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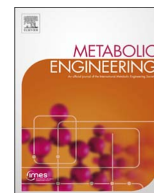

Biosynthesis of plant-derived ginsenoside Rh2 in yeast via repurposing a key promiscuous microbial enzyme

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ABSTRACT

Ginsenoside Rh2 is a potential anticancer drug isolated from medicinal plant ginseng. Fermentative production of ginsenoside Rh2 in yeast has recently been investigated as an alternative strategy compared to extraction from plants. However, the titer was quite low due to low catalytic capability of the key ginseng glycosyltransferase in microorganisms. Herein, we have demonstrated high-level production of ginsenoside Rh2 in *Saccharomyces cerevisiae* via repurposing an inherently promiscuous glycosyltransferase, UGT51. The semi-rationally designed UGT51 presented an ~1800-fold enhanced catalytic efficiency (k_{cat}/K_m) for converting protopanaxadiol to ginsenoside Rh2 *in vitro*. Introducing the mutant glycosyltransferase gene into yeast increased Rh2 production from 0.0032 to 0.39 mg/g dry cell weight (DCW). Further metabolic engineering, including preventing Rh2 degradation and increasing UDP-glucose precursor supply, increased Rh2 production to 2.90 mg/g DCW, which was more than 900-fold higher than the starting strain. Finally, fed-batch fermentation in a 5-L bioreactor led to production of ~300 mg/L Rh2, which was the highest titer reported.

1. Introduction

Ginseng saponins have well-known therapeutic functions, and ginseng plants have been widely cultivated in North America and Asia. Ginsenoside Rh2 is a trace but important saponin isolated from red ginseng (Kitagawa et al., 1983), and it exhibits diverse pharmacological effects, such as anti-oxidation, hepatoprotection, anti-diabetes and anti-tumor (Hwang et al., 2007; Kim et al., 1999; Liu, 2012). The use of Rh2 in combination with anti-tumor drugs remarkably increased the efficacy of tumor suppression and reduced the side effects caused by chemotherapy (Nakata et al., 1998). Despite the versatile bioactivities of ginsenoside Rh2, its content in red ginseng is extremely low (around 0.001%) (Shibata, 2001). Currently, ginsenoside Rh2 is primarily manufactured by chemical or biological deglycosylation of ginsenosides (Bae et al., 2004; Su et al., 2006; Zhang et al., 2001). Because of the long cultivation periods for qualified roots (5–7 years) and the complicated extraction and purification process, the preparation of ginsenoside Rh2 requires considerable time and labor costs.

With the development of synthetic biology, substantial progress has been made in the low-cost production of plant natural products in microbes, including artemisinin (Martin et al., 2003), Taxol (Ajikumar et al., 2010), strictosidine (Brown et al., 2015), and opioids (Galanie et al., 2015). These plant-derived compounds are synthesized in microbial host cells via the establishment of non-inherent synthetic pathways. Recently, microbial production of protopanaxadiol (PPD), the precursor of ginsenoside Rh2, in *S. cerevisiae* was reported (Dai et al., 2013). Subsequently, heterologous production of ginsenoside Rh2 was accomplished by introducing ginseng glycosyltransferase UGTPg45 in *S. cerevisiae*. However, the titer was quite low (~16 mg/L) because of the unsatisfied performance of the key ginseng glycosyltransferase as kinetic parameters measured (Wang et al., 2015). Comparable accumulation of PPD precursor within the engineered yeast strains suggested that the glycosyltransferase involved in the last step was the bottleneck in the whole pathway. Therefore, it was essential to increase glycosyltransferase activity towards Rh2 synthesis.

Besides using plant-derived glycosyltransferase, an alternative way is to use engineered substrate-promiscuous enzymes (Dietrich et al., 2009). Microbial enzymes commonly exhibit 'substrate promiscuity', which represents an evolutionary locus for adapting to varied environments and enables microbes to perform comparable chemical transformations using different substrates (Copley, 2015). Promiscuous microbial enzymes can interact with a variety of substrates that have

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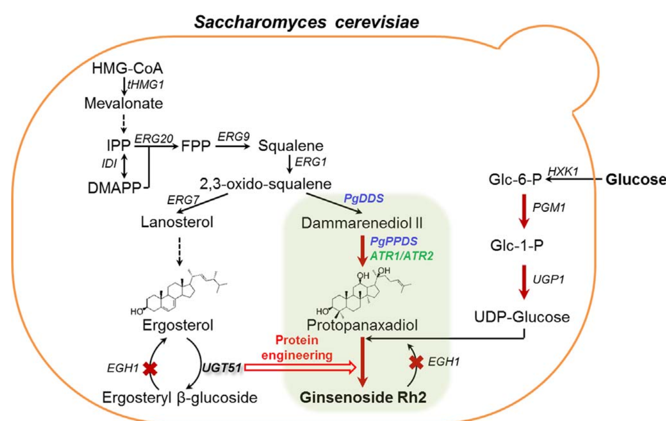

Fig. 1. Designed biosynthetic pathway of ginsenoside Rh2 in yeast. Steryl-glucoside and ginsenoside Rh2 were both derived from 2,3-oxidosqualene via similar reactions, including cyclization, hydroxylation and glycosylation. Yeast genes are shown in black. Genes from *P. ginseng* are shown in blue. Genes from *A. thaliana* are shown in green. Multiple-step reactions are shown as dashed arrows, and overexpressed steps are shown as bold arrows. Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IPP, isopentenylpyrophosphate; DMAPP, dimethylallylpyrophosphate; FPP, farnesyldiphosphate; Glu-6-P, Glucose 6-phosphate; and Glu-1-P, Glucose 1-phosphate.

structures similar to their natural substrates, although the activity may be lower with the non-natural substrates. Nonetheless, these enzymes native to the chassis cells represent good candidates for further directed evolution, which may lead to the desired performance both *in vitro* and *in vivo*.

To identify the promiscuous microbial enzyme candidates for Rh2 synthesis, we performed a comparative analysis of saponins between steryl-glucoside biosynthetic pathway in *S. cerevisiae* and ginsenoside Rh2 biosynthetic pathway from ginseng (Fig. 1). UGT51 is a UDP-glucose: sterol glucosyltransferase from *S. cerevisiae*, and it is responsible for the glucosylation of ergosterol to form ergosteryl-glucoside. Moreover, UGT51 has been reported to enable the conversion of certain sterols into steryl-glucosides (Warnecke et al., 1999). Accordingly, we presumed that the promiscuous UGT51 native to the chassis cell may serve as a promising starting enzyme for microbial production of plant saponins. In the present study, high production of ginsenoside Rh2 represented an excellent example of *de novo* biosynthesis of plant natural products in microbes via the use of an engineered promiscuous microbial enzyme.

2. Materials and methods

2.1. Chemicals, strains and culture conditions

PPD, dammarenediol II and other acceptor compounds (> 98% purity) were obtained from Faces Biochemical (Wuhan, China). Ginsenoside Rh2 was purchased from Sigma-Aldrich (USA), and 2-chloro-4-nitrophenyl β-D-glucopyranoside was synthesized by WuXi AppTec (China).

E. coli BL21 CodonPlus (*DE3*) cells were used as the host cells for the expression of the UGTs. The yeast chassis strain ZD-PPD-016(URA3$^-$) (Dai et al., 2013) was kindly provided by Prof. Zhang at Tianjin Institute of Industrial Biotechnology. The plasmid pUMRI-10 (Xie et al., 2015) was kindly provided by Prof. Yu at Zhejiang University. All plasmids and strains used in this study are listed in Table S1 and S2, and they were constructed as described in the Supplementary Data. All of the primers used for plasmid and strain construction are listed in Table S3.

Shake-flask cultures for the yeast strains are described in the Supplementary Data. The fed-batch fermentations were performed in a 5-L bioreactor (WinPact, USA) with an initial working volume of 2 L. The media for seed and batch fermentation were modified from previously described media (Lenihan et al., 2008), and 5g/L of lysine was added. The seed culture was inoculated with a fermenter with an initial OD_{600} of 0.5. The initial agitation was set to 300 rpm and increased to a maximum of 800 rpm. Fermentation was conducted as previously described (Dai et al., 2013). The glucose and ethanol concentrations were measured with an SBA-40E Biosensor (Institute of Biology, Academy of Sciences, Shandong, China) according to the manufacturer's instructions.

2.2. Production and purification of glucosyltransferase UGT51

The gene fragment of UGT51 excluding the first 721 N-terminal amino acids (Warnecke et al., 1999) was amplified from the chromosome of *S. cerevisiae* S288c as the template using the primer sets UGT51_F (5'-CAAGCATATGTTAATGATTGATGAGAATCCGC-3') and UGT51_R (5'-CTAGCTCGAGTTAAATCATCGTCCACCCTTCA-3'), which incorporated *NdeI* and *XhoI* sites, respectively. The amplified gene was ligated into the pET-28a vector (Novagen). The recombinant plasmid was then transformed into *E. coli* BL21 CodonPlus cells for expression.

The cells were grown at 37 °C in 1 L of LB medium containing 50 μg/mL of kanamycin and 34 μg/mL of chloramphenicol to an OD_{600} of 0.6–0.8. Induction was performed by adding 0.4 mM isopropyl-1-thio-*b*-D-galactopyranoside (IPTG), and further incubation was performed for 20 h at 16 °C. The cells were harvested by centrifugation (5000 × *g*, 4 °C) and resuspended in 100 mL of lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM 1,4-dithiothreitol). Cell disruption was performed by ultrasonication. The cell extracts were applied to a 2-mL Ni Sepharose™ 6 Fast Flow column (GE Healthcare), which was pre-equilibrated in buffer A (20 mM Tris-HCl, pH 8.0, 500 mM NaCl). After extensive washing, fractions were eluted using 5 column volumes of buffer A containing 120 mM imidazole.

2.3. Glycosyltransferase assay

The reaction mixture for the PPD enzymatic assay consisted of 100 μl of 20 mM Tris-HCl buffer (pH 8.0) containing PPD (0.5 mM), UDP-glucose (5 mM), 1% (v/v) Tween80 and 0.2 mg/mL purified glycosyltransferase. For the kinetic study, the concentration of PPD ranged from 0.05 to 1 mM. The mixture was incubated in a thermal shaker at 37 °C and 200 rpm. The reaction was terminated by the addition of the same volume of *n*-butanol. The product was extracted and evaporated, and the residue was dissolved in methanol for the HPLC analysis. The lipids were injected on an Agilent1260 HPLC with UV detection at 203 nm using an Agilent Eclipse XDB-C18 (5 μm, 4.6 × 250 mm) column. The solvent flow rate was 1.0 mL/min and the column temperature was set at 40 °C. The mobile phase consisted of water (A) and acetonitrile (B), and a gradient program of 45–100% B in 0–14 min, 100% B in 14–18 min, and 45% B in 18–20 min was applied.

The reaction mixture for the acceptor specificity determination consisted of 100 μl of 20 mM Tris-HCl buffer (pH 8.0) containing the acceptor (0.5 mM), UDP-glucose (2 mM), 1% (v/v) Tween80 and 0.3 mg/mL purified glycosyltransferase. The mixture was incubated in a thermal shaker at 37 °C and 200 rpm and terminated by the addition of *n*-butanol in 2 h. An HPLC analysis was performed to quantify the conversion of the acceptor in each reaction. The conditions for the HPLC analysis were the same as those mentioned above and excluded the mobile phase. In the case of ergosterol, cholesterol, sitosterol and diosgenin, the mobile phase consisted of acetonitrile, methanol and propanol at the volume ratio of 50:80:10. The mobile phase for protopanaxatriol and mogrol was the same as that of PPD. The mobile phase for estradiol and pregnenolone consisted of water (A) and acetonitrile (B), and a gradient program of 10% B in 0–3 min, 10–100% B in 3–20 min, 100% B in 20–23 min, and 10% B in 23–25 min was applied.

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