Optimization of Rhamnetin Production in *Escherichia coli*

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POMT7, which is an *O*-methyltransferase from poplar, transfers a methyl group to several flavonoids that contain a 7-hydroxyl group. POMT7 has been shown to have a higher affinity toward quercetin, and the reaction product rhamnetin has been shown to inhibit the formation of beta-amyloid. Thus, rhamnetin holds great promise for use in therapeutic applications; however, methods for mass production of this compound are not currently available. In this study, quercetin was biotransformed into rhamnetin using *Escherichia coli* expressing POMT7, with the goal of developing an approach for mass production of rhamnetin. In order to maximize the production of rhamnetin, POMT7 was subcloned into four different *E. coli* expression vectors, each of which was maintained in *E. coli* with a different copy number, and the best expression vector was selected. In addition, the *S*-adenosylmethionine biosynthesis pathway was engineered for optimal cofactor production. Through the combination of optimized POMT7 expression and cofactor production, the production of rhamnetin was increased up to 111 mg/l, which is approximately 2-fold higher compared with the *E. coli* strain containing only POMT7.

Keywords: *S*-Adenosylmethionine, *S*-adenosylmethionine synthetase, biotransformation, *O*-methyltransferase, rhamnetin

Structural modification of natural compounds using biotransformation is an effective way to produce regioselective and/or stereoselective compounds [15]. In most cases, cells or enzymes are used to achieve these modifications. High stability and reactivity of enzymes are important for achieving large production levels of the final product. Although whole cells including microorganisms, plants, or animal cells have been successfully used for biotransformation, a transgenic microorganism expressing a gene with a particular purpose can be more valuable because several genes leading to the synthesis of a final product can be easily introduced into a single strain [16].

Intermediates of natural product biosynthesis have been used as targets for several modification reactions, including hydroxylation, methylation, and glycosylation [6, 12, 17]. Such modifications that involve the attachment or detachment of functional groups can modulate the biological activity of certain compounds. For example, a minor component of the genisenosides, compound K, contains diverse biological activities including antitumor, anti-inflammatory, and anti-allergic activities. Compound K was produced from a crude extract of ginseng by enzymes or biotransformation using a fungus [14]. Sakuranetin (7-*O*-methylnaringenin), containing antifungal activity against *Magnaporthe grisea*, was produced by one-step *O*-methylation from naringenin [7].

Flavonoids are phytochemicals with the basic structure of 1,3-diphenylpropan-1-one (C6-C3-C-6). Flavonoids can be classified into several groups including flavanones, flavones, flavonols, flavans, isoflavones, etc. [4]. The classification of a flavonoid depends on the double bond between carbon 2 and carbon 3, the hydroxyl group at carbon 3, the carbonyl group at carbon 3, the carbonyl group at carbon 4, or the location of the B ring.

The biological activities of flavonoids can be altered by modification reactions. *O*-Methylation of a flavonoid modulates its biological activity [10]. For example, regioselective methylation of naringenin with 4’-specific *O*-methyltransferases produces ponciretin, which displays antibacterial activity against *Helicobacter pylori* [5]. In addition, depending on the methylation position, biological activities can be altered; 7-*O*-methylquercetin inhibits the formation of β-amyloid [8], whereas 3-*O*-methylquercetin contains antiviral activity [1].

The bottleneck in using these *O*-methylated flavonoids as pharmaceuticals or medicinal foods is availability, since most are not synthetically produced but, rather, extracted from a large quantity of specific plants. In order to increase production of *O*-methylated flavonoids, we employed biotransformation using *Escherichia coli* harboring flavonoid

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O-methyltransferase (OMT). The advantages of flavonoid O-methylation using biotransformation are (1) production of regioselective flavonoids, and (2) use of S-adenosylmethionine (SAM), an endogenous cofactor produced by the bacteria and as such that does not need to be provided externally. Although biotransformation has advantages over extraction and purification from plants, the low productivity of biotransformation is still problematic. In order to increase the production of biologically active flavonoids, we employed two approaches. First, we cloned the flavonoid OMT gene in different E. coli expression vectors having different copy numbers in order to identify the best expression platform for this gene. Second, in order to increase the level of the cofactor SAM, an additional gene encoding SAM synthetase was also introduced (Fig. 1A). Using these strategies, we could increase the production of rhamnetin up to 111 mg/l.

RESULTS AND DISCUSSION

Effect of Vector on Production of Rhamnetin Using E. coli Harboring POMT7

Four different E. coli recombinant strains were created by cloning POMT7 into four different E. coli expression vectors, each of which was maintained with a different copy number; conversion of quercetin to rhamnetin was then compared among them. As shown in Fig. 2, E. coli harboring POMT7 in pETDuet showed the highest conversion rate, followed by pRSFDuet, pCDFDuet, and pACYCDuet. E. coli harboring POMT7 in the pRSFDuet vector had a conversion rate that was 85% of that observed with the pETDuet vector. The pRSF vector has the highest copy number in E. coli, pACYCDuet retains 10–12 copies, pCDFDuet retains 20–40, pETDuet retains up to 40, and pRSFDuet retains more than 100 copies. Each POMT7 construct was transformed into E. coli BL21 (DE3).

Biotransformation of Quercetin Using E. coli Transformants

The E. coli transformants were grown in 2 ml of LB medium containing the appropriate antibiotic for each vector. The overnight culture was inoculated into new medium and grown until the OD_600 reached 0.8. At this time, IPTG was added to a final concentration of 1 mM, and the transformants were grown for 20 h at 18°C. The cells were then harvested by centrifugation and resuspended to an OD_600 of 3 in 10 ml of M9 medium containing 2% glucose, 50 µg/ml antibiotics, 1 mM MgSO_4, 0.5% methionine, and 0.1 mM CaCl_2. The methionine was added because it is a precursor of SAM. To test the most effective vector, quercetin was added to a final concentration of 100 µM. The mixture was incubated at 25°C with shaking and harvested after 12 h. The supernatant from the harvested samples was extracted twice with ethyl acetate and dried in a vacuum centrifuge. Each dried sample was dissolved in 60 µl of DMSO and analyzed by HPLC [9].

To test the effect of EcSAM synthetase on the production of rhamnetin, both POMT7 and EcSAM synthetase were cloned into pETDuet and the resulting construct was transformed into E. coli BL21 (DE3). As a control, empty pETDuet was also transformed into E. coli BL21 (DE3). The same medium described above was used to test the effect of EcSAM synthetase on the production of rhamnetin. However, quercetin was added to the reaction mixture at 0, 3, 7, and 24 h and the final concentration of quercetin was 400 µM. For calculation of the rhamnetin production and cell growth, 500 µl of sample was harvested at 3, 7, 12, 24, and 36 h. The cell growth was done by measuring absorbance at 600 nm and the production of rhamnetin was done as described above. Dry cell weight was measured at 36 h; 5 ml of the cell was harvested and washed with distilled water. Cells were then resuspended in 2 ml of distilled water and transferred onto a Whatman filter paper (1.2 mm pore size, 47 mm diameter), which was left to dry at 90°C for 24 h.

MATERIALS AND METHODS

Nucleic Acid Manipulation

Flavone 7-O-methyltransferase from poplar (POMT7), which transfers a methyl group to the 7-hydroxyl group of quercetin, was used [8]. The open reading frame of POMT7 was cloned into the SacI/NotI sites of E. coli expression vectors pACYCDuet, pCDFDuet, pETDuet, and pRSFDuet (EMD Chemicals, Gibbstown, NJ, USA). According to the manufacturer’s manual, these four vectors were maintained at different copy numbers in E. coli. pACYCDuet retains 10–12 copies, pCDFDuet retains 20–40, pETDuet retains up to 40, and pRSFDuet retains more than 100 copies. Each POMT7 construct was transformed into E. coli BL21 (DE3).

The gene for SAM synthetase (EcSAM synthetase; GenBank Accession No. K02129) was cloned from E. coli based on the published sequence [13] and cloned into the second cloning site (BglII/Xhol) of pET7Duet (Fig. 1B). The resulting construct was transformed into E. coli BL21 (DE3).
number and this may have produced a very high metabolic load to *E. coli*, which could have limited the conversion of quercetin into rhamnetin. The lowest copy number plasmid, pACYCDuet, had a conversion rate that was 75% of the conversion rate observed for the pETDuet vector. Thus, the pETDuet vector was used for further studies.

**Coexpression of SAM Synthetase Gene with POMT7**

In order to maximize the production of rhamnetin in *E. coli*, we used a strategy to increase the methyl group supply. OMTs use SAM as a methyl donor. Unlike the enzymatic reaction, SAM does not need to be supplied during biotransformation because endogenous SAM is present. However, increasing the level of SAM during cell culture might increase the production of methylated compounds. SAM is synthesized from methionine and ATP by methionine adenosyltransferase (SAM synthetase). Thus, to increase rhamnetin production in *E. coli*, POMT7 was coexpressed with SAM synthetase.

POMT7 was cloned from *E. coli* based on the published sequence (Markham *et al.* [13]) and cloned into a second cloning site (BglII/XhoI) of pET7Duet containing POMT7 (Fig. 1B). The resulting construct was transformed into *E. coli* BL21 (DE3). Quercetin was then added to the reaction mixture. Initially, 100 µM of quercetin was added and the reaction mixture was incubated for 3 h. Next, 100 µM of quercetin was added again to the reaction mixture at 3, 7, and 24 h. Thus, the final concentration of quercetin in the reaction mixture was 400 µM. Conversion of quercetin to rhamnetin as well as cell growth was monitored by harvesting the supernatant at 3, 7, 12, 24, and 36 h. The growth of *E. coli* harboring either POMT7 or both POMT7 and EcSAM synthetase reached a maximum after 24 h (Fig. 3). *E. coli* harboring only pETDuet also showed similar growth. The dry weights of *E. coli* containing pETDuet, POMT7, or POMT7 and EcSAM synthetase were 34.5, 35, and 34.7 mg, respectively. *E. coli* harboring only POMT7 in the pETDuet vector stopped converting quercetin after 7 h, at which point 180 µM of quercetin was converted into rhamnetin (Fig. 3). However, *E. coli* harboring both POMT7 and EcSAM synthetase continued to convert quercetin to rhamnetin until the quercetin concentration reached 400 µM. After 36 h of incubation, 350 µM of rhamnetin was produced and further incubation did not further increase production. The yield of rhamnetin was 111 mg/l, which was approximately a 2-fold increase compared with the *E. coli* strain containing only POMT7. The production of rhamnetin by *E. coli* containing POMT7 and EcSAM synthetase was 32 mg/g (dry weight) cells.

Overexpression of SAM synthetase in several *Streptomyces* strains has been shown to increase production of antibiotics. In this case, SAM acts as a signal molecule for the production of antibiotics, but not as a methyl group donor [18]. However, in the current study, SAM synthetase was used as a methyl donor.

To increase the production of particular flavonoids in *E. coli*, new *E. coli* strains that have an increased production...
of cofactor have been developed. In a previous study, an *E. coli* strain with increased production of NADPH was used to improve the production of leucocyanidin and catechin from dihydroquercetin by 4-fold and 2-fold, respectively, relative to wild type [2]. In addition, production of the flavonoids naringenin and eriodictyol increased by 6.6- and 4.2-fold, respectively, when an *E. coli* strain engineered to optimize the carbon flux to malonyl-CoA was used [3].

In summary, four different *E. coli* vectors, each of which was maintained in *E. coli* with a different copy number, were used to express POMT7. Among these constructs, POMT7 cloned into the pETDuet vector converted quercetin into rhamnetin most effectively. In order to increase the production of rhamnetin, *SAM synthetase* from *E. coli* was coexpressed along with POMT7 in the pETDuet vector. Using this construct, the production of rhamnetin was increased to 111 mg/l.

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