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Article

Determination of Biosynthetic Pathway of Decursin in Hairy Root Culture of *Angelica gigas*

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To establish the biosynthetic pathway of decursin in *Angelica gigas* Nakai, feeding experiment with stable isotope-labeled precursors were conducted. Umbelliferone and decursin were labeled with deuterium at C-3. The umbelliferone, the decursin, and other commercially available putative precursors, L-phenylalanine-*ring*-d_5 and *trans*-cinnamic acid-d_7, were fed to the hairy root culture of *A. gigas*. Each deuterated compound was incorporated into decursinol, decursinol angelate, and decursin as determined by mass spectrometric analysis. These findings confirmed the coumarin biosynthesis pathway sequence is composed of phenylalanine, cinnamic acid, umbelliferone, decursinol, and decursin.

Key words: Angelica gigas, *decursin, decursinol, deuterium-label, umbelliferone*

Coumarin is one of the plant secondary metabolite classes with interesting biological activities. Decursinol, an isopentenyl conjugate of coumarin, receives considerable attention for its symptom-alleviating activity in Alzheimer patients [Yan *et al*., 2004] and anti-inflammatory activity [Jung *et al*., 2008]. Coumarins, known as phytoalexin involved in the pathogen defense, are widespread in plants, among which umbelliferae is especially rich in coumarins [Conrath *et al*., 2002]. One of the species, *Angelica gigas* Nakai, commonly known as to-danggwi in Korea, is endemic to the Korean peninsula and is cultivated in the fields. Decursin and decursinol in the root of the plant is now utilized as a health-food supplement in Korea. With the progression of demographic change toward older population in the developed countries, the need for decursinol to produce nutraceutical and medicine for treating the senility-related symptoms is growing. Although the chemical synthesis of decursinol is not difficult [Lim *et al*., 2001], demand is for the plant-derived materials, especially for the nutraceutical application. Therefore, understanding of the chemistry and the biology of the decursinol biosynthesis in *A. gigas* would play an important role in securing sufficient supply of the "natural" compound from *A. gigas*.

Biosynthetic pathway of decursin in *A. gigas* could be formulated as shown in Fig. 1, deduced from the unit steps known in other plants [Brown, 1981]. Cinnamic acid, derived from phenylalanine through the action of phenylalanine ammonia lyase, would be hydroxylated on C-3 on the aromatic ring to give *p*-coumaric acid. Hydroxylation at C-2, and intramolecular esterification gives umbelliferone. Prenylation with isopentenyl pyrophosphate would result in 7-demethylsuberosin. Oxidative ring closure catalyzed by a cytochrome P450 acting on the isopentenyl side chain would directly provide decursinol. However, the nature of the cyclization process is not yet fully understood. In the root of *A. gigas*, concentration of the free decursinol is very low [Ahn *et al*., 2008]. Instead, it is accumulated as an ester of angelic acid or 3-methyl-2-pentenoic acid latter being known as decursin.

Site of coumarin biosynthesis has been studied; the biosynthesis both in actively growing leaves and roots has been revealed. For example, furanocoumarin was synthesiszed in the leaves of *Angelica archangelica* [Härmälä *et al*., 1992], whereas simple coumarins were formed in the roots [Steck and Bailey, 1969]. Also

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Abbreviations: EIMS, electron impact mass spectrometry; LC-ESIMS, liquid chromatography-electrospray ionization mass spectrometry.

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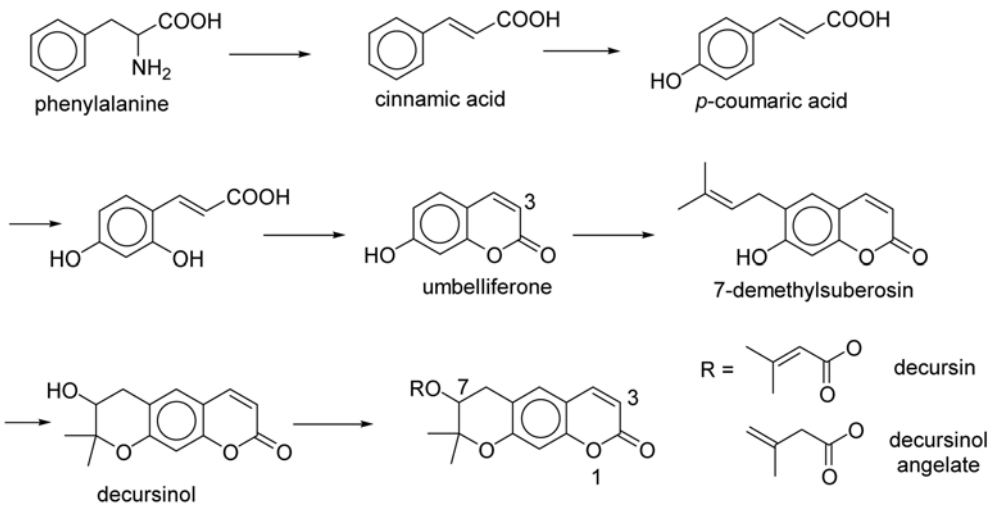

Fig. 1. Proposed biosynthetic pathway of decursin in *A. gigas*. The individual steps were derived from the studies of other plants.

noteworthy is that the site of biosynthesis correlates with that of storage or exudation of coumarins. In the case of *A. gigas*, decursin and decursinol angelate are found in the root.

To improve the production of the Angelica coumarins, the application of biotechnology such as metabolic engineering is necessary. Towards this goal, identification of the biosynthetic pathway would be the cornerstone of the ensuing study. Therefore, the biosynthetic pathway of decursin starting from phenylalanine was established in the present study through the feeding of the deuterium-labeled intermediates to the hairy root culture of *A. gigas*. The experiment confirmed that the pathway formulated from the unit step studied in other plant system was also operating in *A. gigas*.

Fig. 2. Axenic culture of seedling on antibiotics-free MS medium.

Materials and Methods

Reagents. MS medium premix (Catalog number M0222) was from Duchefa (Haarlem, Netherland). Other biochemicals and antibiotics were from Sigma. L-Phenylalanine-*ring*-d_5 (98 atom % D) was from Cambridge Isotope Laboratories (Andover, MA), and *trans*-cinnamic acid-d_7 (98 atom % D) from Aldrich.

Aseptic culture of plant. Seeds of *A. gigas* were harvested from the cultivating field near Hoengsung, Korea. The seeds were washed in running water for 3 to 4 days. The seed coats were then removed, and soaked in 70% ethanol for 1 min, 2% sodium hypochlorite for 10 min, and finally in 0.1% mercuric chloride for 10 min. The treated seeds were washed with sterile water five times, and then transferred on to a solid MS medium (10 ppm streptomycin sulfate, 5 ppm ampicillin, and 3% sucrose and 0.65% agar) for germination. After reaching 2-4 leaf stage, the seedlings were transferred on to the antibiotics-free MS medium (Fig. 2).

Hairy root culture. *Agrobacterium rhizogenes* strains (KTCC 2743, 2744, and 2703) were individually maintained on the solid YEP medium (Bacto-peptone 10 g/L, Bacto-yeast extract 10 g/L, NaCl 5 g/L, and 1.5% agar). Culturing was initiated by growing the bacterium in a liquid YEP medium for 1 day on a gyrotatory shaker at 250 rpm. Axenic leaves from the plantlet described above were cut into 0.5-1 cm pieces and placed in 5 mL liquid MS medium (3% sucrose without hormone) containing 20 μL bacterial culture and 50 μM acetosyringone. The mixture was shaken for 24 h at 100 rpm under darkness. The leaves were then incubated on a solid MS medium (3% sucrose without hormone) with 250 mg/L carbenicilin until the hairy root emerged. For maintenance, 4 to 6 root tips, cut into 2-5 cm pieces, were sub-cultured every 2 to 3 weeks in 20 mL MS medium

(3% sucrose without hormone) in 100 mL Erlenmeyer flask at 100 rpm. All cultures were maintained at 25ºC.

Decursinol-*3-d*. Decursinol (0.258 g) was mixed with 50-fold molar ratio of ^2H_2O in CCl_4 and let stand for 30 min. The mixture was dried under reduced pressure. The solid was dissolved in acetone, added with 0.213 g methyl iodide and 0.138 g anhydrous potassium carbonate, and finally refluxed on a 60-70ºC water bath for about 6 h. The solvent was removed under reduced pressure, and the solid was dissolved in dichloromethane and chromatographed on Silica gel 60 column with dichloromethane as the eluting solvent to obtain fully methylated coumaric acid. Treatment of methyl decursinol in NaOMe/MeOD by 18 h of reflux yielded 62% decursinol [Zinn *et al*., 1963].

Decursinol-*3-d*. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.90 (s), 7.42 (1H, s), 6.71 (1H, s), 6.22 (trace of signal), 5.25 (0.8H, br s), 3.69 (1H, br t), 2.98 (3H, dd, *J*=16.5, 4.7), 2.67 (1H, dd, *J*=12.7, 4.7), 1.29 (3H, s), 1.22 (3H, s); ^{13}C NMR (DMSO-d_6, 50 MHz) δ 160.3, 156.4, 153.5, 144.0, 117.9, 112.1, 103.1, 78.6, 67.3, 30.2, 25.4, 21.1; EIMS (70 eV) *m/z* 248 [M+1] (20), 247 [M] (68), 246 (4), 177 (100), 176 (88).

Umbelliferone-*3-d*. Phenol functionality of umbelliferone was methylated by CH_3I/ K_2CO_3 under the acetone reflux, and deuteriation and deprotection were executed as described above.

Umbelliferone-*3-d*. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 10.53 (1H, s), 7.92 (1H, s), 7.51 (1H, d, *J*=8.4), 6.18 (trace of signal), 6.77 (1H, d, *J*=8.4), 6.70 (1H, s); ^{13}C NMR (DMSO-d_6, 50 MHz) δ 161.2, 160.3, 155.5, 144.4, 129.7, 113.1, 111.4 (trace), 111.2, 102.1; EIMS (70 eV) *m/z* 164 (22), 163 (100), 162 (9.64), 135 (78).

Feeding of precursors and analyses of the products. The culture medium was replaced with a half-strength MS medium without sugar before the feeding. Each precursor (0.2 to 6.2 mg) in 200 μL of 70 % ethanol was fed to the flask containing 2-week-old hairy root culture. The incubation was continued for additional 3 to 4 days, and the roots were harvested and dried at room temperature. The dried sample was ground to powder with a mortar and a pestle. About 0.1 g of the material was extracted with 5 mL methanol in an ultrasonicator for 60 min. The mixture was filtered through a 0.45-μm filter and directly analyzed by a LC-ESIMS as described below.

Analyses. Determination of decursinol was performed by HPLC (HP1100; column, Eclipse XDB-C18 2.1 mm × 150 mm; detection, 320 and 254 nm; flow rate, 0.2 mL/min). The solvent gradient was composed of B (0.1% acetic acid in acetonitrile) in A (0.1% acetic acid in water); 0 to 10 min 15% B, to 30 min 30% B, to 35 min 30% B, to 65 min 50% B. LC-ESIMS was performed on a Micromass Quattro LC Triple Quadrupole Tandem Mass Spectrometer equipped with HPLC described above.

Results and Discussion

Hairy root culture. Among the *Agrobacterium* strains, *A. rhizogens* KTCC 2743 induced the most vigorously growing hairy root strain, which was found by HPLC analysis to produce decursin. The hairy root strain was thus used for the ensuing feeding experiments.

Synthesis of labeled compounds. Labeling of coumaric acid, umbelliferone, and decursinol was performed in CH_3O^-/CH_3OD after the appropriate protection on the hydroxyl groups. However, coumaric acid was recovered as unlabeled. ^{1}H and ^{13}C NMR analyses of the labeled compounds showed that deuterium was introduced into the expected sites of umbelliferone and decursinol. For decursinol-*3-d*, proton signal at δ 7.90 appeared as a singlet accompanied by a trace of a doublet signal (*J*=9.5 Hz), and a signal at d 6.22 collapsed into a singlet. In the ^{13}C NMR spectrum, C-3 signal at d 112.3 was missing due to the presence of deuterium. Therefore, the labeling at C-3 was confirmed. Similarly, labeling of umbelliferone at C-3 with deuterium was confirmed by the loss of a signal at δ 6.18 and the collapse of the doublet (*J*=9.5) at δ 7.92 into a singlet. The labeling pattern was further confirmed by EIMS. Analysis of the mass spectrum of the labeled umbelliferone showed the distribution of the unlabeled/monodeuterated/dideuterated compound was 7/85/8 after correction for [M+1] due to the natural abundance of ^{13}C. For decursinol, the ratio was estimated as 5/83/12. The position of the second label was not determined.

Feeding experiment. The standard compounds were run on HPLC, and LC-MS (direct injection), and the retention time of each compound in question was determined: coumaric acid 2.14, *trans*-cinnamic acid 31.0, umbelliferone 4.36, decursin 23.7, and decursin (or decursinol angelate) 44.0 (or 47.0) min. ESIMS of the standard compounds consistently gave [M+H] peaks. Feeding of cinnamic acid at 4.4 mg per flask was detrimental to the hairy root. Therefore, the medium composition was changed into a half-strength MS without sugar, and the concentration of the acid was lowered to 1.1 mg per flask, which resulted in no significant change in the appearance of the roots. Umbelliferone at 2.6 mg per flask also retarded the growth of the root.

Phenylalanine-*ring*-d_5 would give doubly-labeled decursinol and decursin. About 25% of decursinol was indeed doubly-labeled, whereas only 6% was doubly-labeled in decursin (Table 1 and Fig. 3), likely to have arisen from the dilution with the internal decursin. From the feeding of cinnamic acid-d_7, no labeled decursin was

Table 1. Feeding of decursin biosynthesis precursors to *A. gigas* hairy root culture

Precursors	Relative abundance of products (corrected for ^{13}C natural abundance)					
	Decursinol (*m/z*)			Decursin (*m/z*)		
	247 [MH]	248 [MH-*d*]	249 [MH-d_2]	329 [MH]	330 [MH-*d*]	331 [MH-d_2]
Phenylalanine-*ring*-d_5	100	15	33	100	20	8
trans-Cinnamic acid-d_7	nd	nd	nd	100	~0	0
Umbelliferone-3-*d*	42	100	8	100	31	3
Decursinol-3-*d*	23	100	12	100	43	6

nd, not detected.

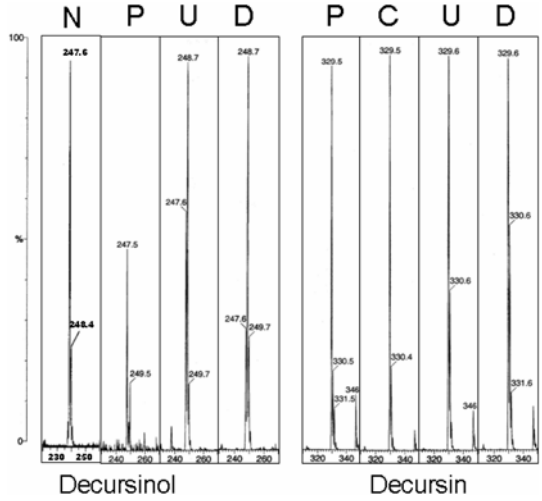

Fig. 3. EISMS of decursinol and decursin (or decusinol angelate) obtained by feeding of deuterium-labeled precursors. The plant extracts were separated on HPLC. N, non-fed; P, phenylalanine-*ring*-d_5; C, cinnamic acid-d_7; U, umbelliferone-*3*-*d*; D, decursinol-*7*-*d*.

found (Table 1). This lack of incorporation of cinnamic acid was probably due to the lower cellular metabolism caused by the toxicity of the acid, or failure of the acid transportation into the root; polar compounds are well known for their difficulty in trans-membrane uptake. Cinnamic acid-d_7 (*m/z* 155) was not recovered in the root extract after incubation.

Feeding of the key intermediate umbelliferone-*3*-*d* would result in singly-labeled products. Indeed, singly-labeled decursin (or decursinol angelate) and decursinol were found (Table 1 and Fig. 3). However, due to the additional labeling of umbelliferone at a site other than C-3 (refer to the preceding section), presumably at carbon(s) ortho to phenolic hydroxy group, the decursinol fraction contained about 5% of the doubly-labeled compound.

When the deuterium-labeled decursinol was used as a precursor, a singly-labeled decursin (or decursinol angelate) was found as expected (Table 1 and Fig. 3). Higher proportion of the monodeuteriated species in decursinol than in decursin could be explained if the separate sites of decursinol biosynthesis and decursin storage are presumed. In other words, if the biosynthesis of decursinol occurred in the cytosol and then slowly transferred to the organelle such as vacuol to be stored as decursin, the labeled compound would be diluted in the storage organelle.

Conclusion. The results of the current feeding experiment confirmed the proposed biosynthetic pathway of decursin in the root of *A. gigas*. The exogeneously fed phenylalaine and umbelliferone were respectively converted into decursinol and decursin, and decursinol into decursin. Failure in the incorporation of cinnamic acid into decursin suggested the lack of uptake of the acid. The cell-free extract of the hairy root or the feeding of cinnamic acid ester could be used to overcome the low uptake of the acid. While some enzymes such as phenylalanine ammonia lyase and phenol hydroxylases are well characterized, prenylation of umbelliferone, cyclization of prenyl side chain, and acyl transfer reaction have not been studied in detail. These later phase reactions would be the ideal targets for both molecular biological and biochemical studies of this plant.

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