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*-methyltranferase, 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 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 nargingenin 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 biotransfomation using *Esherichia coli* harboring flavonoid

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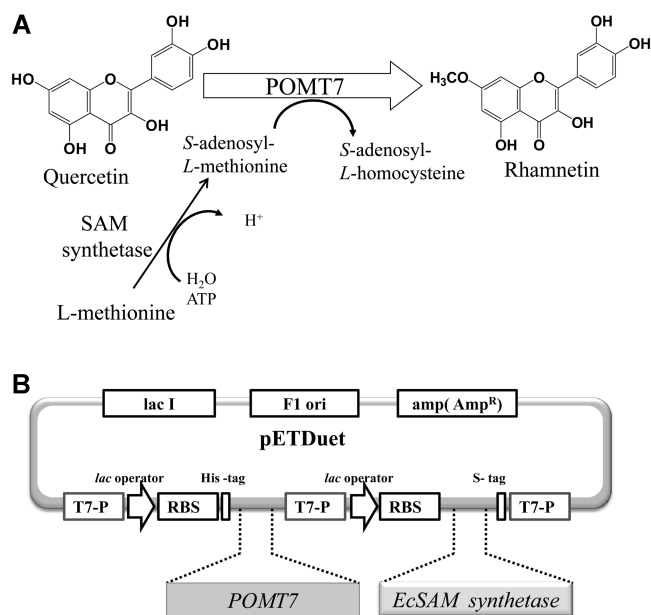Phone: +82 2 450 3764; Fax: +82 2 3437 6106;
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Fig. 1. *POMT7* in rhamnetin production, and the gene construct in vector pETDuet.
A. Production of rhamnetin from quercetin. The cosubstrate *S*-adenosyl-L-methionine was synthesized from L-methionine by SAM synthetase. *SAM synthetase* from *E. coli* along with *POMT7* was overexpressed in *E. coli*. **B.** Construct of *POMT7* and *Ec-SAM synthetase* in *E. coli* expression vector pETDuet.

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.

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 *Sac*I/*Not*I 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 (*Bgl*II/*Xho*I) of pET7Duet (Fig. 1B). The resulting 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 synthase 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.

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
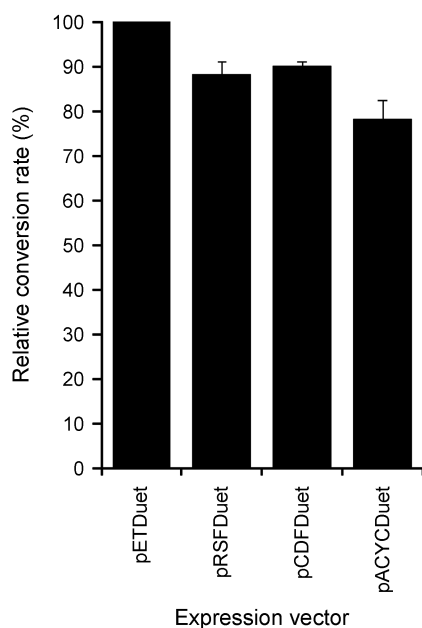
Fig. 2. Effect of different *E. coli* expression vectors on the production of rhamnetin.

POMT7 was cloned into four different vectors, pETDuet, pRSFDuet, pCDFDuet, or pACYCDuet. Conversion of quercetin into rhamnetin was examined in each *E. coli* transformant. Data are the mean ± standard deviation (SD) of three replicates.

Fig. 3. Production of rhamnetin with *E. coli* harboring only *POMT7* (closed circle) or *E. coli* harboring both *POMT7* and *EcSAM synthetase* (open circle).

Quercetin (100 μM) was added to the reaction mixture at 0, 3, 7, and 24 h. Conversion of quercetin into rhamnetin was monitored by harvesting the supernatant at 3, 7, 12, 24, and 36 h. Growth of cells [*E. coli* harboring only *POMT7* (closed triangle) or *E. coli* harboring both *POMT7* and *EcSAM synthetase* (open triangle)] was also monitored by measuring the absorbance at 600 nm at each time point. Data are the means ± standard deviations (SD) of three replicates.

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*. *SAM synthetase* (*EcSAM synthetase*) was cloned from *E. coli* based on the published sequence (Markham *et al*. [13]) and cloned into a second cloning site (*Bgl*II/*Xho*I) 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
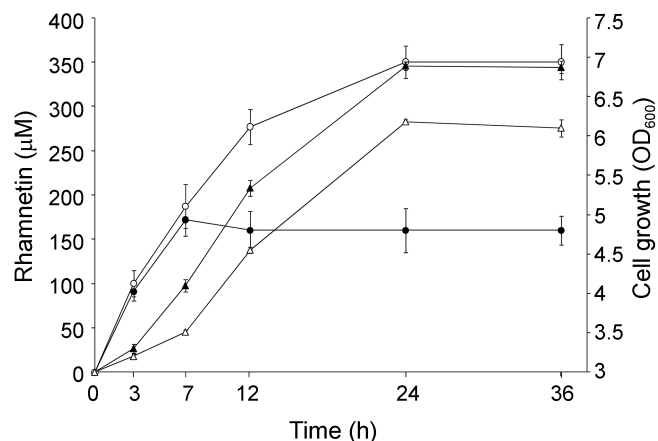
mixture at 3, 7, and 24 h. Thus, the final concentration of quercetin in the reaction mixture was 400 μM. Conversion of quercetin to rhamnetin as well as cell growth was monitored by harvesting the supernatant at 3, 7, 12, 24, and 36 h. The growth of *E. coli* harboring either *POMT7* or both *POMT7* 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 syntethase* 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 increase 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.

Acknowledgments

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