and SaFNS) did not contain a transmembrane structure, and fourteen FNS contained a transmembrane structure.

A three-dimensional protein model of FNS/FLS from different sources was constructed with Modeller 9.18, and the binding of FNS/FLS to the substrate (hesperetin) was simulated with AutoDock Vina1.1.2 and PyMol 1.7 (Fig. 3). The binding energy of CalFNS was the minimum of − 38.49 kJ/mol, and the binding energy of DaFNS was the maximum of 2.09 kJ/mol. The binding energy of FNS/FLS was below 0 except DaFNS. The results indicated that the enzymes existed the possibility of spontaneous reactions, and the binding of FNS/FLS to hesperetin was mainly by the hydrogen bonds of amino acid residues of the protein (carboxyl of aspartic acid and histidine, hydroxyl of threonine and tyrosine, and carbonyl of asparagine) with 5,7-hydroxy, 3′-hydroxy, and C-ring carbonyl of hesperetin.

Recombinant E. coli **was constructed and protein expression was examined by SDS-PAGE**

According to the results of bioinformatics analysis, eight FNS/FLS from different sources were selected to construct nine kinds of expression plasmids (pAnFNS, pCaFLS, pCrFLS, pInFLS, pLoFLS, pPoFNS, pPoFNS-GST, pViFLS, and pScFNS). These plasmids were constructed and introduced into *E. coli* BL21(DE3). pET-28a(+) plasmid without the FNS/FLS gene was introduced into *E. coli* BL21(DE3) as the control group. Induction of heterologous protein expression by IPTG and detection of protein expression using SDS-PAGE. SDS-PAGE results showed that the expected bands were observed in the broth of six recombinant *E. coli* strain (DE3/pAnFNS, DE3/pCaFLS, DE3/pCrFLS, DE3/pPoFNS-GST, DE3/pViFLS, and DE3/pScFNS). The expected bands were not found in DE3/pET-28a(+), DE3/pInFLS, DE3/pLoFLS and DE3/pPoFNS recombinant *E. coli* strain (Fig. 4).

Characterization and quantification of substances in fermented broth of recombinant E. coli

These recombinant *E. coli* strains were used to synthesize diosmetin from hesperetin. The LC-MS results demonstrated that the mass-to-charge ratio of the diosmetin reference material was 299.0623 $[M - H]^-$. The molecular weight of diosmetin detected in recombinant *E. coli* strains broth was the same as that of the diosmetin reference material (Fig. 5). In addition, a substance with a mass-to-charge ratio of 317.0697 was found in the recombinant *E. coli* broth, and its molecular weight accorded with the predicted intermediate product (4'-O-Methyl taxifolin). The UPLC results revealed that diosmetin could be detected in four recombinant *E. coli* strain (DE3/pAnFNS, DE3/pCaFLS, DE3/pCrFLS, and DE3/pPoFNS-GST) broths, and the content of diosmetin in DE3/pAnFNS broth was the highest, reaching 39.6 mg/L, which was about 20-fold higher than that of the sub high recombinant *E. coli* strains (DE3/pCaFLS) broth. And the diosmetin was not detected in other six kinds of recombinant *E. coli* strains (DE3/pET-28a(+), DE3/pInFLS, DE3/pLoFLS, DE3/pPoFNS, DE3/pViFLS, and DE3/pScFNS) broth (Fig. 6). The UPLC results were calculated by a reference material curve with a credible concentration interval of 0.1–100 mg/L. These results suggest that the diosmetin synthesis proposed in this study was considered reasonable and feasible. In addition, the content of the reaction substrate (hesperetin) and the predicted intermediate (4'-O-Methyl taxifolin) in recombinant *E. coli* broth were determined. 4'-O-Methyl taxifolin was detected in four recombinant *E. coli* (DE3/pAnFNS, DE3/pCaFLS, DE3/pCrFLS, and DE3/pPoFNS-GST) broth. And the total of the three flavonoids (diosmetin, hesperetin and 4'-O-Methyl taxifolin) in all of the recombinant *E. coli* broth were not significantly difference.

Discussion

Previous studies reported that FNS/FLS shows potential for application in catalyzing the conversion of flavanones to flavones (Cheng et al. 2014; Zhou et al. 2017). The results of bioinformatics analysis revealed that the isoelectric points of nine FNS/FLS were around 7.5. Lee et al. (2010) reported that an enzymatic activity and stability at the isoelectric point attachment would be weakened with reduced solubility. Due to the optimum pH of E. coli growth was 7.5, pH of the culture medium was adjusted to 7.5. These FNS/FLS were considered presented disadvantages to biosynthesize diosmetin under the experimental conditions. The proteins with an instability index of above 40 were considered to be unstable and might degrade rapidly in *E. coli* (Gunaratne et al. 2019). So that seventeen FNS/FLS were considered unstable due to instability index above 40. These FNS/FLS These FNS could not exhibit their proper catalytic activity due to poor stability under experimental conditions. The results of bioinformatics analysis also indicated that SaFNS which grand average of hydropathicity was above 0 cannot be expressed and modified correctly to reflect the activity in *E. coli* (Målen et al. 2008). The results of physicochemical properties indicated that some of the enzymes from multiple sources have defect in terms of stability and solubility, so they are unsuitable for diosmetin biosynthesis. In subsequent experiments, InFLS, LoFLS, PoFNS, and ScFNS were selected to verify the hypothesis. Results showed that no significant diosmetin was detected by UPLC in these recombinant *E. coli* broths.

The results of conservative domain analysis show that the almost all FNS and FLS present both 2OG-Fe(II)_Oxy conservative domain and DIOX conservative domain, while FNS present a conserved domain belonging to cytochrome P450, which was reported to be related to the eukaryotic microsomal membrane (Davydov et al. 2015). These results were consistent with the description in the Uniprot database (Uniprot 2019; Uniprot 2019; Uniprot 2019). However, some inconsistent information with the description in the database was also found in the study. PaFNS was considered to belong to FNS, due to PaFNS present

cytochrome P450 conserved domains rather than 2OG-Fe(II)_Oxy conservative domain and DIOX conservative domain. Based on the published literature and analysis results in this study, our conclusion was that FLS and FNSI played a role in 2OG-Fe(II)_Oxy and DIOX conserved domains. FNSII participated in the cytochrome P450 monooxygene conserved domain. EpFLS and ViFLS might not be able to catalyze diosmetin synthesis because of the lack of 2OG-Fe(II) conservative domain. In subsequent experiments, ViFLS was chosen to verify the influence of conservative domains. Results showed that no significant diosmetin was detected by UPLC in DE3/pViFLS broths.

The results of transmembrane structure analysis indicated that FLS and FNSI did not include the transmembrane structure, which was consistent with the existing published literature (Huang et al. 2014). Zhu et al. (2014) explored the relationship between transmembrane structures and enzyme activities and found that FNS has a transmembrane structure, which was supposed to be inappropriate for expression and modification in *E. coli*. In subsequent experiments, ScFNS was chosen to verify the influence of transmembrane structures, and UPLC results showed that no significant diosmetin was detected by UPLC in DE3/pScFNS broths.

By establishing a 3D model of FNS/FLS and using the established model for docking and conformation optimization with substrate molecules, we obtained the dominant conformations of FNS/FLS and hesperetin binding from different sources and analyzed their binding sites. The results of binding sites between enzymes and substrates were consistent with the influence of amino acid residues on the activity in published literature. The acidic residues of amino acids, such as asparagine and histidine, play an irreplaceable role in reactions (Wellmann et al. 2002). Therefore, the enzyme based on the combination of acidic residues and hesperetin, such as AnFNS, effectively catalyzes diosmetin synthesis.

The SDS-PAGE showed that expected bands were not observed in the DE3/plnFLS, DE3/pLoFLS and DE3/pPoFNS broth, indicating that these proteins could not be stable in recombinant *E. coli.* The result was consistent with the bioinformatics analysis, because the instability index of these proteins were above 40. The expected bands were found in DE3/pViFLS and DE3/pScFNS broth, but diosmetin was not produced. It was presumed that these enzymes can be synthesized by recombinant *E. coli*, but cannot form the correct structure with activity to catalyze the synthesis of diosmetin from hesperetin. For DE3/pViFLS, the result agreed with the finding that ViFLS could not catalyze diosmetin synthesis because of the absence of 2OG-Fe(II)_Oxy conservative domain through bioinformatic analysis. Downey et al. (2003) isolated two FLS with 80% homology from *Vitis vinifera L*. at different flowering stages. The type and consistency of flavonols at different flowering stages were related to the ratio of two FLS. In this study, ViFLS had no activity in synthesizing diosmetin independently because of the lacking 2OG-Fe(II)_Oxy conservative domain. The substrate was converted into other substances instead of diosmetin. For DE3/pScFNS, it was presumed to be inactive in a heterologous expression because of the presence of transmembrane domains associated with eukaryotic microsomes (Davydov et al. 2015). This finding was consistent with the report of Zhu et al. (2014), who showed that the transmembrane structure truncation of eukaryotic enzymes can significantly improve the activity of enzymes in heterologous expression. No expected band was observed in DE3/pPoFNS, but the expected band could be observed in DE3/pPoFNS-GST. Pei et al. (2016) studied enzyme modification and found that a GST tag can improve the stability and hydrophilicity of the enzyme and significantly increase the activity of the enzyme with poor stability and hydrophilicity. Therefore, we speculated that the GST tag can make PoFNS more favorable for heterologous expression in *E. coli* by increasing the stability and aqueous solubility of PoFNS.

The results of LC-MS and UPLC definite showed that FNS/FLS which were heterologous expressed in *E. coli* could synthesize diosmetin by catalyzing hesperetin. And the mass spectral characteristics of diosmetin which was detected in the recombinant *E. coli* broth were consistent with the diosmetin reference material and public literature (Huang et al. 2013). The concentrations of diosmetin detected in FNS/FLS broth from different sources were different. The concentration of diosmetin in DE3/pAnFNS broth was significantly higher than that in other recombinant *E. coli* broths, reaching 39.6 mg/L. Leonard et al. (2006) and Park et al. (2011) also reported that the completion of apigenin biosynthesis with similar FNS, and the apigenin content in the broth in these studies (15.3 mg/L) was less than the diosmetin content in this study. The difference could be ascribed to the substances, such as cinnamic acid as precursors, used in these studies, and the synthetic routes were more complex or different host bacteria. In the published literature on apigenin biosynthesis by FLS (Prescott et al. 2002), the content of apigenin in the broth was similar to that of diosmetin in the broth in this study. The ability of FLS to catalyze flavone synthesis was weaker than that of FNS because of a large number of flavanols, such as dihydrokaempferol produced during FLS catalysis (Han et al. 2014). In the present study, LC-MS demonstrated that a substance with a molecular weight of 318.28 was detected, and this result was consistent with the hypothetical intermediate product of hydroxylated hesperetin (4'-O-Methyl taxifolin), and 4'-O-Methyl taxifolin was also found in the UPLC results. The result that flavones and flavanols can be produced when FNS/FLS catalyzes flavanone substrate was consistent with the report of Turnbull et al. (2004). Meanwhile we also determined that the added 4'-O-methyl taxifolin could not be catalyzed to diosmetin. The phenomenon similar to the results of Martens et al. (2004) on the catalytic properties of FNS. But differently, Martens et al suggested that the phenomenon arises because this reaction requires sequential hydroxylation - elimination at the C-3 position. While we supposed that two kinds of products, 2-hydroxyflavone and 3-hydroxyflavone, were actually produced during the reaction. 2-hydroxyflavone underwent elimination reaction to form diosmetin as a result of structural instability. 3-hydroxyflavone was retained (Fig. 7). Britsch (1990) also suggested that the catalytic intermediate of FNS should be 2-hydroxyflavone. Although FNS was highly similar to FLS in protein structure, there was a preference difference in hydroxylation position probably due to the difference in the relative position of its active center to the flavonoid parent ring, which makes the ratio of diosmetin to 4'-O-methyl taxifolin in the catalytic products of different enzymes quite different. This speculation has research value for the control of flavone biosynthetic byproducts. Martens et al (2003) showed that FLS could catalyze 2S naringenin to (+)-Trans DHK, which can also catalyze (+)-Trans DHK to kaempferol. So that we believe the literature could support the speculation that FLS can catalyze C-2 and C-3 positions.

Declarations

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Authors' contributions: Zhen Wang: Writing - Original draft, Conceptualization, Methodology, Performed the expriments. Juan Liu: Methodology, Date curation. Feiyao Xiao: Methodology. Huang Xu: Date curation. Miaomiao Tian: Resources, Visualization. Shenghua Ding: Writing - Review & editing, Methodology. Yang Shan: Conceptualization, Methodology, Supervision.

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Figures

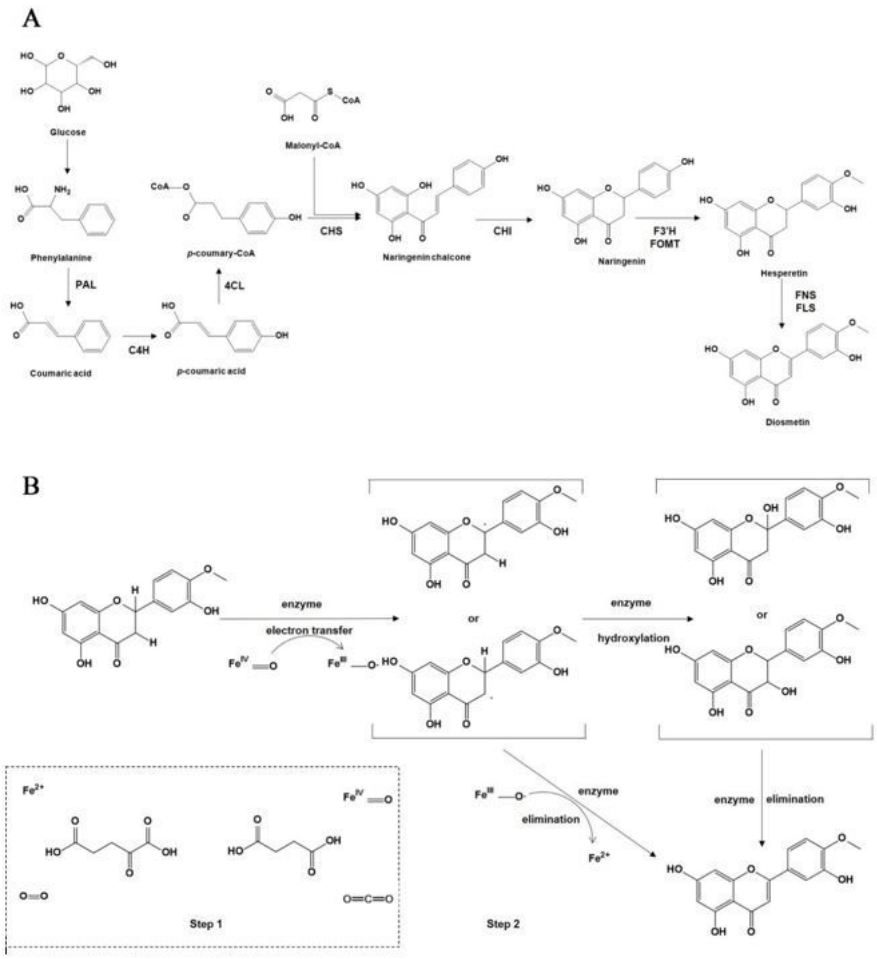

Figure 1

Biosynthesis of diosmetin Note: A: Biogenic synthesis of flavone compound, PAL phenylalanine ammonia lyase, C4H cinnamic acid-4-hydroxylase, 4CL 4-acyl CoA ligase, CHS chalcone synthase, CHI chalcone isomerase, F3'H flavonoid 3'-monooxygenase, FOMT flavone O-methyltransferase, FNS flavone synthase, FLS flavonol synthase. B: Possible mechanism of synthesis of diosmetin from hesperetin catalyzed by FNS/FLS

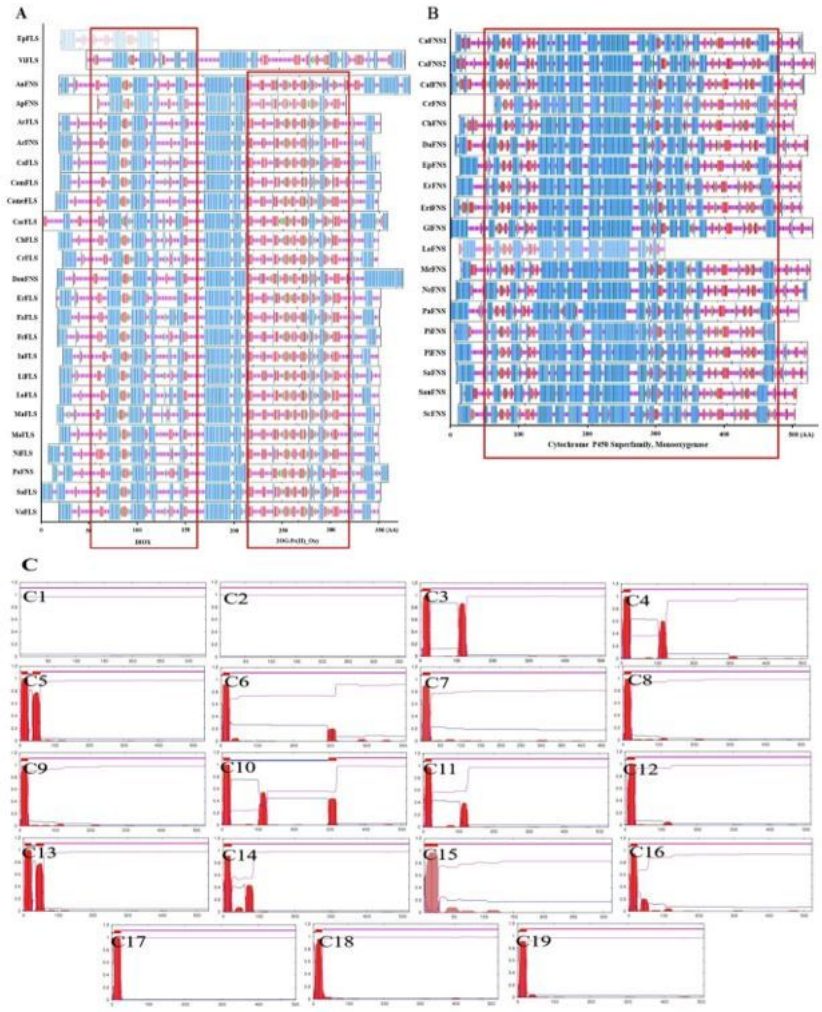

Figure 2

Secondary structure, conservative domain, and transmembrane structure of FNS/FLS Note: A: The enzymes with DIOX or 2OG-Fe(II)_Oxy conserved domain. B: The enzymes with Cytochrome P450 Superfamily Monooxygenase conserved domain. C: transmembrane structure of FNS/FLS, C1 CrFLS, C2 AnFNS, C3 ChFNS, C4 SauFNS, C5 CaFNS1, C6 PlFNS, C7 PiFNS, C8 EpFNS, C9 EriFNS, C10 DaFNS, C11 GlFNS, C12 CalFNS, C13 CaFNS2, C14 MeFNS, C15 LoFNS, C16 NeFNS, C17 ScFNS, C18 PaFNS, C19 ErFNS

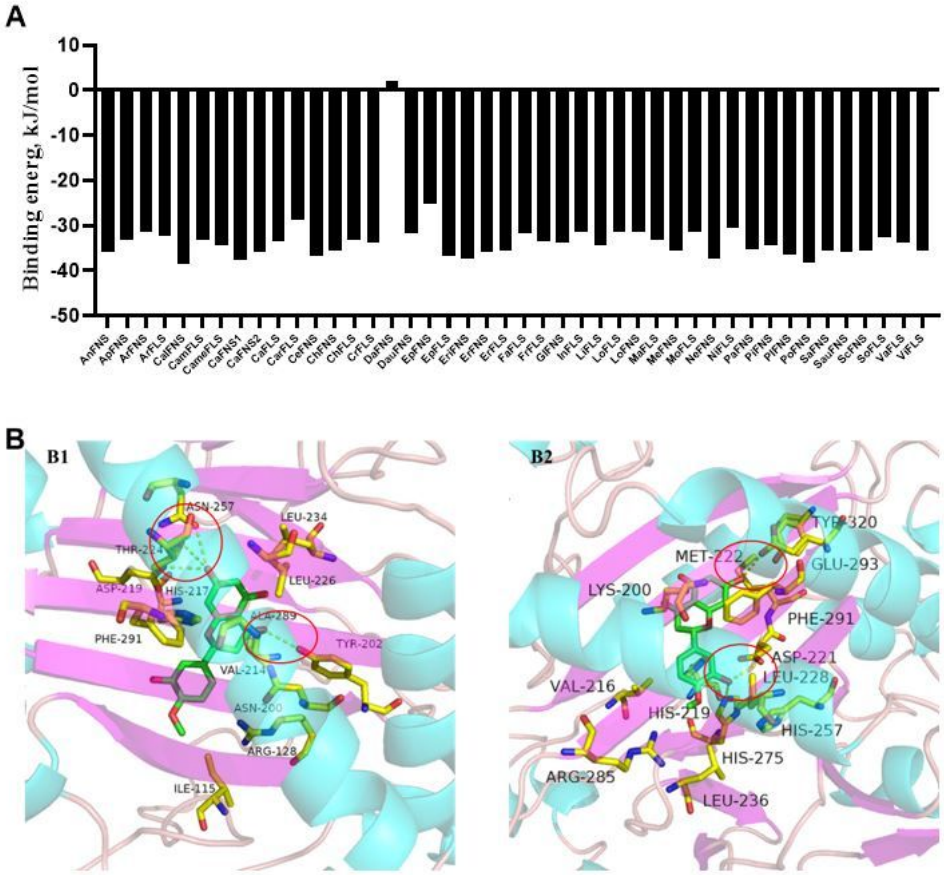

Figure 3

Computer/molecular simulation of the binding of FNS/FLS with hesperetin Note: A: Binding energy of FNS/FLS with hesperetin. B: Dominant conformation of FNS/FLS combined with hesperetin B2 ArFLS, B2 AnFNS

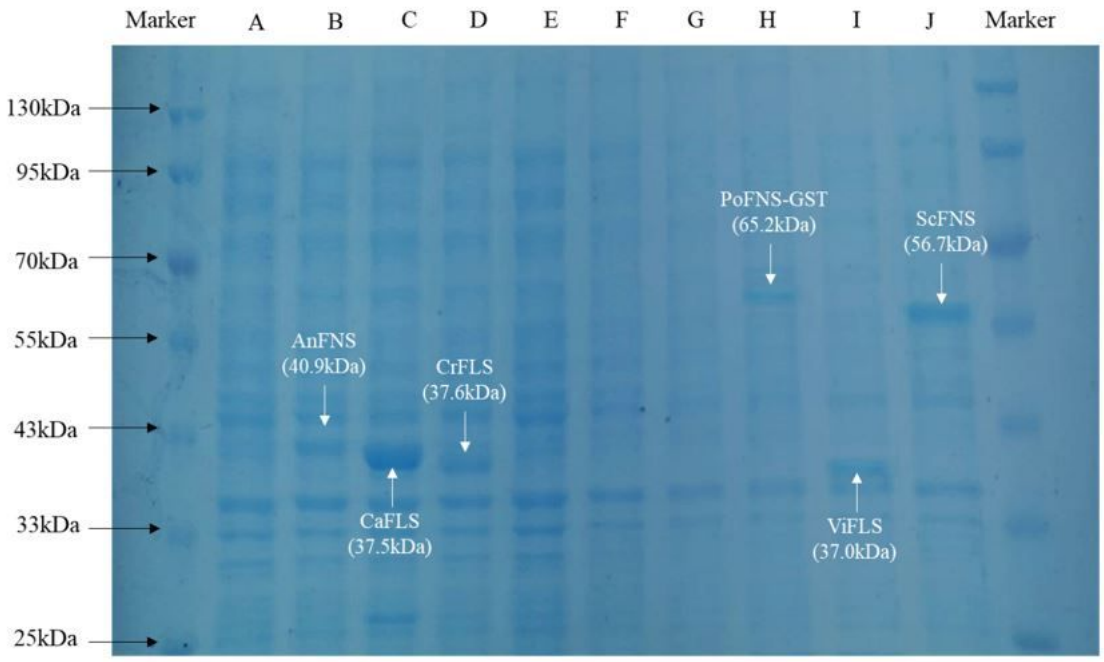

Figure 4

SDS-PAGE analysis of intracellular soluble proteins in recombinant E. coli strain Note: SDS-PAGE analysis of intracellular soluble proteins in recombinant E. coli strain (A DE3/pET-28a(+), B DE3/pAnFNS, C DE3/pCaFLS, D DE3/pCrFLS , E DE3/pInFLS , F DE3/pLoFLS , G DE3/pPoFNS , H DE3/pPoFNS-GST, I

DE3/pViFLS , J DE3/pScFNS)

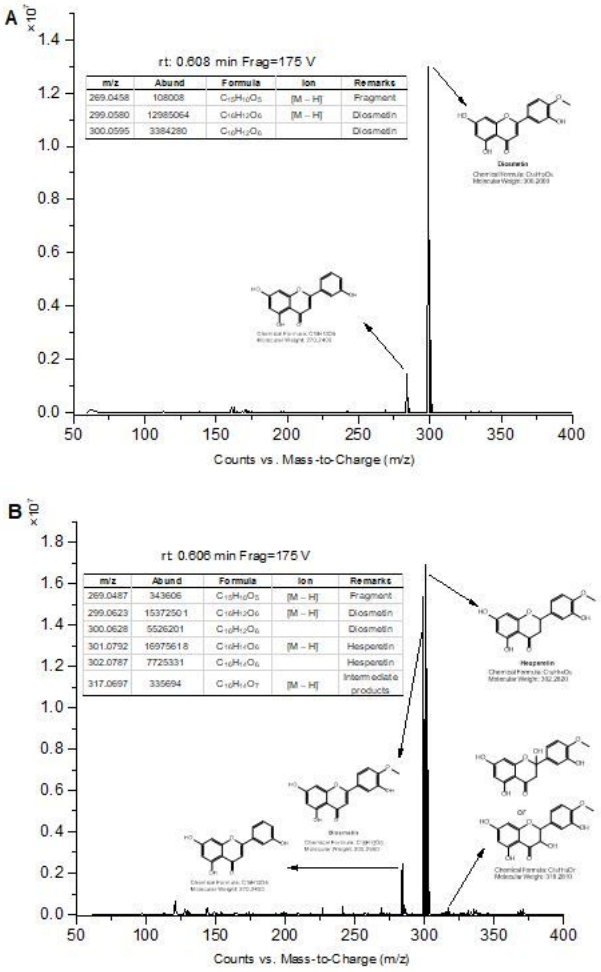

Figure 5

LC-MS spectrum of recombinant E. coli strain broth Note: A diosmetin reference material, B DE3/pAnFNS recombinant Escherichia coli broth.

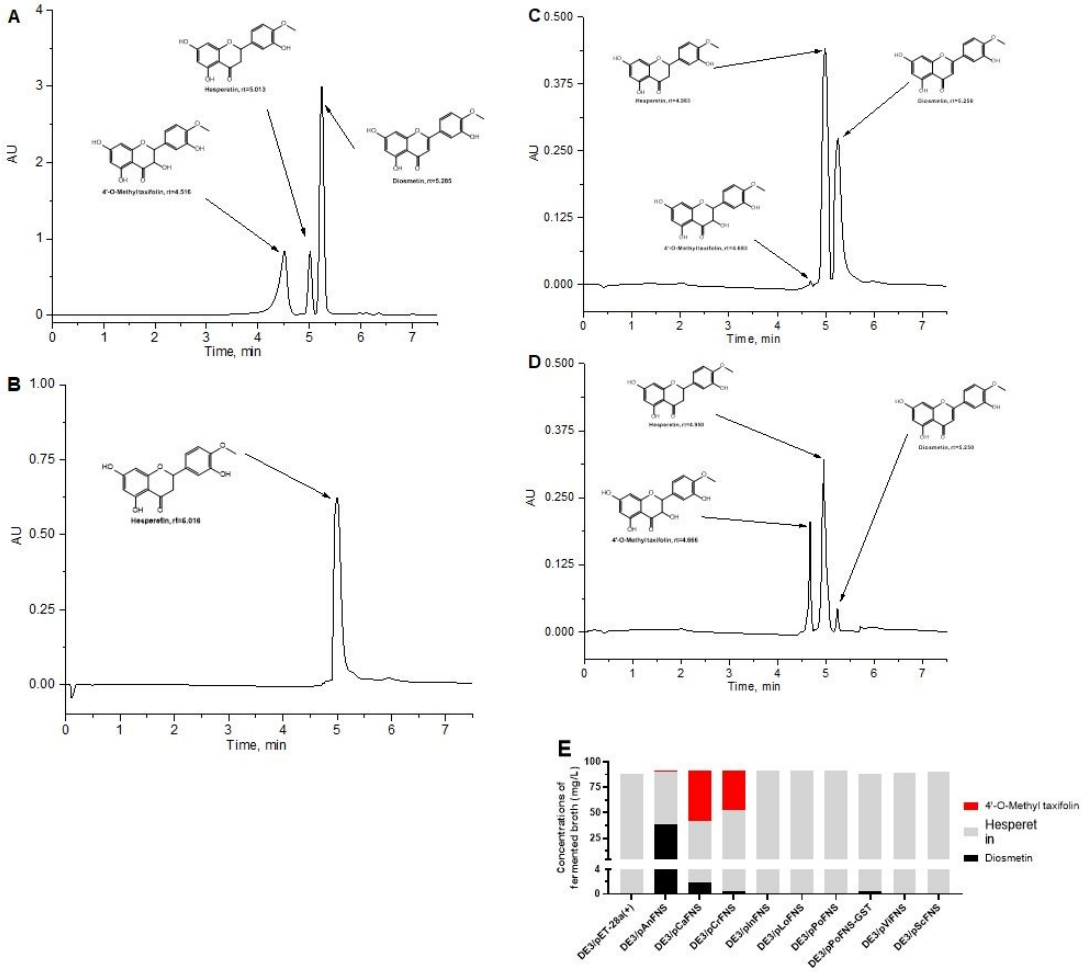

Figure 6

UPLC results of recombinant E. coli strain broth Note: A UPLC spectrum of 4'-O-Methyl taxifolin, hesperetin and diosmetin reference material at 330 nm, B UPLC spectrum of DE3/p28a(+) recombinant Escherichia coli broth at 330 nm, C UPLC spectrum of DE3/pAnFNS recombinant Escherichia coli broth at 330 nm, D UPLC spectrum of DE3/pCaFLS recombinant Escherichia coli broth at 330 nm, E Concentration of diosmetin, 4'-O-Methyl taxifolin, hesperetin in recombinant Escherichia coli strains broth.

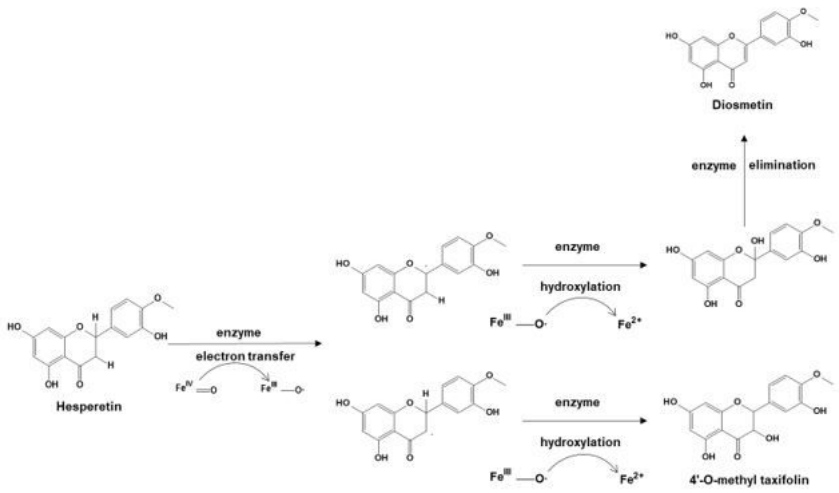

Figure 7

The process of hesperetin transformed into diosmetin