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ARTICLE

Microbial synthesis of hydroxytyrosol and hydroxysalidroside

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Abstract Plant-derived phenolic compounds, such as hydroxytyrosol and hydroxysalidroside, have a beneficial impact on human health owing to their antioxidant activity. In this study, we used *Escherichia coli* to synthesize hydroxytyrosol. Tyrosine decarboxylase from *Papaver somniferum*, tyrosine oxidase from *Micrococcus luteus*, and 4-hydroxyphenylacetate 3-monooxygenase from *E. coli* were transformed into the bacterial cell. The resulting transformant successfully synthesized hydroxytyrosol. Furthermore, we used the engineered *E. coli* strains to synthesize ~ 268.3 mg/L hydroxytyrosol. Three uridine diphosphate-dependent glycosyltransferases (UGTs), which were previously shown to convert tyrosol into salidroside, were tested to synthesize hydroxysalidroside, and one of UGTs was used to synthesize hydroxysalidroside from hydroxytyrosol. Finally, *E. coli* harboring this UGT converted approximately 50% of hydroxytyrosol into hydroxysalidroside.

Keywords Hydroxysalidroside · Hydroxytyrosol · Metabolic engineering

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Introduction

Fruits and vegetables are a rich source of phenolic compounds; for example, coffee provides chlorogenic acid, grapes provide resveratrol, and olives provide tyrosol and hydroxytyrosol. The consumption of these compounds through a daily diet can help prevent several diseases. To this end, plant-derived phenolic compounds were originally considered to possess antioxidant activity. However, recent studies have shown that these natural phenolic compounds have diverse range of biological activities, exhibiting antimicrobial, anticancer, anti-inflammation effects, and aiding in the prevention of cardiovascular diseases [1–3].

Hydroxytyrosol, a major component of olive, is one such phenolic compound that is one of the most powerful natural antioxidants [4]. Hydroxytyrosol has been shown to prevent bone loss and can be used for the treatment of osteoporosis [5]. Moreover, this compound promotes the reduction in low-density lipoprotein [6, 7], reduces the age-related neurodegeneration [8], and has a neuroprotective effect on diabetic neuropathy [9]. Thus, due to its inherent health benefits, hydroxytyrosol is considered a "super" nutrient.

Hydroxysalidroside is the glucoside of hydroxytyrosol, and salidroside is an important compound derived from the Chinese medicinal plant *Rhodiola sp* [10]. Hydroxysalidroside was first isolated from *Picrorhiza scrophulariiflora* followed by *Lepisorus contortus* and is a known antioxidant [11, 12]. Moreover, hydroxysalidroside is reported to exert a neuroprotective effect against H_2O_2 and 6-hydroxydopamine [13].

Hydroxytyrosol, classified as a phenylethanoid, has a C6-C2 carbon skeleton and is synthesized from tyrosine. The rational biosynthetic pathway of hydroxytyrosol is as

follows: decarboxylation and deamination of tyrosine by tyrosine decarboxylase (TDC) and tyramine oxidase (TYO), respectively, leads to the formation of 2-(4-hydroxyphenyl) acetaldehyde. In a microbial system such as *Escherichia coli*, 2-(4-hydroxyphenyl)acetaldehyde is spontaneously converted into tyrosol. The hydroxylation of tyrosol results in the formation of hydroxytyrosol. Moreover, the conversion of tyrosine into 2,4-dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase (TH) at the initial stage could also lead to the synthesis of hydroxytyrosol [14]. Previously, hydroxytyrosol was synthesized by using *TH*, DOPA decarboxylase, and *TYO* [15]. To this end, genes required for the synthesis of cofactor tetrahydrobiopterin of TH have been introduced in *E. coli* to increase the conversion of tyrosine into DOPA. On the other hand, hydroxysalidroside has not been synthesized biologically because no uridine-dependent glycosyltransferase (UGT) has been discovered for the conversion of hydroxytyrosol into hydroxysalidroside.

In this study, we synthesized hydroxytyrosol and hydroxysalidroside in *E. coli*. 4-Hydroxyphenylacetate 3-monooxygenase (*HpaBC*) from *E. coli* along with *TDC* and *TYO* was employed to synthesize hydroxytyrosol. HpaBC was used to convert tyrosine into L-DOPA [16]. To synthesize hydroxysalidroside, we took the advantage of the structural similarity between tyrosol and hydroxytyrosol to identify a UGT that converts hydroxytyrosol into hydroxysalidroside. Furthermore, by increasing the endogenous tyrosine content in *E. coli* by overexpressing genes involved in tyrosine biosynthesis, we aimed to increase the final yield of hydroxytyrosol and hydroxysalidroside.

Materials and methods

Constructs and *E. coli* strain

Tyrosine decarboxylase (TDC from *Papaver somniferum*; GenBank U08598.1) and tyrosine oxidase (TYO from *Micrococcus luteus*; GenBank AB010716.1) were synthesized after codon optimization for *E. coli* (Bioneer, Daejeon, Korea) using the published nucleotide sequences. TDC was subcloned into the BamHI/HindIII site of the pCDF-Duet1 vector (MilliporeSigma, MA, USA), and the resulting construct was named pC-TDC. TYO was introduced into the second cloning site (NdeI/XhoI) of pC-TDC, and the resulting construct was named pC-TDC-TYO.

HpaBC from *E. coli* (Gene ID: 7156703 and 7155545) was cloned by polymerase chain reaction using 5′-ATGAATTCGATGAAACCAGAAGATTTCCGC-3′ (EcoRI restriction site is underlined) and CATGCGGCCGCTTAAATCGCAGCTTCCATT (NotI restriction site is underlined) as primers. The resulting product was subcloned in the EocRI/NotI site of pET-Duet1 (MilliporeSigma). pA-aroG-tyrA, pA-aroG^{fbr}-tyrA^{fbr}, and pA-aroG^{fbr}-ppsA-tktA-tyrA^{fbr} were cloned previously [17]. The list of constructs is shown in Table 1. The *E. coli* strain, B-TP, in which both *tyrR* and *pheA* were deleted was previously generated [17].

Production and structural determination of reaction products

Hydroxytyrosol production was carried out according to the protocol described by Kim et al. [17]. Hydroxysalidroside was synthesized using *E. coli* harboring UGT85A1. Hydroxytyrosol was fed into the culture of *E. coli* harboring UGT85A1. Analysis of reaction products was performed using high performance liquid chromatography (HPLC) [18].

The structure of the reaction product was determined using nuclear magnetic resonance spectroscopy (NMR) [19]. NMR data were as follows; δ of ^{1}H NMR of hydroxytyrosol in MeOD-d_4 (ppm); 6.69 (1H, d, J = 8.0 Hz, H-7), 6.67 (1H, d, J = 1.7 Hz, H-4), 6.54 (1H, dd, J = 8.0, 1.7 Hz, H-8), 3.69 (2H, t, J = 7.3 Hz, H-1), 2.68 (2H, t, J = 7.3 Hz, H-2). δ of ^{1}H NMR of hydroxysalidroside in DMSO-d_6 (ppm); 6.59 (1H, d, J = 8.0 Hz, H-7), 6.58 (1H, d, J = 2.2 Hz, H-4), 6.44 (1H, dd, J = 8.0, 2.2 Hz, H-8), 4.12 (1H, d, J = 7.8 Hz, H-glc1), 3.82 (1H, m, H-1a), 3.62 (1H, d, J = 11.5 Hz, H-glc6a), 3.51 (1H, m, H-1b), 3.39 (1H, dd, J = 11.7, 5.8 Hz, H-glc6b), 3.10 (1H, t, J = 8.8 Hz, H-glc2), 3.03-3.06 (1H, ddd, J = 9.6, 5.9, 2.0 Hz, H-glc5), 3.00 (1H, m, H-glc3), 2.92 (1H, t, J = 8.4 Hz, H-glc4), 2.63 (2H, m, H-2).

Results and discussion

Engineering *E. coli* for the synthesis of hydroxytyrosol

Hydroxytyrosol is synthesized from tyrosol by a one-step reaction. Conversion of tyrosine into DOPA (3,4-dihydroxyphenylalanine) could lead to the synthesis of hydroxytyrosol. Previous reports have shown that HpaBC from *E. coli* can be used to convert tyrosine into L-DOPA [16]. Thus, we tested whether HpaBC could convert tyrosol into hydroxytyrosol. *E. coli* expressing HpaBC was used, and it was found that this strain converted tyrosol into hydroxytyrosol (data not shown).

Next, we tested the biosynthesis of tyrosol using TDC and TYO. *TDC* and *TYO* were synthesized after codon optimization and subcloned into the pCDF vector. The construct (pC-TDC-TYO) was then transformed into

Table 1 Plasmids, *Escherichia coli* strains, and primers used in this study

Plasmids or *E. coli* strain	Relevant properties or genetic marker	Source or reference
Plasmids		
pACYCDuet	P15A ori, Cm^r	Novagen
pCDFDuet	CloDE13 ori, Str^r	Novagen
pA-aroG-tyrA	pACYCDuet carrying *aroG* and *tyrA*	[17]
pA-$aroG^{fbr}$-$tyrA^{fbr}$	pACYCDuet carrying feedback inhibition free of *aroG* and *tyrA*	[17]
pA-$aroG^{fbr}$-ppsA-tktA-$tyrA^{fbr}$	pACYCDuet carrying *ppsA, tktA*, and feedback inhibition free of *aroG* and *tyrA*	[17]
pC-TDC-TYO	pCDFDuet carrying tyrosine decarboxylase (TDC) from *Papaver somniferum* and tyrosine oxidase (TYO) from *Micrococcus luteus*	This study
pE-HpaBC	pET-duet carrying HpaBC from *Escherichia coli*	This study
Strains		
BL21 (DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm lon* (DE3)	
B-TP	BL21(DE3) *ΔtyrR::FRT-ΔPheA::FRT-*kan^R*-FRT[17]*	[17]
B-TPF	BL21(DE3) *ΔtyrR::FRT-ΔPheA::FRT-ΔfeaB::FRT-*kan^R*-FRT*	[21]
B-TYS1	BL21 (DE3) harboring pC-TDC-TYO	This study
B-TYS2	BL21 (DE3) harboring pC-TDC-TYO, and pE-HpaBC	This study
B-TYS3	BL21 (DE3) harboring pA-aroG-tyrA, pC-TDC-TYO, and pE-HpaBC	This study
B-TYS4	BL21 (DE3) harboring pA-$aroG^{fbr}$-$tyrA^{fbr}$, pC-TDC-TYO, and pE-HpaBC	This study
B-TYS5	B-TP harboring pA-$aroG^{fbr}$-$tyrA^{fbr}$, pC-TDC-TYO, and pE-HpaBC	This study
B-TYS6	B-TPF harboring pA-$aroG^{fbr}$-$tyrA^{fbr}$, pC-TDC-TYO, and pE-HpaBC	This study

E. coli (B-TYS1 in Table 1), and the production of tyrosol in B-TYS1 was examined. As shown in Fig. 1A, a new peak which had the retention time similar to a standard tyrosol was observed.

Next, we introduced *HpaBC* into the B-TYS1 strain and examined the production of hydroxytyrosol using the resulting strain, B-TYS2. The reaction product showed the same HPLC retention time as well as the expected molecular mass of hydroxytyrosol (Fig. 1B). Moreover, the structure of this was determined using proton NMR. It remains unclear as to whether HpaBC in involved in the conversion of tyrosine into DOPA or the conversion of tyrosol into hydroxytyrosol, or both. However, our goal here was to synthesize hydroxytyrosol.

We attempted to increase the production of hydroxytyrosol in *E. coli* by manipulating the tyrosine biosynthesis pathway in *E. coli*. Two constructs, both of which contained genes for tyrosine biosynthesis, were co-expressed along with pC-TDC-TYO. The first construct contains 2-dehydro-3-deoxyphosphoheptonate aldolase (*aroG*) and chorismate mutase/prephenate dehydrogenase (*tyrA*). AroG is at the entry point of shikimate pathway and catalyzes the formation of 7-phosphate-2-dehydro-3-deoxy-D-arabino-heptulosonate (DAHP) from erythrose-4-phosphate and phosphoenolpyruvate. TyrA catalyzes the conversion of chorismate to prephenate and 4-hydroxyphenylpyruvate, which eventually converts tyrosine by tyrosine aminotransferase (tyrB and aspC). The final product, tyrosine, inhibits aroG and tyrA. Thus, feedback inhibition that was free of aroG and tyrA (pA-$aroG^{fbr}$-$tyrA^{fbr}$) was used in the second construct [20].

Each construct was transformed into B-TYS2, and the resulting strains B-TYS3 and B-TYS4 were used for hydroxytyrosol production. B-TYS2-4 produced 22.3, 44.2, and 79.2 mg/L of hydroxytyrosol, respectively, suggesting that the productivity of hydroxytyrosol was related to the tyrosine content in the cell (Fig. 2A). We also used the *E. coli* mutant B-TP in which the *tyrR* and *pheA* genes were deleted, TyrR represses genes in phenylalanine, tryptophan, and tyrosine biosynthesis, while PheA drives prephenate for phenylalanine synthesis instead of tyrosine. We used another *E. coli* mutant B-TPF, in which *feaB* was deleted along with *tyrR* and *pheA*. FeaB encodes phenylacetaldehyde dehydrogenase which converts 4-hydroxyphenylacetaldehyde into 4-hydroxyphenyllactate instead of tyrosol, thereby lowering the hydroxytyrosol production. B-TP and B-TPF mutants were transformed with pA-$aroG^{fbr}$-$tyrA^{fbr}$, pC-TDC-TYO, and pE-HpaBC. The resulting transformants, B-TYS5 and B-TYS6, were tested for hydroxytyrosol production. B-TYS5 produced approximately 239.2 mg/L and B-TYS6 produced 260.7 mg/L hydroxytyrosol, both of which were much higher than the

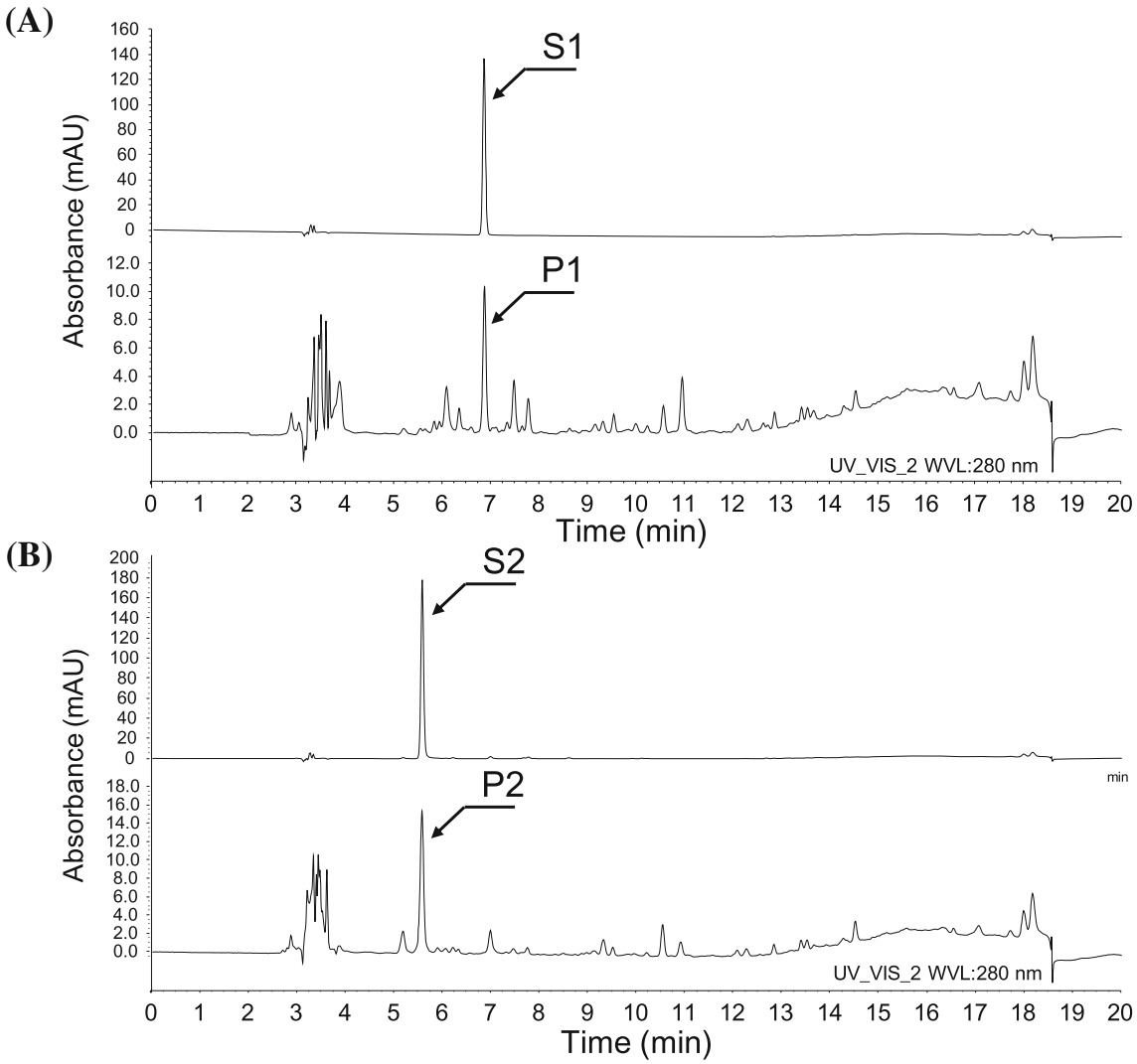

Fig. 1 Synthesis of tyrosol (**A**) and hydroxytyrosol (**B**) using B-TYS1 and B-TYS2, respectively. S1, standard tyrosol; P1, reaction product obtained using B-TYS1; S2, standard hydroxytyrosol; P2, reaction product using B-TYS2

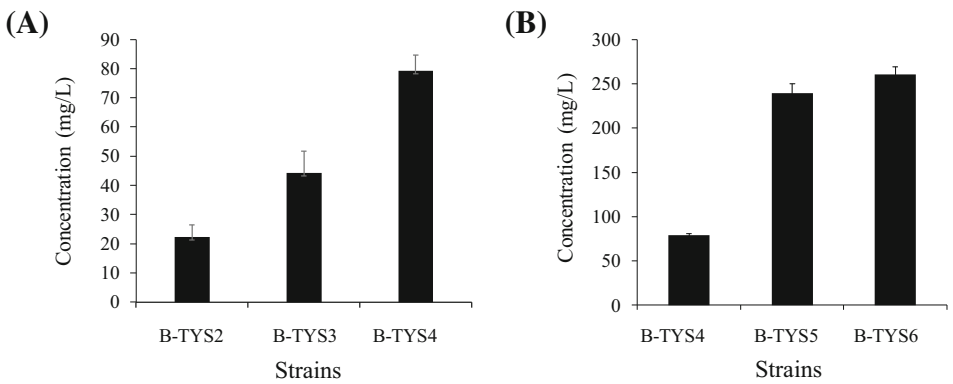

Fig. 2 Production of hydroxytyrosol using *E. coli* strains harboring different constructs (**A**) or *E. coli* mutants (**B**)

wild type (Fig. 2B). We then monitored the production of hydroxytyrosol using B-TYS6 for 36 h (Fig. 3). Hydroxytyrosol production increases constantly from 6 to 24 h, while the cells grew from 0 to 12 h. At 30 h, the production of hydroxytyrosol reached 268.3 mg/L.

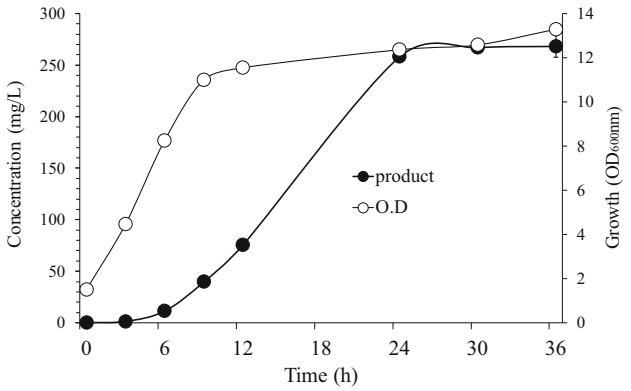

Fig. 3 Monitoring of hydroxytyrosol production using strain B-TYS6

Synthesis of hydroxysalidroside using *E. coli* harboring UGT85A1

We previously synthesized salidroside from tyrosol using UGT85A1 [21]. Two more UGTs (UGT73C5 and UGT73C6) have also been found to synthesize salidroside from tyrosol. Due to the structural similarity between hydroxytyrosol and tyrosol, these UGTs could transfer glucose from UDP-glucose to hydroxytyrosol to synthesize hydroxysalidroside. Thus, *E. coli* strains harboring UGT85A1, UGT73C5, or UGT73C6 were used to examine the production of hydroxysalidroside from hydroxytyrosol. After 24 h of incubation, the culture filtrate from each transformant was analyzed using HPLC. The transformant harboring UGT85A1 showed one new peak, while other two transformants showed two peaks (Fig. 4). However, the mass of both reaction products was 323.809 Da, which matched the predicted molecular mass of the compound in which one glucose molecule is attached to hydroxytyrosol. The glucose residue can be attached to either the phenolic hydroxyl group or alcohol group of hydroxytyrosol. Next, we determined the structure of the product obtained from the three *E. coli* transformants using NMR. NOESY experiments were performed to determine the glycosylation position, and partial NOE correlations are shown in Fig. 5. The glucose moiety was attached at 1-OH as indicated by an NOE cross-peak between H-glc1 and H-1. Therefore, we concluded the structure of the reaction product was that of hydroxysalidroside. *E. coli* harboring UGT85A1 converted approximately 45% of hydroxytyrosol into hydroxysalidroside. *E. coli* harboring UGT73C6 produced more by-product (2-glucosyloxy-4-(2-hydroxylethyl) phenol or 2-glucosyloxy-5-(2-hydroxylethyl)

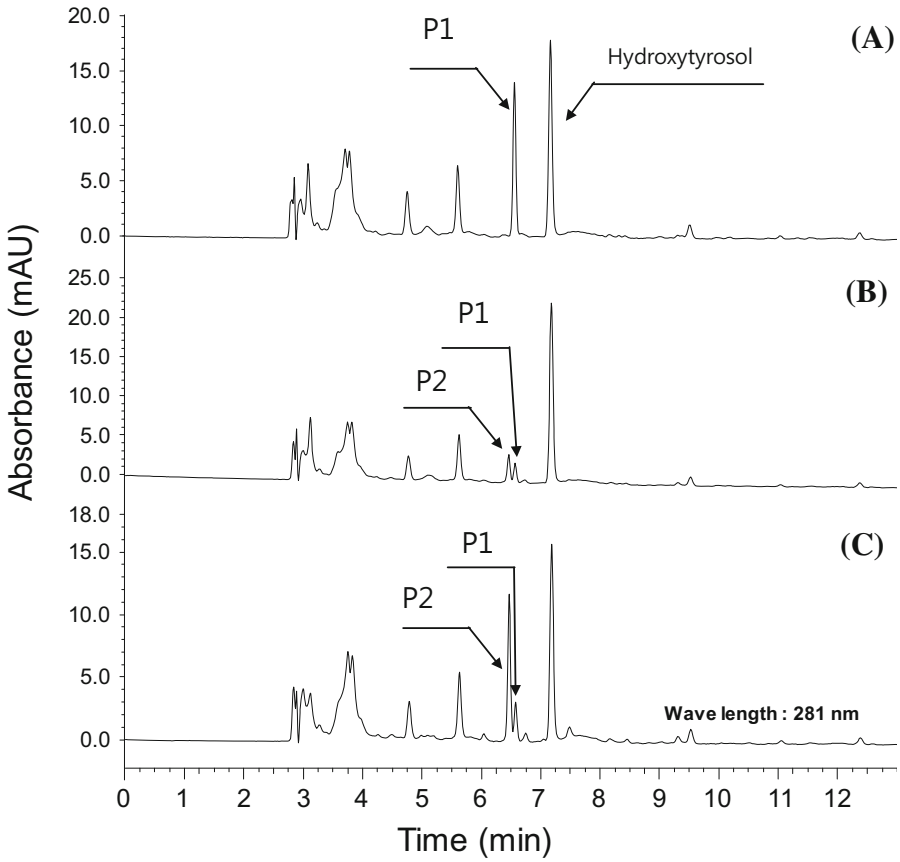

Fig. 4 Biological synthesis of hydroxysalidroside using *E. coli* harboring UGT85A1 (**A**), UGT73C5 (**B**), or UGT73C6 (**C**). P1 was determined to be hydroxysalidroside. P2 is likely to be (2-hydroxy-5-hydroxyethyl)phenyl-*O*-β-D-glucopyranoside or (2-hydroxy-4-hydroxyethyl)phenyl-*O*-β-D-glucopyranoside

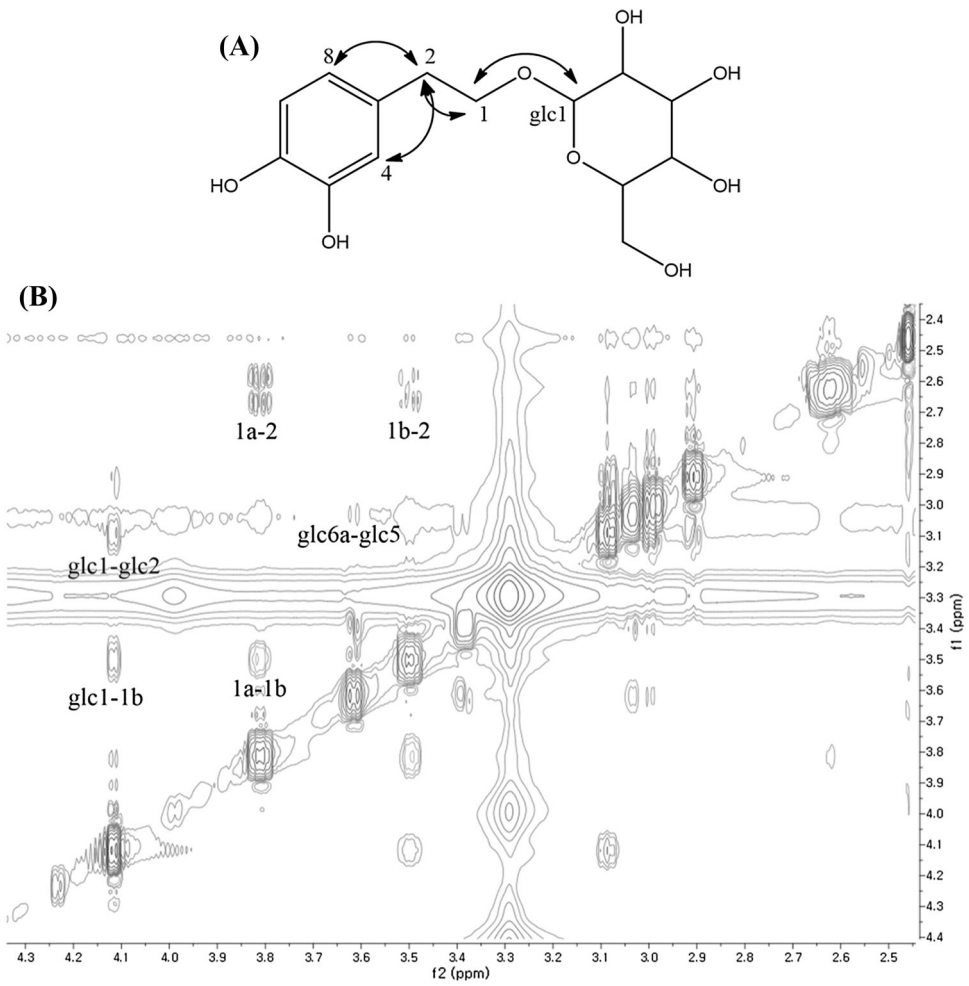

Fig. 5 (**A**) Partial NOE correlations marked with arrows. The cross-peak between H-1 and H-glc1 in the NOESY spectrum clarified the hydroxysalidroside structure. (**B**) Partial NOESY spectrum showing important cross-peaks to determine the structure of the reaction product

phenol than hydroxysalidroside. The ratio of by-product to hydroxysalidroside was 2:8 with an overall conversion rate of 45%. *E. coli* harboring UGT73C5 converted only 15% of hydroxytyrosol into two products, while the by-product was produced to greater extent.

Next, we transformed UGT85A1 into *E. coli* B-TYS6 and the resulting transformant was used to examine hydroxysalidroside production, but observed no production of hydroxysalidroside. However, because salidroside was synthesized, we speculated that the glycosylation step was inhibited by hydroxylation and examined whether hydroxysalidroside could be synthesized from tyrosol. *E. coli* harboring HpaBC and UGT85A1 was used to synthesize hydroxysalidroside from tyrosol. This *E. coli* did not convert tyrosol into hydroxysalidroside, producing only hydroxytyrosol. It seemed that metabolite(s) inhibits UGT85A1 during this reaction. Therefore, two-step reactions were used for the synthesis of hydroxysalidroside. We synthesized hydroxytyrosol using B-TYS6, and the filtrate was then extracted and mixed with *E. coli* harboring UGT85A1. Using this approach, ~ 45% of hydroxytyrosol was converted into hydroxysalidroside.

Previous reports have shown that tyrosine hydroxylase instead of HpaBC along with TDC and TYO was used to produce hydroxytyrosol [15]. TDC and TYO recycle cofactor for tyrosine hydroxylase and synthesized ~ 29.3 mg/L hydroxytyrosol (when 1 mM tyrosine was fed) or 12.3 mg without tyrosine. We used the HpaBC and tried to supply more tyrosine in *E. coli* by engineering the tyrosine biosynthetic pathway. Through these strategies, we could synthesize ~ 268.3 mg/L hydroxytyrosol. Jung et al. [21] also synthesized hydroxytyrosol using *AAS* (aromatic aldehyde synthase), which is bifunctional, possessing both TDC and TYO activities [22]. Using AAS and HpaBC, we managed to synthesize 208 mg/L hydroxytyrosol. Thus, for the synthesis of hydroxytyrosol, the current system was better than the previously system that used *AAS* and *HpaBC*.

Due to the structural similarity between tyrosol and hydroxytyrosol, we used three UGTs (UGT73C5, UGT73C6, and UGT85A1) that converted tyrosol to salidroside. These three UGTs regioselectively synthesized salidrosides from tyrosol [22]. UGT85A1 regioselectively synthesized hydroxysalidroside, while UGT73C5 and

UGT73C6 produced not only hydroxysalidroside but also other by-product (probably (2-hydroxy-5-hydroxyethyl)phenyl-*O*-β-D-glucopyranoside or (2-hydroxy-4-hydroxyethyl)phenyl-*O*-β-D-glucopyranoside). The natural substrate of these three UGTs differed significantly. UGT73C6 is known to transfer a glucose residue to 7-OH group of kaempferol 3-*O*-rhamnoside and quercetin 3-*O*-rhamnoside [23]. UGT73C5 used brassinosteroid as a substrate [24], while UGT85A1 is involved in cytokinin metabolism in *Arabidopsis thaliana* [25]. These three UGTs have a broad substrate range and differing regioselectivity, which made it possible to synthesize hydroxysalidroside or other glucosylate compounds.

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