NOTE

Biosynthesis of fraxetin from three different substrates using engineered Escherichia coli

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Abstract
Fraxetin, which is a simple coumarin, is a phytochemical present in medicinal plants, such as Fraxinus rhynchophylla, and Cortex Fraxini. In plants, it serves as a controller of iron homeostasis. The health-enhancing activities of fraxetin, such as anticancer, neuroprotective and antibacterial activities, are known. Scopoletin 8-hydroxylase (S8H) is a key enzyme involved in the synthesis of fraxetin from scopoletin. Scopoletin can be synthesized either from esculetin by O-methylation or from ferulic acid by feruloyl CoA 6′-hydroxylase (F6′H) and 4-coumaric acid CoA ligase (4CL).

To enable fraxetin synthesis, the fraxetin biosynthesis pathway was introduced into Escherichia coli. Three distinct routes, from ferulic acid, esculetin, and scopoletin, were designed for the synthesis of fraxetin. In the first approach, E. coli strain harboring S8H was used and found to synthesize 84.8 μM fraxetin from 100 μM scopoletin. Two E. coli strains were used for the other two approaches because these approaches required at least two enzymatic reactions. Through this approach, 41.4 μM fraxetin was synthesized from 100 μM esculetin, while 33.3 μM fraxetin was synthesized from 100 μM ferulic acid.

Keywords: Coumarin, Fraxetin, Scopoletin 8-hydroxylase

Introduction

Coumarins (benzo-alpha-pyrones) were first isolated from the tonka bean (Dipteryx odorata) in 1820. Since then, their presence has been detected in various parts of different plants, including the fruit (e.g., in Bael fruit or Aegle marmelos), seed (e.g., in tonka beans or Calophyllum inophyllum), root (e.g., in Ferulago campestris), and leaf (e.g., Murraya paniculata) [1]. All coumarins have a hydroxy or methoxy group at position 7. Scopoletin, esculetin, umbelliferone, fraxetin, as well as their respective glycosides, are termed simple coumarins; they are widespread in higher plants [2]. These coumarins play a pivotal role in protecting plants against pathogens [3]; furthermore, a simple coumarin, such as fraxetin, was found to modulate vital physiological processes such as iron homeostasis [4]. As naturally occurring phytochemicals, coumarins possess health-enhancing properties, including anticancer [1], neuroprotective [5], and antibacterial properties [6].

Coumarins were synthesized from hydroxycinnamic acids, such as p-coumaric acid, caffeic acid, and ferulic acid, in plants; p-coumaric acid, caffeic acid, and ferulic acid resulted in the synthesis of umbelliferone, esculetin, and scopoletin, respectively. The key enzyme for coumarin biosynthesis was p-coumaroyl CoA 2′-hydroxylase (C2′H) or feruloyl CoA 6′-hydroxylase (F6′H); the corresponding genes were cloned in Arabidopsis thaliana [7], Ruta graveolens [8], Ipomoea batatas [9], Manihot esculenta [10], Angelica decursiva [11], and Peucedanum praeruptorum [12]. This enzyme is a 2-oxoglutarate-dependent dioxygenase; the hydroxylation of hydroxycinnamoyl-CoA resulted in the formation of a pyrone ring.

Fraxetin belongs to the family of simple coumarins and is synthesized from scopoletin by the hydroxylation of its carbon at position 8. Fraxetin is involved in iron metabolism in plants [4, 13]. Similar to other phytochemicals, fraxetin was found to exert beneficial effects in humans. These included antitumor [10, 14], neuroprotective [15],...
antihyperglycemic [16], and anti-inflammatory [17] effects.

Since the metabolic pathway responsible for the synthesis of simple coumarins is well established, these compounds have been synthesized in *E. coli*. Scopoletin, esculetin, umbelliferone, skimming (umbelliferone 7-O-glucoside), and herniarin (7-O-methyl umbellifereone) were synthesized in *E. coli* [18, 19]. For the fraxetin synthesis process in *E. coli*, two routes were postulated (Fig. 1). The first route involved the synthesis of esculetin from ferulic acid, followed by 8-hydroxylation. In the present study, one coumarin, namely fraxetin, was synthesized using *E. coli* via these two routes.

**Materials and methods**

**Plasmid construction**

Reverse transcription polymerase chain reaction (RT-PCR) was used to clone cDNA of scopoletin 8-hydroxylase (*S8H*) from *Arabidopsis thaliana* (*AtS8H*; GenBank: DQ446698.1). Two primers 5′-aagaattcaATGGGTATC AATTTCAGAGA-3′ and 5′-aagggccgcgTCACTCGGC ACGTG-3′ were used (restriction sites for EcoRI and NotI have been underlined). Additionally, the *S8H* homologue from *Oryza sativa* (*OsS8H*; GenBank: XM_026024461) was cloned by RT-PCR using two primers: 5′-aagaattcaATGCCGTCGGCTAGCAG-3′ and 5′-aagggccgCTAATCTAGACTAGCCGGCGG-3′ (restriction sites for EcoRI and NotI have been underlined). *AtS8H* was digested using the EcoRI and NotI sites and subcloned into pGEX 5X-3 (pG-AtS8H), pET-duet1 (pE-AtS8H), and pCDF-duet1 (pC-AtS8H). *OsS8H* was subcloned into EcoRI/NotI sites of pGEX 5X-3 (pG-OsS8H).

F6′H2 from *Ipomoea batatas* (*IbF6′H2*; GenBank: AB636154) and 4CL (*Os4CL*; 4-coumarate: CoA ligase) from *O. sativa* had been previously cloned using RT-PCR [18]. F6′H2 was first cloned into pET-duet1 (EcoRI/NotI) using PCR and, then, *Os4CL* was subcloned into pET-duet1 containing F6′H2 to generate pE-ibF6′H2-Os4CL (*NotI/Xhol*). Subsequently, *Os4CL* was re-amplified with a forward primer, adding a NotI site and ribosomal-binding site (RBS), and a reverse primer, containing a Xhol site. Thereafter, *Os4CL* was subcloned into the NotI/Xhol sites of pET-duet1 containing ibF6′H2 to generate pE-ibF6′H2-Os4CL controlled by a single promoter (operon). The ibF6′H-Os4CL operon was subcloned into pGEX 5X-3 (*EcoRI/Xhol*).

*POMT7* (flavone 7-O-methyltransferase) [20] and *POMT9* from *Populus deltoids* [21] and *ROMT9* (flavonoid 3′-O-methyltransferase) from *O. sativa* [22] have also been cloned previously. These genes were subcloned into pGEX 5X-3 vector.

**Production and analysis of metabolites**

For the synthesis of fraxetin from scopoletin, an overnight culture of an *E. coli* transformant containing pG-OsS8H, pG-AtS8H, pC-AtS8H, pET-AtS8H, or pR-AtS8H was inoculated into fresh LB medium containing 50 μg/mL ampicillin and grown at 37 °C until the OD₆₀₀ reached 0.8; following this, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the medium at a final concentration of 0.1 mM or 1 mM and incubated at 18 °C for 16 h. Cells were harvested and the cell concentration was adjusted to an OD₆₀₀ of 3.0. The cells were resuspended in M9 medium containing 2% glucose, ampicillin (50 μg/mL), and either 0.1 mM or 1 mM IPTG.

![Biosynthetic pathways of fraxetin from esculetin and ferulic acid. POMT7 is an O-methyltransferase, which converts esculetin into scopoletin. AtS8H is a scopoletin 8-hydroxylase. Os4CL catalyzes the attachment of CoA to ferulic acid. IbF6′H encodes feruloyl CoA 6′-hydroxylase (F6′H), which converts feruloyl CoA into scopoletin.](image-url)
in a test tube. A total concentration of 100 μM of the substrate (esculetin, isoscopoletin, scopoletin, or scoparone) was added, and the resulting culture was incubated at 30 °C for 24 h. An *E. coli* transformant containing the pG-AtS8H construct was employed to determine the substrate (scopoletin) concentration. The cell concentration was adjusted to an OD600 of 3.0. The substrate was added to the appropriate M9 medium at 0.1, 0.2, 0.3, or 0.5 mM. The reaction culture was incubated at 30 °C for 24 h.

The *E. coli* transformant harboring ROMT9 was used to methylate esculetin to scopoletin, isoscopoletin, and scoparone. Three reaction products were purified using thin layer chromatography (silica gel 60 F254, Millipore). A mixture of benzene and ethyl acetate (3:1) was used as a solvent. The *E. coli* transformant harboring POMT9 was used to synthesize scopoletin from esculetin. The methylation reaction using *E. coli* was carried out as described by Kim et al. [20].

Analysis of the reaction products was carried out using Thermo Ultimate 3000 HPLC [23]. Mass spectrometry and proton nuclear magnetic resonance (NMR) were performed as previously described [24, 25]. The 1H NMR of fraxetin in acetone-d₆ (in ppm) is δ 3.87 (3H, s, 6-OCH₃), 6.15 (1H, d, J=9.3 Hz, H-3), 6.76 (1H, s, H-5), 7.91 (1H, d, J=9.3 Hz, H-4) [26].

**Results and discussion**

**Biotransformation of scopoletin into fraxetin using *E. coli* harboring scopoletin 8-hydroxylase**

Fraxetin is 8-hydroxy scopoletin. S8H from *A. thaliana* (AtS8H) and its homologue from rice (OsS8H) were cloned as a glutathione S-transferase fusion protein and expressed in *E. coli*. Scopoletin was tested, along with other structurally-related coumarins, such as esculetin, isoscopoletin, and scoparone. These four compounds have esculetin derivatives. Three methylated esculetins (isoscopoletin, scopoletin, and scoparone) were synthesized using *E. coli* harboring ROMT9, purified, and used as substrates.

*E. coli* harboring AtS8H or OsS8H was tested for the conversion of esculetin, isoscopoletin, scopoletin, and scoparone by the administration of each compound. *E. coli* harboring OsS8H did not convert any coumarins used. However, for *E. coli* harboring AtS8H, scopoletin and isoscopoletin were converted into novel compounds that had retention times different from those of the corresponding substrates (Fig. 2). Other substrates

![Fig. 2](image-url)
(esculetin and scoparone) did not generate any new product. The molecular mass of the products from scopoletin and isoscopoletin was 207.937 Da, which is the molecular mass obtained if hydroxylation occurs. S8H utilized one methylated esculetins (scopoletin and isoscopoletin) as a substrate and did not utilize dimethylated (scoparone) or unmethylated esculetin. Scopoletin was a better substrate than isoscopoletin; 84.8% of scopoletin was converted, as opposed to the conversion of only 55% isoscopoletin. To determine the structure of the biotransformation product from scopoletin, the reaction product was purified, and its structure was analyzed using proton NMR. The reaction product was determined to be fraxetin (see Materials and Methods). E. coli harboring different constructs (pG-AtS8H, pE-AtS8H, pR-AtS8H, or pC-AtS8H) synthesized the approximately same amount fraxetin from scopoletin.

To optimize the initial concentration of scopoletin and the final yield of fraxetin, E. coli harboring AtS8H was prepared at an OD600 of 3.0 after induction of AtS8H. Subsequently, four different concentrations of scopoletin (100, 200, 300, and 500 μM) were added. The highest rate of conversion of scopoletin into fraxetin was seen at 100 μM scopoletin; 84.8 μM fraxetin was synthesized (84.8% conversion rate). However, fraxetin production was highest at 200 μM scopoletin; approximately 139.5 μM fraxetin was synthesized (69.8% conversion rate). Above 200 μM scopoletin, the production level of fraxetin registered a decline. The optimum initial cell concentrations were also determined. Five initial cell concentrations (OD600 = 1.0, 2.0, 3.0, 4.0, and 5.0) were tested and 200 μM scopoletin was administered. As the initial cell concentration increased, the conversion of scopoletin also registered a concomitant increase. At an OD600 of 5.0, approximately 152.0 μM of scopoletin was converted into fraxetin.

**Synthesis of fraxetin from esculetin and ferulic acid**

Fraxetin may also be synthesized from esculetin. Two enzymatic reactions are required; the first is the conversion of esculetin into scopoletin by an O-methyltransferase (OMT), and the second is the synthesis of fraxetin from scopoletin by S8H. For the synthesis of scopoletin from esculetin, three OMT genes (POMT7, POMT9, and ROMT9) were evaluated. E. coli harboring POMT7 synthesized 56.8 μM scopoletin from 100 μM esculetin (Fig. 3a). However, E. coli harboring ROMT9 produced three methylated esculetins (isoscopoletin, scopoletin, and scoparone), with isoscopoletin as a major product. The ratio of isoscopoletin, scopoletin, and scoparone was 83:13:3. POMT9 generated almost the same amounts of isoscopoletin (38.1 μM) and scopoletin (37.4 μM) from 100 μM esculetin. Therefore, E. coli harboring POMT7 was utilized to synthesize scopoletin from esculetin.

A two-step reaction was conducted using two E. coli transformants to augment the final yield of fraxetin. The first reaction was carried out using E. coli harboring POMT7. Approximately 56.9 μM scopoletin was synthesized from 100 μM esculetin (Fig. 3a). Further incubation did not result in the conversion of more esculetin. Thereafter, the culture filtrate from the first reaction was combined with E. coli harboring AtS8H. Approximately 41.4 μM fraxetin was synthesized from 56.9 μM scopoletin (Fig. 3b), indicating that there was approximately 72.7% conversion from the synthesized fraxetin.

**Fig. 3** Synthesis of fraxetin from esculetin using two E. coli transformants. a Conversion of esculetin into scopoletin using E. coli harboring POMT7. E. coli harboring POMT7 was administered esculetin (S1), following which the reaction product was analyzed. P1 denotes the reaction product from scopoletin. b Synthesis of fraxetin from scopoletin using E. coli harboring AtS8H. The culture filtrate from E. coli harboring POMT7 was administered to the E. coli harboring AtS8H, and the reaction product was analyzed.
Fraxetin was successfully synthesized from esculetin by a two-step reaction. The final yield of fraxetin synthesized from esculetin was lower than that from scopoletin; moreover, the conversion rate of scopoletin into fraxetin in the two-step reaction was lower than that seen for the direct conversion. This could possibly be attributed to the metabolite(s) in the first step inhibiting the second reaction. It was attempted herein to synthesize fraxetin from esculetin using an *E. coli* transformant harboring both POMT7 and AtS8H. Only 3.4 μM fraxetin and 17.2 μM scopoletin were synthesized from 100 μM esculetin.

Next, fraxetin was synthesized from ferulic acid. Three enzymatic reactions are required for this. Previously, scopoletin was successfully synthesized from ferulic acid using *E. coli* harboring *IbF6‘H2* and *Os4CL*. It was reasoned that introducing AtS8H into *E. coli* harboring *IbF6‘H2* and *Os4CL* could result in the synthesis of fraxetin from ferulic acid. Three genes (*IbF6‘H*, *Os4CL*, and *AtS8H*) were introduced into *E. coli*, and the resulting transformant was administered ferulic acid. The *E. coli* transformant synthesized fraxetin from ferulic acid. To optimize fraxetin synthesis, several initial ferulic acid concentrations (100, 200, 300, and 500 μM) were tested. The synthesis of fraxetin was optimal at 100 μM of initial ferulic acid, and approximately 33.3 μM fraxetin was synthesized (Fig. 4). Unreacted ferulic acid and scopoletin were accumulated at the higher concentrations of ferulic acid.

Fraxetin was synthesized from three different substrates (scopoletin, esculetin, and ferulic acid). As shown in Fig. 1, more enzymes are required when fraxetin is synthesized from ferulic acid or esculetin than when it is synthesized from scopoletin. Consequently, the final yield of fraxetin was higher (84.8 μM) when it was synthesized from scopoletin (100 μM). Its yield was decreased when synthesis was carried out from esculetin (41.4 μM) or ferulic acid (33.3 μM).

An attempt was made to synthesize fraxetin from esculetin or ferulic acid. One *E. coli* transformant harboring both POMT7 and AtS8H synthesized a lower amount of fraxetin from esculetin than the other two *E. coli* transformants, each of which conducted one reaction. However, fraxetin was successfully synthesized from ferulic acid using one *E. coli* transformant harboring three genes (*Os4CL, IbF6‘H2*, and *AtS8H*). Esculetin may compete with scopoletin for AtS8H. In the *E. coli* transformant harboring both POMT7 and AtS8H, esculetin served as a substrate for POMT7 and an inhibitor of AtS8H. Therefore, following the synthesis of scopoletin by POMT7, AtS8H could not utilize scopoletin because it was inhibited by esculetin. Conversely, when two independent *E. coli* transformants were used, more scopoletin synthesized by the first *E. coli* transformant harboring *POMT7* was present in the medium and was converted into fraxetin by the second *E. coli* transformant harboring *AtS8H*. When fraxetin was synthesized from ferulic acid, only scopoletin was synthesized; therefore, it was possible to synthesize fraxetin using *E. coli* harboring *Os4CL, IbF6‘H2*, and *AtS8H*.

**Acknowledgements**

The present study was supported by grants from the Next-Generation BioGreen 21 Program (PJ01326001), Rural Development Administration, Republic of Korea.

**Authors’ contributions**

SHA and JHA designed the experiments. SHA, GSC, and JHA performed the experiments and analyzed the data. SHA, GSC, and JHA wrote the manuscript. All authors read and approved the final manuscript.

**Funding**

Funding was received from the Next-Generation BioGreen 21 Program, Rural Development Administration (PJ01326001).

**Availability of data and materials**

All data generated or analyzed during the present study are included in this published article.

**Competing interests**

The authors declare that they have no competing interests.

Received: 5 August 2020    Accepted: 9 September 2020  
Published online: 14 September 2020
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