Enzymatic synthesis of myricetin 3-O-galactoside through a whole-cell biocatalyst

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ABSTRACT

Objective: Myricetin 3-O-galactoside is an active compound with pharmaceutical potential. The insufficient supply of this compound becomes a bottleneck in the druggability study of myricetin 3-O-galactoside. Thus, it is necessary to develop a biosynthetic process for myricetin 3-O-galactoside through metabolic engineering.

Methods: Two genes OcsUSB1 and OcUGE1 encoding sucrose synthase and UDP-glucose 4-epimerase were introduced into BL21(DE3) to reconstruct a UDP-O-galactose (UDP-Gal)-biosynthetic pathway in Escherichia coli. The resultant chassis strain was able to produce UDP-Gal. Subsequently, a flavonol 3-O-galactosyltransferase DkFGT gene was transformed into the chassis strain producing UDP-Gal. An artificial pathway for myricetin 3-O-galactoside biosynthesis was thus constructed in E. coli.

Results: The obtained engineered strain was demonstrated to be capable of producing myricetin 3-O-galactoside, reaching 29.7 mg/L.

Conclusion: Biosynthesis of myricetin 3-O-galactoside through engineered E. coli could be achieved. This result lays the foundation for the large-scale preparation of myricetin 3-O-galactoside.© 2020 Tianjin Press of Chinese Herbal Medicines. Published by ELSEVIER B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Myricetin 3-O-galactoside is an important flavonol glycoside in which a galactosyl group was linked to 3-OH group of myricetin through a galactosidic bond (Devi et al., 2015). Myricetin 3-O-galactoside is widely distributed in plants, like Oenothera lavenulaefolia T. and G. (Kagan, 1967), Myrtus communis Linn. (Hayder et al., 2008), Davilla elliptica St. Hil. (de Oliveira et al., 2015; Campos et al., 2013), Catha edulis Forssk (Al-Meshal et al., 1986), Solanum melongena L. (Singh, Luthria, et al., 2009; Singh, Wilson, et al., 2009), Camellia sinensis (L.) O. Ktze (Hilal & Engelhardt, 2009), Betula pendula Roth (Lavola et al., 1997; Laitinen et al., 2002) and Betulae folium Roth (Pietta et al., 1989). Besides having better water solubility and oral bioavailability, myricetin 3-O-galactoside shows a variety of activities, such as antioxidant activity (Hayder et al., 2008; Singh, Luthria, et al., 2009; Singh, Wilson, et al., 2009), anti-inflammatory effect (de Oliveira et al., 2015), antinociceptive action (de Oliveira et al., 2015; Campos et al., 2013) and antigenotoxic potential (Hayder et al., 2008). Myricetin 3-O-galactoside is thus regarded as an active molecule with pharmaceutical potential.

Plant extraction is still the main method to obtain myricetin 3-O-galactoside. However, the content of myricetin 3-O-galactoside in plants is low (Al-Meshal et al., 1986; Singh, Luthria, et al., 2009; Singh, Wilson, et al., 2009). In addition, the large-scale production of myricetin 3-O-galactoside requires a lot of plants, which brings significant ecological and environmental pressure. These adverse conditions limit the supply of myricetin 3-O-galactoside, thus affecting the druggability study of myricetin 3-O-galactoside. In this way, it is necessary to establish a green alternative for the scale-up preparation of myricetin 3-O-galactoside. Because enzyme-assisted biosynthesis of natural products has made great strides recently, it has attracted our attention to preparing myricetin 3-O-galactoside by enzymatic synthesis (Schmidt-Dannert & Lopez-Gallego, 2016).

The galactosylation of myricetin to form myricetin 3-O-galactoside is catalyzed by a flavonol 3-O-galactosyltransferase (F3GalTase), which usually uses uridine diphosphate (UDP)-
galactose (UDP-Gal) as the sugar donor. Many F3GalTases had been isolated from varied plant species and then functionally characterized (Ikegami et al., 2009; Miller et al., 1999). These F3GalTases were thus introduced into host cells like Escherichia coli to construct whole-cell biocatalysts for 3-0-galactosylation of flavonols such as myricetin, quercetin and kaempferol. Up to date, the biosynthesis of quercetin 3-O-galactoside and kaempferol 3-O-galactoside through the whole-cell biocatalysis system had been achieved (Kim et al., 2015). The enzymatic synthesis of myricetin 3-O-galactoside through engineered cells, however, have been documented in scarcity.

Herein, an E. coli chassis producing UDP-Gal was reconstructed firstly. Subsequently, a flavonol 3-O-galactosyltransferase was introduced into this microbial chassis, thereby generating an engineered strain. The resulting engineered E. coli was used as the whole-cell biocatalysts for myricetin 3-O-galactoside production using myricetin as the substrate. Results demonstrated that the engineered strain was able to convert myricetin to myricetin 3-O-galactoside.

2. Materials and methods

2.1. Plasmids and strains

A synthetic flavonol 3-O-galactosyltransferase gene DkFGT from Diospyros kaki Thunb. (Gene ID AB435084 (Ikegami et al., 2009)), was cloned into Xhol/Ndel digested pACYCDuet-1 to form a recombinant plasmid pA-DkFGT. Using pA-DkFGT as the template for PCR amplification, a 1380 bp linear DkFGT gene was inserted SacI/HindIII site of pCold-TF (Takara, Kyoto, Japan) to obtain a recombinant expression plasmid pTF-DkFGT using Seamless Assembly Cloning Kit (CloneSmarter Tech. USA). Plasmids pCDFDuet-OcSUS1 containing sucrose synthase gene (Li & Kong, 2016) and pET28a-OcUGE1 harboring UDP-glucose 4-epimerase gene (Yin & Kong, 2016) were constructed previously in our laboratory. Strains Trans1-T1 (TransGen Biotech Co., Ltd., Beijing, China) and BL21 (DE3) were used as the cloning and expression host, respectively. The detailed description of plasmids and strains was summarized in the supplementary Table S1.

2.2. Chemicals

The chemicals used for galactosylation assay were listed in the supplementary Table S2.

2.3. Protein expression and purification

The plasmid pTF-DkFGT was transformed into BL21(DE3) to yield a recombinant strain BL21(DE3) [pTF-DkFGT]. The recombinant strain was inoculated overnight into LB medium with 50 μg/mL ampicillin at 37 °C. Subsequently, 2 mL overnight culture was refreshed with 200 mL LB medium and continued to grow until OD600 reached 0.5 at 37 °C. These cells were then kept at 15 °C for 30 min. IPTG at a final concentration of 0.4 mmol/L was added into the strains to induce the protein expression with shaking at 160 rpm at 15 °C for 24 h. The induced cells were harvested by centrifugation at 8000 g for 5 min. The resulting cell pellets were re-suspended in PBS buffer (0.02 mol/L, pH 8.0) and then disrupted by sonication on ice. The cell homogenate was clarified by centrifugation at 10,000 rpm for 15 min. The resultant supernatants were analyzed by SDS-PAGE or subject to protein purification using Ni-NTA Superflow™ resin (Qiagen, Hamburg, Germany). The concentration quantification of the purified proteins was carried out using Super-Bradford Protein Assay Kit (CWBio. Co., Ltd., Beijing, China).

2.4. Galactosylation assay

In vitro galactosylation assays were performed in 100 μL PBS buffer (20 mmol/L, pH 6.0 for myricetin and pH 7.0 for other chemicals) containing sugar acceptor (1 mmol/L), UDP-Gal (0.5 mmol/L) and 20 μg purified galactosyltransferase. Galactosylation assays have been lasted for 2 h at 50 °C. The reactions were terminated by the addition of methanol of equal volume (100 μL). The mixtures were then clarified by centrifugation at 12,000 rpm for 5 min. The samples were passed through a filter (0.22 μm) and the resulting filtrates were analyzed by thin-layer chromatography (TLC). The specific TLC conditions for each sugar acceptor were summarized in supplementary Table S3. Further confirmation of galactosylated flavonols (myricetin, quercetin and kaempferol) was performed with a prominence HPLC system (Shimadzu, Kyoto, Japan) equipped a CAPCELL PAK C18 MGII S5 column (150 × 4.6 mm id, 5 μm particle size) using a mobile phase mixture of acetonitrile (eluente B) and ddH2O (eluente A). The flow rate was maintained at 1.0 mL/min and a sample volume of 30 μL was injected. High resolution-electrospray ionization-mass spectrometry (HR-ESI-MS) data were obtained on a Thermo Scientific Exactive Orbitrap LC-Mass Spectrometer (Thermo Scientific, Waltham, MA, USA).

2.5. Whole-cell biosynthesis of myricetin 3-O-galactoside

Three plasmids, pCDFDuet-OcSUS1, pET28a-OcUGE1 and pA-DkFGT, were co-transformed into BL21 (DE3) to yield an engineered strain. The engineered strain was grown overnight at 37 °C in 20 mL LB medium with appropriate antibiotics. One milliliter overnight culture was subsequently inoculated into 100 mL fresh TB medium (12 g/L tryptone, 24 g/L yeast extract, 4 mL glyc erol and 100 mL phosphate buffer (KH₂PO₄: 0.17 mol/L; K₂HPO₄: 0.72 mol/L)) and continued to grow at 37 °C until OD₆₀₀ reached 0.6. At this time, IPTG of 100 mmol/L (400 μL) was added into the cell culture to start induction. The whole induction process lasted for 20 h at 22 °C. Next, the induced culture was harvested by centrifugation (8000 rpm, 8 min) and the resultant pellet was resuspended with 6 mL TB medium. One milliliter suspension was separated by centrifugation (12,000 rpm, 2 min) and the resulting pellet was resuspended by 500 μL TB medium, into which myricetin (5 μL, 100 mmol/L) and sucrose (20%) were added. The resultant mixture was incubated for 48 h at 30 °C with a continuous shaking of 240 rpm. The reagents were extracted by adding equal volume of ethyl acetate and the resultant extract was analyzed by HPLC as mentioned above.

3. Results and discussion

3.1. Results and discussion

Initially, the heterologous expression of DkFGT was tested in BL21(DE3) [pA-DkFGT]. However, DkFGT was induced to form a small amount of soluble protein and most of the expressed proteins were in the form of inclusion body. The inclusion body form is not conductive to protein purification of DkFGT. To improve the soluble expression, DkFGT gene was cloned into the plasmid pCold-TF. The pCold-TF is a cold shock expression vector equipped a CAPCELL PAK C18 MGII S5 column (150 × 4.6 mm id, 5 μm particle size) using a mobile phase mixture of acetonitrile (eluente B) and ddH2O (eluente A). The flow rate was maintained at 1.0 mL/min and a sample volume of 30 μL was injected. High resolution-electrospray ionization-mass spectrometry (HR-ESI-MS) data were obtained on a Thermo Scientific Exactive Orbitrap LC-Mass Spectrometer (Thermo Scientific, Waltham, MA, USA).

The concentration quantification of the purified proteins was carried out using Super-Bradford Protein Assay Kit (CWBio. Co., Ltd., Beijing, China).

3.2. Soluble expression and purification of DkFGT

The plasmid pTF-DkFGT was transformed into BL21(DE3) to yield a recombinant strain BL21(DE3) [pTF-DkFGT]. The recombinant strain was inoculated overnight into LB medium with 50 μg/mL ampicillin at 37 °C. Subsequently, 2 mL overnight culture was refreshed with 200 mL LB medium and continued to grow until OD₆₀₀ reached 0.5 at 37 °C. These cells were then kept at 15 °C for 30 min. IPTG at a final concentration of 0.4 mmol/L was added into the strains to induce the protein expression with shaking at 160 rpm at 15 °C for 24 h. The induced cells were harvested by centrifugation at 8000 g for 5 min. The resulting cell pellets were re-suspended in PBS buffer (0.02 mol/L, pH 8.0) and then disrupted by sonication on ice. The cell homogenate was clarified by centrifugation at 10,000 rpm for 15 min. The resultant supernatants were analyzed by SDS-PAGE or subject to protein purification using Ni-NTA Superflow™ resin (Qiagen, Hamburg, Germany). The concentration quantification of the purified proteins was carried out using Super-Bradford Protein Assay Kit (CWBio. Co., Ltd., Beijing, China).
(DE3) in soluble protein forms of about 100 kDa (TF tag plus DkFGT). The soluble protein was then purified to near homogeneity with protein concentration of 3.62 mg/mL.

3.2. Galactosylation of myricetin

With the help of chaperone TF, almost all recombinant proteins of DkFGT existed in the form of a soluble fusion protein with TF. It was uncertain whether the fusion protein was active. Hence, the flavonol 3-O-galactosyltransferase activity of DkFGT-TF fusion was tested in vitro using three flavonols (myricetin, quercetin and kaempferol) as the substrates. As shown in Fig. 2, when myricetin was incubated with DkFGT, a new peak with the retention time of 10 min was present in HPLC profile (Fig. 2A). The new peak displayed the characteristic UV spectrum of myricetin 3-O-galactoside, indicating this new compound is a derivative of myricetin (Fig. 2B). Further co-injection of this compound with the authentical myricetin 3-O-galactoside revealed their identical retention time (Fig. 2A). Moreover, this new molecule displayed the pseudomolecular [M−H]− ion at m/z 479.06918 in the HR-ESI-MS, consistent with that of the reference myricetin 3-O-galactoside standard (Fig. 2C and D). These data, together with the catalytic behavior of flavonol 3-O-galactosyltransferases, revealed that the new compound was myricetin 3-O-galactoside. In addition, two other flavonols (kaempferol and quercetin) were demonstrated to be galactosylated by DkFGT-TF fusion protein to form kaempferol 3-O-galactoside (trifolin) and quercetin 3-O-galactoside (hyperoside) (Figs. 3 and 4), respectively. These data collectively revealed that DkFGT-TF fusion protein was active as a flavonol 3-O-galactosyltransferase.

Previously, DkFGT was demonstrated to be specific for flavonols (Ikegami et al., 2009). To understand the substrate range of DkFGT more comprehensively, more compounds, including flavonoids, steroids, phenolic acids and so on, were used to react with the purified DkFGT-TF. However, none of other compounds listed in Table S2 can react with DkFGT.

Besides the acceptor substrate specificity, the sugar donor promiscuity of DkFGT was also evaluated. Due to the limited availability of sugar donors, only two donors, namely UDP-D-glucose (UDP-Glc) and UDP-Gal, were selected for activity screening in this investigation. Three flavonols (myricetin, kaempferol and quercetin) displayed no activity with UDP-Glc under the action of DkFGT. These results confirmed again that DkFGT was a flavonol specific galactosyltransferase.

3.3. Whole-cell biosynthesis of myricetin 3-O-galactoside

The recombinant strain BL21(DE3) [pTF-DkFGT] was used as a whole-cell biocatalyst to react with myricetin. Results showed that almost no products were produced. It is speculated that UDP-Gal may be under supplied. It appeared that the insufficient supply of UDP-Gal was the limiting factor in the biosynthesis of myricetin 3-O-galactoside. A chassis strain producing UDP-Gal was thus constructed. Specifically, two genes, namely OcSUS1 and OcUGE1, were introduced into BL21(DE3) in the form of plasmids pCDFduet-
OcSUS1 and pET28a-OcUGE1. OcSUS1 encodes a sucrose synthase while OcUGE1 encodes a UDP-glucose epimerase. OcSUS1 can utilize sucrose supplemented in medium to form UDP-Glc, which is subsequently converted into UDP-Gal under the action of OcUGE1. Thus, the chassis cell harboring OcSUS1 and OcUGE1 genes was able to produce UDP-Gal. When DkFGT was introduced into this chassis cell in the form of the plasmid pTF-DkFGT, the expression of DkFGT would decrease the soluble expression of other two genes OcSUS1 and OcUGE1. Therefore, pA-DkFGT instead of pTF-DkFGT was introduced into the chassis cell producing UDP-Gal. An artificial pathway for myricetin 3-O-galactoside biosynthesis was thus reconstructed in E. coli (Fig. 5A). The engineered E. coli was grown in LB medium supplemented with myricetin, which then entered the cell through cell membrane. After induced by IPTG, the expressed DkFGT was able to transfer the galactosyl group from the resultant UDP-Gal to myricetin, thereby generating myricetin 3-O-galactoside (Fig. 5B). The yield of myricetin 3-O-galactoside was quantified to 29.7 mg/L. This result lays the foundation for the large-scale preparation of myricetin 3-O-galactoside through engineered strains.

E. coli is rich in nucleotide sugars such as UDP-Glc, UDP-glucuronic acid, UDP-N-acetylglucosamine and TDP-L-rhamnose (Pabst et al., 2010). E. coli expressing OcSUS1 and OcUGE1 cannot only produce UDP-Glc, but also catalyze the conversion of exogenous sucrose and TDP to form TDP-Glc (Li & Kong, 2016). Hence, E. coli expressing OcSUS1 and OcUGE1 can be used as a nucleotide sugar library to screen the donor selectivity of DkFGT. As shown in Fig. 5, the strain containing DkFGT, OcSUS1 and OcUGE1 can only
glycosylate myricetin to 3-O-galactoside, displaying strict donor specificity towards UDP-Gal.

To further explore the underlying causes of specific recognition towards UDP-Gal, DkFGT was aligned with other six glycosyltransferases (GTs) using BioEdit Sequence Alignment Editor with default parameters. Among the seven GTs, DkFGT and F3GalTase (Miller et al., 1999) specifically recognize UDP-Gal, while the other five GTs, AtUGT78D1 (Mo et al., 2016), AtUGT78D2 (Lim et al., 2004), AtUGT89B1 (Lim et al., 2004), UGT78k1 (Parajuli et al., 2015) and VvGT1 (Offen et al., 2006), prefer UDP-Glc. Multiple alignment of the seven sequences revealed that the well-known plant secondary product glycosyltransferase (PSPG) motif was highly conserved (Fig. 6) (Jadhav et al., 2012). Moreover, one conserved amino acid responsible for the direct interaction with hydroxyls of sugar donors was observed: aspartic acid (Asp, D)-374 (the order in VvGT1) (Offen et al., 2006). The amino acid next to PSPG motif varied in GTs. In GTs with preference for UDP-Gal, including DkFGT and F3GalTase, the amino acid is histidine (His, H). The amino acid, however, is changed to asparagine (N) or glutamine (Q) in GTs using UDP-Glc as the favoured donor (Fig. 6). These results...
revealed that H-378 (the order in DkFGT) might play important role in donor selection for GTs, consistent with the previous report (Kubo et al., 2004). However, more data are required to demonstrate the exact function of H-378 in determining donor preference of GTs.

4. Conclusion

In this study, we use three genes including OcSUS1, OcUGE1 and DkFGT to successfully reconstruct a myricetin 3-O-galactoside biosynthetic pathway in Escherichia coli, and the yield could reach 29.7 mg/ml in current conditions. This result lays the foundation for the large-scale preparation of myricetin 3-O-galactoside.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chmed.2020.03.009.

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