Flavonoids are a growing class of bioactive natural products with distinct and interesting bioactivity both in vitro and in vivo. The extraction of flavonoids from plant sources is limited by their low natural abundance and commonly results in a mixture of products that are difficult to separate. However, due to recent advances, the microbial production of plant natural products has developed as a promising alternative for flavonoid production. Through optimization of media, induction temperature, induction point, and substrate delay time, we demonstrate the highest conversion of naringenin to eriodictyol (62.7 ± 2.7 mg/L) to date, using the native E. coli hydroxylase complex, HpaBC. We also show the first evidence of in vivo HpaBC activity towards the monohydroxylated flavan-3-ol afzelechin with catechin product titers of 34.7 ± 1.5 mg/L. This work confirms the wide applicability of HpaBC towards realizing efficient de novo production of various orthohydroxylated flavonoids and flavonoid derived products in E. coli. © 2015 American Institute of Chemical Engineers Biotechnol. Prog., 000:000–000, 2015

Keywords: hydroxylation, non-P450 hydroxylase, pathway optimization, flavonoids, catechin

Introduction

The biosynthesis of flavonoids and flavonoid-derived compounds has gained much attention in the recent literature due to their well-established bioactive properties as antioxidant, anti-inflammatory, and anticancer agents. Due to their low natural abundance, current methods of extraction and purification from plant biomass represent a limiting and unsustainable source of flavonoids and flavonoid-derived compounds. This motivates the development of efficient and industrially scalable microbial production processes. One of the key hurdles limiting the high titer de novo synthesis of complex flavonoid compounds is obtaining soluble and active expression of the membrane associated plant cytochrome P450 monooxygenases responsible for the ortho- and para-hydroxylation of flavonoid compounds. To address this hurdle, much effort has been focused on finding suitable enzyme replacements for the P450 catalyzed hydroxylation steps. The need for hydroxylation at the para position has been achieved through expression of tyrosine ammonia lyase (TAL) to convert tyrosine into the para-hydroxylated flavonoid precursor, p-coumaric acid.

Additional ortho-hydroxylation had proven to be much more troublesome until the discovery of a non-P450 hydroxylase, HpaBC, native to E. coli and P. aeruginosa with high activity towards phenylpropanoic acid substrates. This operon, although present in the E. coli genome, is not natively expressed. However, when cloned and expressed from a high copy plasmid, it demonstrates gram scale conversion of a variety of substrates including p-coumaric acid, tyrosol, coniferaldehyde, umbelliferone, and resveratrol to their corresponding ortho-hydroxylated counterparts.

Figure 1 highlights the substrates tested in this manuscript. Inefficient conversion of naringenin to eriodictyol has also been reported with final titers of only 16.7 mg/L using a two-step fermentation protocol, where the cells were concentrated 3.3-fold prior to substrate addition. This low titer eriodictyol conversion supports the need for development of optimization methods to create industrially feasible production strains and processes.

In this work, we will present a simplified fermentation protocol for the in vivo conversion of naringenin to eriodictyol at higher titer (62.7 ± 2.7 mg/L) than has previously been reported in the literature. To accomplish this task, we have screened a variety of genetic and fermentation
conditions including media, temperature, promoter strengths, substrate timing, and induction points. Using optimum conditions, we confirm the previously published high titer conversion of p-coumaric acid to caffeic acid resulting in titers of 3.5 g/L caffeic acid and demonstrate the ability of the HpaBC hydroxylase complex to act on afzelechin to form catechin. This work supports the main metabolic engineering goal of complete de novo synthesis of complex flavonoid compounds, such as catechins and anthocyanins from simple carbon sources without the need for substrate supplementation.18,19

Methods

Bacterial strains, vectors, and media

E. coli DH5α was used to propagate all plasmids, while the BL21 star™ (DE3) was used as the host for flavonoid production. The ePathBrick vector, pETM6, was used as the basis for all plasmid construction and pathway expression.20 Luria broth (LB) Lennox modification (Sigma), Difco M9 Salts (BD), and Andrew’s Magic Media21,22 (AMM) were used where noted. When added, glucose and glycerol were supplemented at 20 g/L.

Construction of the HpaBC expression vector

The HpaB and HpaC genes were amplified from E. coli DH5α genomic DNA using primers P1–P4. The resulting amplicons and ePathBrick destination vector pETM6 were digested with NdeI and SpeI and ligated to form pETM6-HpaB and pETM6-HpaC, respectively. pETM6-HpaBC was then assembled using ePathBrick cloning protocols20 resulting in HpaBC in monocistronic form. The vector, pETM6-HpaBC, will be available from addgene.org.

Fermentation protocol

A single colony was pre-inoculated in Andrew’s Magic Media (AMM) supplemented with 80 μg/mL ampicillin and grown at 37°C overnight (14 h). The overnight culture was inoculated at 2% into 2 mL of AMM in a 48-well plate (5 mL, VWR) and grown at 37°C until induction. The cultures were induced when noted with 0.1 or 1 mM IPTG and transferred to 37, 30, or 20°C for 48 h. Substrate (300 mg/L) was added at induction except where noted. Fermentation broth was mixed with an equal volume of absolute ethanol and vortexed for 10 s prior to centrifugation (10 min, 20,000g). The supernatant was used for HPLC analysis. All experiments were preformed in duplicate.

HPLC analysis

Analysis was carried out using Agilent 1200 series HPLC equipped with a ZORBAX SB-18 column (5 μm, 4.6 × 150 mm) and a diode array detector. The mobile phase was acetonitrile (solvent A) and water (solvent B) (both contain 0.1% formic acid) at a flow rate of 1 mL/min. HPLC program was as follows: 10–40% A (0–10 min) and 40–60% A (10–15 min). Absorbance at 280 nm was monitored in all cases. Titer of products was determined using authentic standards while (+)-afzelechin was quantified using the (+)-catechin calibration curve. Error bars represent ±1 standard deviation of biological duplicate. Significance of data was determined using a two-tailed unpaired t test with a 95% confidence interval.

Purification of afzelechin

A single colony of E. coli BL21 Star™ (DE3) Δpgi Δppc containing plasmid p16823 was pre inoculated in 10 mL of Andrew’s Magic Media (AMM 2% glucose) supplemented with 80 μg/mL ampicillin and 50 μg/mL kanamycin and grown at 37°C overnight (14 h). The overnight culture was used to inoculate 500 mL of AMM 2% glucose in a 2 L baffled shake flask and grown at 37°C until induction. The culture was induced at 8 h with 1 mM IPTG and transferred to 30°C. Naringenin (400 mg/L) was added 1-h postinduction. After 2 days, the culture was centrifuged (30 min, 7100g) and the supernatant was concentrated to a wet solid using a Buchi Rotovapor R-210 under reduced pressure. The resulting solid was extracted with methanol and concentrated for purification by preparative HPLC (Shimadzu Prominance Series) using a Waters Nova-Pak C18 column (60 Å, 19 mm × 200 mm, 6 μm) at room temperature. One milliliter of concentrated sample in methanol was injected. The mobile phases were acetonitrile (A) and water (B), both containing 0.1% formic acid. The following gradient was used at a flow rate of 10 mL/min: 10–40% A (0–35 min) and 40–60% A (35–53 min). The peak containing afzelechin (15.5 min) was collected and evaporated under reduced pressure. An example chromatogram from preparative HPLC is shown in Supporting Information Figure 1. The resulting solid was resuspended in absolute ethanol and confirmed to contain afzelechin by LCMS, Supporting Information Figure 2.

Results and Discussion

Determination of optimum media and induction temperature

To begin the optimization, we chose three induction temperatures (20, 30, and 37°C), three growth media (M9, LB, AMM), and two supplemental carbon sources (glucose, glycerol). The combination of these three factors resulted in a screen of 18 initial conditions to determine the optimal conditions for the conversion of naringenin to eriodictyol (Figure 2). This data shows a clear preference towards AMM–glycerol with near identical performance between 30 and
37°C. The preference towards higher temperature for the HpaBC complex suggests high stability and the ability to express soluble, highly active protein even at elevated temperatures. Furthermore, the retention of activity at reduced temperature (30°C) demonstrates potential compatibility with the plant-derived flavonoid pathway genes that show higher activity at reduced temperatures. Interestingly, the use of LB media supplemented with either glucose or glycerol demonstrated very low substrate conversion but significant browning of the media was observed on plates and in liquid media indicating activity of HpaBC in vivo. These initial results led us to choose AMM–glycerol at a constant temperature of 37°C for the remainder of the study.

**Induction point optimization yields highest conversion to date**

Building on the optimization from Figure 2, we decided to look at induction point sensitivity for two different inducer concentrations. Here we induced with either 1.0 or 0.1 mM IPTG hourly from 0 to 10 h. The results (Figure 3) show high sensitivity prior to the optimum induction point for both inducer concentrations, while post optimum the 1 mM IPTG induction shows slight instability, with the 0.1 mM IPTG induction showing a slow decline in conversion with later induction points. Optimum titers of 56.2 ± 2.1 mg/L were obtained with induction points 4–5 h postinoculation. These results support the use of lower IPTG concentrations, which reduces the protein expression burden and results in more stable and predictable conversion.

**Improvements in conversion through substrate delay**

Previous literature reports and personal observations have noted a reduction of cell growth in the presence of phenylpropanoic acids and flavonoid compounds. In an attempt to minimize this negative effect, we studied the effect of a delay between protein induction with IPTG and addition of substrate (p-coumaric acid or naringenin). In the case of naringenin, we saw minimal insignificant (P > 0.05) improvements with the application of substrate delay presumably due to the minimal effect on growth with feeding only 300 mg/L of naringenin. Using a 1-h substrate delay a top titer of 62.7 ± 2.7 mg/L eriodictyol was achieved, Figure 4b. To the authors’ knowledge, this represents the highest titer reported to date for the direct conversion of naringenin to eriodictyol in vivo. It should also be noted that approximately 90% of eriodictyol formation occurred in the first 24 h postinoculation.

Furthermore, we attempted this method of substrate delay for the conversion of p-coumaric acid to caffic acid. This conversion has been reported to be highly efficient with in vivo titers of 3.82 g/L reported in recent literature. Upon initial testing without substrate delay, we noticed a severe decrease in growth with feeding amounts higher than 3 g/L of p-coumaric acid. Upon varying the substrate delay, we saw a maximum titer of 3.46 ± 0.05 g/L caffic acid production from a feed of 4 g/L p-coumaric acid with a 2-h substrate delay, a 2.2-fold improvement over the zero-hour substrate delay control, Figure 4a. In an attempt to minimize p-coumaric and caffic acid degradation, a supplement of 100 mg/L of ascorbic acid was added resulting in an insignificant slight decrease (P > 0.05) in titers. These findings support the previous literature reports and confirm the high affinity of HpaBC for p-coumaric acid in vivo.

**Activity of HpaBC towards afzelechin**

To further investigate the substrate flexibility of HpaBC, we fed the monohydroxylated flavan-3-ol, afzelechin, under optimal conditions (4 h induction with 0.1 mM IPTG and a 2 h substrate delay). HpaBC demonstrated weak activity towards afzelechin resulting in final titers of 34.7 ± 1.5 mg/L catechin after 48 h from an initial feed of 300 mg/L afzelechin. These results were further confirmed through LC/MS identification of only afzelechin in the substrate feed and both afzelechin and catechin present after 48 h of fermentation (Supporting Information Figures 2 and 3). We also did not detect activity towards the substrates cinnamic acid or pinocembrin, which is consistent with previous literature reports. Additionally, no activity was detected towards a non-natural fluorinated flavanone (4'-fluoro,5,7-dihydroxyflavanone) or aureusidin. These results demonstrate the selectivity of HpaBC towards substrates with a para-hydroxylation of the benzyl-ring.

**Modification of transcriptional landscape leads to reduced titers**

In an attempt to further improve conversion of naringenin, we applied the recently published ePathOptimize technique to randomly vary the strength of the T7 promoters controlling expression of HpaB and HpaC. Using a library comprised of five reduced strength promoters, the genes were randomly assembled resulting in 25 possible promoter combinations. Ninety mutants were tested for the ability to convert naringenin to eriodictyol in vivo with the top mutant being the previously constructed consensus T7 control (Supporting Information Figure 4). We hypothesize this method of optimization was unsuccessful due to the limited number of gene overexpressions present in this strain. We predict higher sensitivity to transcriptional balancing for systems with increased transcriptional burden associated with overexpression of larger biosynthetic pathways. This result also confirms the need for system specific pathway optimization strategies with diverse points of effect to fully maximize the potential of any microbial system.
Conclusion

We have presented a simplified protocol for the in vivo ortho-hydroxylation of naringenin to eriodictyol at higher titers than previously reported for the direct conversion. Furthermore, we have detailed the bioconversion optimization with respect to media, temperature, induction point, and substrate delay. We have also demonstrated the ability of HpaBC to act on afzelechin opening the door for site-specific hydroxylation of a variety of flavonoids with good potential to have interesting bioactivity. This work demonstrates the ability for HpaBC to hydroxylate many intermediate products throughout the flavonoid pathway indicating a high potential to converge on a single end product rather than a mixture of products with various degrees of hydroxylation. Future applications incorporating HpaBC into microbial biosynthesis schemes will enable the efficient de novo production of many ortho-hydroxylated aromatic products through its high activity and promiscuous substrate utilization of simple aromatic amino acids though complex flavones and flavan-3-ols.

Acknowledgements

The authors would like to thank proteomics core director Dr. Dmitri V. Zagorevski for assistance with LC/MS analysis. The authors would like to acknowledge funding from the RPI biocatalysis and metabolic engineering constellation fund.

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Figure 3. Induction point optimization.

Moderate sensitivity to induction point was observed with similar trends apparent with 1 or 0.1 mM IPTG. All data obtained in AMM-glycerol at 37°C. Error bars represent ±1 SD of biological duplicate (n = 2).

Figure 4. Effect of substrate delay on final titer.

(a) Significant improvements were obtained in conversion of phenylpropanoic acids (0 hour vs. 2 hour, p-value < 0.01), while (b) only moderate improvements were realized for flavanone conversion (p-value > 0.05). Data obtained using AMM-glycerol at 37°C. Error bars represent ±1 SD of biological duplicate (n = 2).


Manuscript received Sep. 24, 2015, and revision received Oct. 9, 2015.