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De novo biosynthesis of Gastrodin in *Escherichia coli*

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Tyrosol (PubChem CID: 10393)
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4-Methylumbelliferone (PubChem CID: 5280567)
4-Methylumbelliferyl β-D-glucoside (PubChem CID: 2733779)
4-Hydroxybenzoic acid β-D-glucopyranoside (PubChem CID: 440186)
4-Formylphenyl β-D-glucopyranoside (PubChem CID: 7573800)

ABSTRACT

Gastrodin, a phenolic glycoside, is the key ingredient of *Gastrodia elata*, a notable herbal plant that has been used to treat various conditions in oriental countries for centuries. Gastrodin is extensively used clinically for its sedative, hypnotic, anticonvulsive and neuroprotective properties in China. Gastrodin is usually produced by plant extraction or chemical synthesis, which has many disadvantages. Herein, we report unprecedented microbial synthesis of gastrodin via an artificial pathway. A *Nocardia* carboxylic acid reductase, endogenous alcohol dehydrogenases and a *Rhodiola* glycosyltransferase UGT73B6 transformed 4-hydroxybenzoic acid, an intermediate of ubiquinone biosynthesis, into gastrodin in *Escherichia coli*. Pathway genes were overexpressed to enhance metabolic flux toward precursor 4-hydroxybenzyl alcohol. Furthermore, the catalytic properties of the UGT73B6 toward phenolic alcohols were improved through directed evolution. The finally engineered strain produced 545 mg l^{-1} gastrodin in 48 h. This work creates a new route to produce gastrodin, instead of plant extractions and chemical synthesis.

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1. Introduction

The Chinese orchid *Gastrodia elata* Blume is a famous and precious herbal plant that has been used in oriental countries to treat a variety of central nervous system and cardiovascular symptoms for over 2000 years (Wang et al., 2007; Zhou, 1991). Gastrodin (**1**), namely, 4-hydroxymethylphenyl β-D-glucopyranoside was identified as the major active ingredient of *G. elata* Blume, and has been extensively used to treat various diseases such as headache, dizziness, vertigo, and convulsion, with no side effects reported in patients (Hsieh et al., 2001; Wang et al., 2007; Zhou, 1991). In addition, gastrodin reportedly exhibits other biological effects, including antioxidative activity (Liu and Mori, 1992), anti-obesity (Park et al., 2011), anti-inflammation (Ahn et al., 2007), anxiolytic activity (Jung et al., 2006), anti-epilepsy (Ojemann et al., 2006), neuroprotection (Han et al., 2014) and memory improvement (Wu et al., 1996).

Gastrodin is usually produced by direct extraction from *G. elata* Blume rhizomes or chemical synthesis (Wang et al., 2007; Zhou, 1991; Zhou et al., 1980), which has many disadvantages. In recent years, microorganisms have been engineered to produce a range of economically important plant derived natural products, including terpenoids (Ajikumar et al., 2010; Ro et al., 2006), alkaloids (Nakagawa et al., 2011; Thodey et al., 2014), flavonoids (Leonard

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et al., 2008; Lim et al., 2011; Santos et al., 2011; Yan et al., 2008), and many other phenolic compounds (Bongaerts et al., 2001; Lin et al., 2013; Lin and Yan, 2012; Yao et al., 2013). However, gastrodin production in microorganisms from renewable feedstocks has been hampered by the incomplete understanding of its native biosynthetic pathway.

It is known that *G. elata* Blume accumulates *p*-coumaric acid, 4-hydroxybenzaldehyde, 4-hydroxybenzyl alcohol (**2**), vanillyl alcohol and vanillin, besides gastrodin (Duan et al., 2013; Li and Chen, 2004; Yang et al., 2007). The pattern and structural similarities of these metabolites suggest gastrodin is formed via 4-hydroxybenzaldehyde and 4-hydroxybenzyl alcohol. The biosynthetic pathway of vanillin and 4-hydroxylbenzaldehyde in *Vanilla,* another orchid, has been well studied. The most specific step of vanillin biosynthesis is a chain shortening of ferulic acid, a precursor in lignin formation in plants that derives from L-phenylalanine via cinnamic acid, *p*-coumaric acid, and caffeic acid, involving deamination, hydroxylation and an O-methylation (Gallage et al., 2014; Podstolski et al., 2002). 4-Hydroxybenzaldehyde has been proposed to derive from *p*-coumaric acid in a chain shortening mechanism, although the responsible enzyme remains unknown (Gallage et al., 2014; Podstolski et al., 2002).

Gastrodin formation would also require a uridine sugar glycosyltransferase (UGT) catalyzing the glucosylation of 4-hydroxybenzyl alcohol; this has not yet been reported. In a previous study, we found that a *Rhodiola*-derived glycoltransferase (GT) UGT73B6 could glucosylate both aromatic and aliphatic alcohols of tyrosol (**3**), a 4-hydroxybenzyl alcohol homolog to yield salidroside (**4**) and icariside D2 (**5**), respectively (Bai et al., 2014). We also demonstrated that one of the limiting steps for high yield production of the phenolic glycosides in engineered *E. coli* was the low GT capacity.

In recent years, GT engineering has become an important research field in improving catalytic efficiency and altering substrate specificity. Rational engineering, such as point mutation and domain-swapping, which depends on high sequence similarity or enzyme crystal structures, has been applied successfully in engineering both bacterial and plant derived GTs (Chang et al., 2011; Harle et al., 2011; Hoffmeister et al., 2002; Krauth et al., 2009; Osmani et al., 2009; Wang, 2009). Directed evolution is an alternative tool to investigate which parts of a GT sequence is involved in defining substrate specificity and obtains GTs with improved activity and desired catalytic characteristics. In the context of natural product biosynthesis, the approach has only been used to create mutants of bacteria derived GT OleD involved in the biosynthesis of the antibiotic oleandomycin, making staggering progress in creating catalysts with both improved catalytic efficiency and broadened substrate specificity (Chang et al., 2011; Gantt et al., 2011; Williams and Thorson, 2008; Williams et al., 2007). No such studies have been reported for plant GTs.

Here, we report unprecedented microbial biosynthesis of gastrodin in *E. coli* from glucose using an artificial pathway depicted in Fig. 1. In recombinant *E. coli* strains, a carboxylic acid reductase (CAR) from *Nocardia iowensis* (He et al., 2004), endogenous alcohol dehydrogenases (ADHs) (Bai et al., 2014) of *E. coli* and a *Rhodiola* UGT73B6 catalyzed the formation of gastrodin from 4-hydroxybenzoic acid (**6**), a key intermediate of ubiquinone biosynthesis in *E. coli* derived from chorismate (Siebert et al., 1994; Zhang et al., 2015). Furthermore, the enzymatic activity and regioselectivity of glucosyltransferase UGT73B6 toward aromatic alcohols was also successfully improved

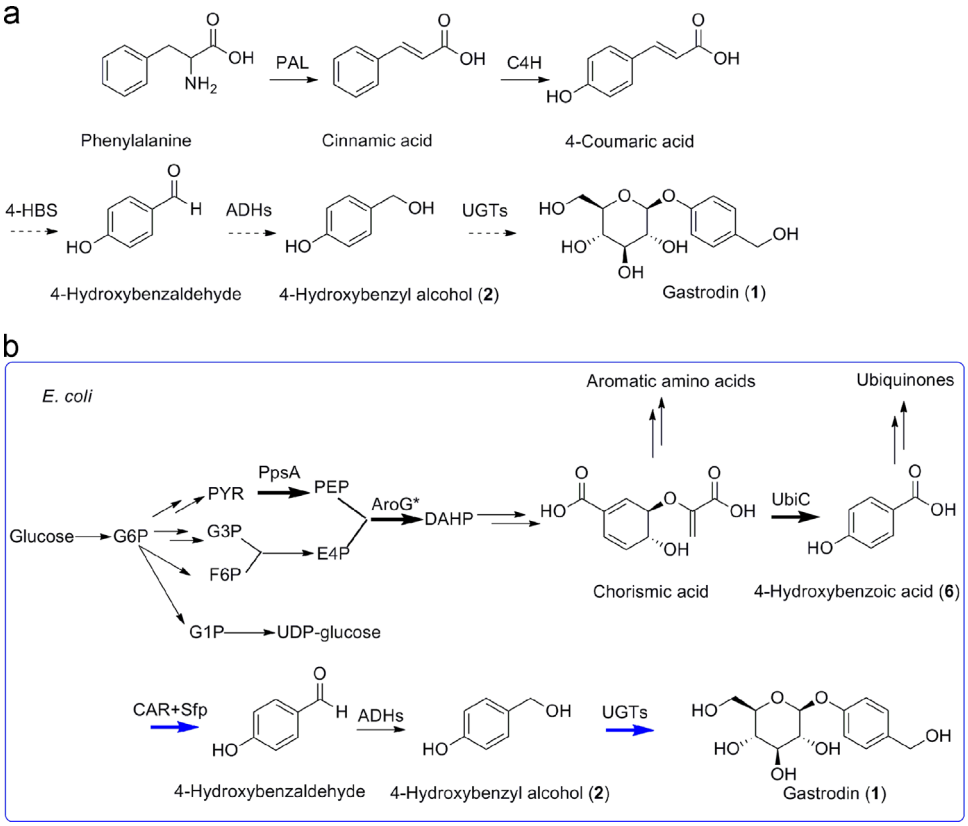

Fig. 1. Biosynthesis of gastrodin (a) Proposed biosynthetic pathway of gastrodin (**1**) in plants, (b) the artificial pathway to **1** from glucose constructed in *E. coli*. Single arrows represent one-step conversions, while single dashed arrows indicate no known enzymes available in the pathway. Double arrows represent multi-step conversions. Bold arrows indicate gene overexpression. Blue and bold arrows represent heterologous gene overexpression. Abbreviations: PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4-HBS, 4-hydroxybenzaldehyde synthase; ADH, alcohol dehydrogenase; G6P, 6-phosphate-D-glucose; PYR, pyruvate; G3P, glyceraldehyde-3-phosphate; F6P, fructose-6-phosphate; PEP, phosphoenolpyruvate; E4P, erythrose 4-phosphate; UDP-glc, UDP-glucose; DAHP, 3-deoxy-arabino-heptulonate-7-phosphate; AroG*, feedback resistant mutant of AroG. (For interpretation of the references to color in this figure legend,the reader is referred to the web version of this article).

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