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8-Dimethylallylnaringenin 2′-hydroxylase, the crucial cytochrome P450 mono-oxygenase for lavandulylated flavanone formation in *Sophora flavescens* cultured cells

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Abstract

8-Dimethylallylnaringenin (8-DMAN) 2′-hydroxylase, which is indispensable for the formation of a lavandulylated flavanone, sophoraflavanone G, was detected in cell suspension cultures of *Sophora flavescens*. The enzyme catalyzes the 2′-hydroxylation of 8-DMAN to leachianone G, and is tightly bound to the membrane. It required NADPH and molecular oxygen as cofactors, and was inhibited by several cytochrome P450 inhibitors such as carbon monoxide and cytochrome c, indicating that the reaction is mediated by a cytochrome P450 monooxygenase. The optimum pH of 8-DMAN 2′-hydroxylase was 8.5, and the enzyme hydroxylated only 8-DMAN. Apparent K*m* values for 8-DMAN and NADPH of the enzyme were 55 and 34 μM, respectively.

Keywords: *Sophora flavescens*; Leguminosae; Cell suspension cultures; Biosynthesis; Cytochrome P450 mono-oxygenase; Prenylated flavanones; 8-Dimethylallylnaringenin; Leachianone G; Sophoraflavanone G

1. Introduction

Prenylated flavonoids are widely distributed in leguminous, moraceous, berberidaceous and iridaceous plants (Barron and Ibrahim, 1996). In most cases, one or two dimethylallyl moieties are bound to the A or B rings of flavonoids, and sometimes cyclization of a double bond of this side chain with a neighboring phenolic OH function forms a chromene ring as seen in euchrestaflavanone c and osajin in *Euchresta japonica* (Shirataki et al., 1982). Furthermore, it is characteristic of prenylated flavonoids in *Sophora flavescens*, a leguminous plant, to possess a lavandulyl group, an irregular monoterpenoid unit, at the C-6 or C-8 position of 2′-hydroxyflavanone (Hatayama and Komatsu, 1971; Wu et al., 1985a,b,c, 1986). For elucidation of the formation mechanism of this unique prenyl group, we have established cell suspension cultures of *S. flavescens* which produced sophoraflavanone G (SFG), one of the main lavandulylated flavanones in the intact plant (Yamamoto et al., 1991). We have also found that the production of SFG in the cultured cells was stimulated by the addition of some elicitors (Yamamoto et al., 1995) as well as cork tissues (Yamamoto et al., 1996, 1999). Recently, we reported naringenin 8-dimethylallyltransferase in the microsomal fraction from *S. flavescens* cultured cells that prenylated C-8 of (−)-(2*S*)-naringenin to afford (−)-(2*S*)-8-dimethylallylnaringenin (8-DMAN; sophoraflavanone B) as the sole prenylated product (Yamamoto et al., 2000a). The enzyme prenylated naringenin more effectively compared to other flavanone derivatives. Interestingly, when 2′-hydroxynaringenin was used as a prenyl acceptor, the formation of SFG, a lavandulylated flavanone, together with that of 2′-hydroxy-8-dimethylallylnaringenin (leachianone G, LG) was observed. At that time, any naringenin 2′-hydroxylation activity could not be detected in *S. flavescens* cells. These data suggest that the 2′-hydroxy group of flavanone plays an important role for the formation of a lavandulyl group in *S. flavescens* cells, and that a lavandulyl group is formed by two-step dimethylallylations, between which 2′-hydroxylation is inserted (Fig. 1).

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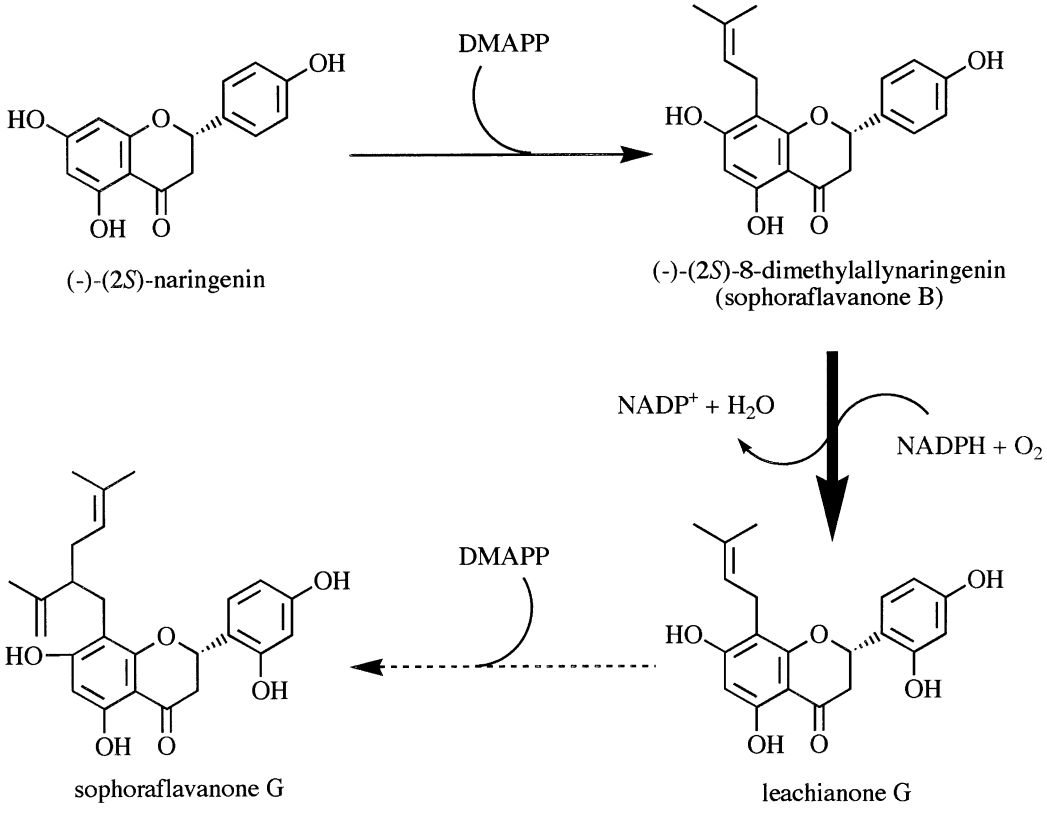

Fig. 1. Proposed biosynthetic route to sophoraflavanone G from naringenin in *Sophora flavescens* cultured cells. DMAPP: dimethylallyl diphosphate.

In the present paper, we report the detection and characterization of 8-DMAN 2′-hydroxylase, essential for the formation of lavandulylated flavanones, in *S. flavescens* cultured cells.

2. Results and discussion

2.1. Detection of 8-DMAN 2′-hydroxylase activity

When crude cell-free extracts or the microsomal fraction from *S. flavescens* cell suspension cultures were incubated with (−)-(2*S*)-8-DMAN and NADPH, the formation of a new compound whose retention time and UV absorption pattern were completely identical to those of leachianone G (LG) was observed in HPLC-photodiode array analysis. The reaction product was isolated on a preparative scale, and was identified as LG by the direct comparison of its EI-MS spectrum with that of authentic LG. More than 90% of the hydroxylation activity still remained in the twice-washed 100,000×*g* pellets, indicating that the hydroxylase was tightly bound to the membrane fraction of the cells (data not shown). The activity depended on the presence of the active enzyme, 8-DMAN and NADPH, and the latter could not be replaced by NADH, FAD or FMN (Table 1).

Table 1
Activity and cofactor requirement of 8-DMAN 2′-hydroxylase from cultured cells of *Sophora flavescens*[a]

Conditions	Relative activity (%)
Complete assay	100.0
Heat-denatured enzyme	0.0
- 8-DMAN	0.0
- Protein	0.0
- NADPH	0.0
- 1 mM NADPH + 1 mM NADH	16.2
- 1 mM NADPH + 1 mM FAD	7.3
- 1 mM NADPH + 1 mM FMN	9.5

[a] The complete assay contained microsomal protein (430 μg), 1 mM (S)-8-DMAN, and 1 mM NADPH. Incubations were carried out as described in Experimental (incubation time: 120 min). 8-DMAN 2′-hydroxylase activity in the complete assay was 13.3 pkat/mg protein.

2.2. Biochemical properties of 8-DMAN 2′-hydroxylase

The pH optimum of 2′-hydroxylase activity was 8.5 in Tris–HCl buffer. The activity in glycine-NaOH buffer at pH 8.5 was only 60% of that observed in Tris–HCl-buffer (data not shown). The hydroxylation of 8-DMAN was linear with time up to 60 min and with protein concentration from 0.06 mg to 0.6 mg in the standard assay. The substrate specificity of 8-DMAN 2′-hydroxylase for

several flavonoids including chemically synthesized dimethylallylated naringenins (Tahara et al., 1994) was investigated. Among these, only 8-DMAN was hydroxylated, but other flavonoids examined, kaempferol, quercetin, apigenin, genistein, naringenin, 6-dimethylallylnaringenin, 3′-dimethylallylnaringenin, 3′,6-di-(dimethylallyl)naringenin, 6″,6″-dimethyl-4″,5″-dihydropyrano-[2″,3″:6,7]naringenin and 6″,6″-dimethyl-4″,5″-dihydropyrano-[2″,3″:7,8]naringenin were not hydroxylated (data not shown). The optimal concentrations of (−)-(2*S*)-8-DMAN and NADPH for enzymatic hydroxylation were between 0.5 and 1 mM, and the apparent K_m values for 8-DMAN and NADPH were calculated as 55 and 34 μM, respectively (Figs. 2 and 3).

2.3. Involvement of cytochrome P450 in 8-DMAN 2′-hydroxylation

In addition to NADPH, 8-DMAN 2′-hydroxylase required molecular oxygen (Table 2). Replacement of O_2 in the reaction mixture by N_2 prior to the incubation led to a significant decrease in the activity. When O_2 in the reaction mixture was completely removed by the addition of an excess of glucose, glucose oxidase and catalase (Kochs and Grisebach, 1987), the formation of LG was hardly detectable (Table 2). These findings suggested the participation of cytochrome P450 haemoprotein in the hydroxylation of 8-DMAN. To confirm this hypothesis, the effects of several cytochrome P450 inhibitors were examined (Table 3). Carbon monoxide, a well-known inhibitor of cytochrome P450 (Ortiz de Montellano and Correia, 1995), strongly inhibited the

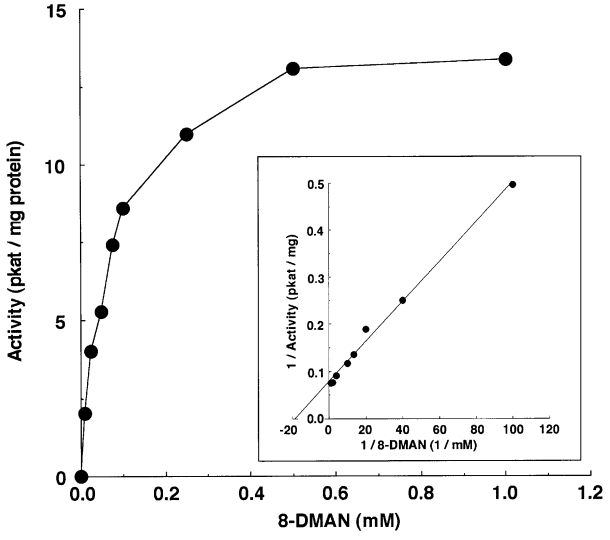

Fig. 2. Dependence of 8-DMAN 2′-hydroxylase on the concentration of (−)-(2*S*)-8-dimethylallylnaringenin (DMAN) measured with the microsomal fraction of *Sophora flavescens*. Insert: Lineweaver-Burk plot with varying concentrations (0.01–1 mM) to calculate the apparent K_m value for (−)-(2*S*)-8-DMAN.

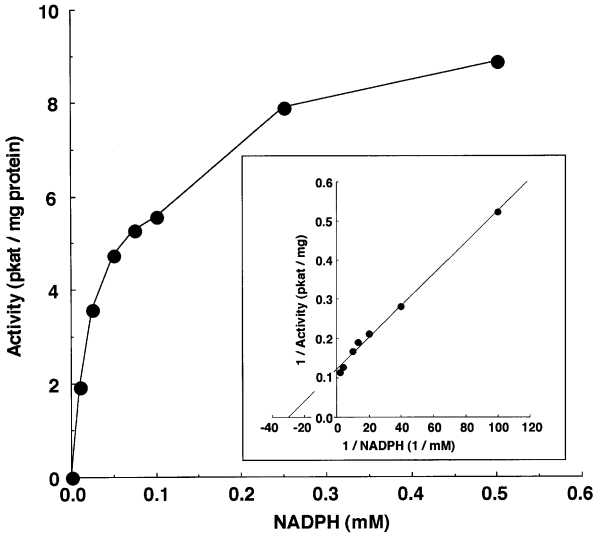

Fig. 3. Dependence of 8-DMAN 2′-hydroxylase on the concentration of NADPH measured with the microsomal fraction of *Sophora flavescens*. Insert: Lineweaver–Burk plot with varying concentrations (0.01–0.5 mM) to calculate the apparent K_m value for NADPH.

Table 2
Oxygen dependence of 8-DMAN 2′-hydroxylase activity[a]

Conditions	Relative activity (%)
Air bubbled	100.0
N_2 bubbled	13.7
+ Glucose + glucose oxidase + catalase	8.8
+ Glucose + heat-denatured glucose oxidase + catalase	89.4

[a] Incubations were carried out as described in Experimental. 8-DMAN 2′-hydroxylase activity in air bubbled control was 12.6 pkat/mg protein

Table 3
Effects of cytochrome P450 inhibitors on 8-DMAN 2′-hydroxylase activity[a]

Inhibitor	Relative activity (%)
Air (dark)	100.0
CO/O_2 (9:1, dark)	14.5
CO/O_2 (9:1, light)	77.0
None	100.0
Ancymidol 1 mM	94.7
Metyrapone 1 mM	76.0
Miconazole 1 mM	63.7
Tropolon 1 mM	69.9
Cytochrome 10 mM	29.1
KCN 1 mM	109.0

[a] Incubations were carried out as described in Experimental. White light (20,000 lx) was used for the reversion experiment. 8-DMAN 2′-hydroxylase activity in the control assay (Air, Dark) was 15.5 pkat/mg protein.

hydroxylation in the dark, and this inhibition was partially reversible by the illumination. Cytochrome c, which competitively removes electrons from the NADPH-cytochrome c reductase complex (Ortiz de Montellano and Correia 1995), was the strongest inhibitor tested. Even at 10 μM cytochrome c, the activity was inhibited by about 70%. Other inhibitors, ancymidol (Graebe 1987), metyrapone and miconazole (Ortiz de Montellano and Correia 1995) and tropolone (Rueffer and Zenk, 1994) showed less pronounced inhibition of 8-DMAN 2′-hydroxylation similar to their effects on other plant cytochrome P450 mono-oxygenases (Tang and Suga 1994; Petersen, 1997; Yamamoto et al., 2000b). Potassium cyanide, an inhibitor of peroxidase, hardly affected the activity (Saunders et al., 1964). These results indicate that 8-DMAN 2′-hydroxylase belongs to the group of cytochrome P450 monooxygenases.

Several studies have been done on the flavonoid hydroxlases, most of which are cytochome P450-dependent. 2′- and 3′-Hydroxylases from *Cicer arietinum* were specific solely for 4′-methoxyisoflavones but the substrate specificity for flavanones was not described (Clemens et al., 1993; Overkamp et al., 2000). Heterologous expression in yeast demonstrated that CYP81E1, a cytochrome P450 cDNA cloned from *Glycyrrhiza echinata,* is isoflavone 2′-hydroxylase, which catalyzed the hydroxylation of isoflavones such as daidzein and formononetin but did not that of flavanones (Akashi et al., 1998). Furthermore, up to the present time, nothing has been reported about flavanone 2′-hydroxylase. The cytochrome P450-dependent hydroxylase investigated in this paper, which catalyzes the hydroxylation of 8-dimethylallynaringenin alone, accordingly, is the first example of the 2′-hydroxylase of prenylated flavanones.

Recently we demonstrated that the presence of 2′-OH function of naringenin is indispensable for the formation of SFG in *S. flavescens* cultured cells (Yamamoto et al., 2000a). We also speculated that a lavandulyl group at C-8 of naringenin is formed by phased dimethylallylations, between which the hydroxylation at C-2′ intervenes, since naringenin 2′-hydroxylase activity was hardly detectable in *Sophora* cells. In this paper, we demonstrated that 8-DMAN was converted to LG by membrane-bound 8-DMAN 2′-hydroxylase from *S. flavescens* cultured cells and that 8-DMAN 2′-hydroxylase is a member of the cytochrome P450 mono-oxygenase family. It follows from these results that SFG is biosynthesized from naringenin via 8-DMAN and LG, successively, by naringenin 8-dimethylallyltransferase, 8-DMAN 2′-hydroxylase and an uncharacterized membrane-bound lavandulyl moiety synthase (Yamamoto et al., 2000a) in *S. flavescens* cells (Fig. 1).

In leguminous plants, prenylation of flavonoids is known to take place in the plastids (Biggs et al., 1990). Furthermore, their dimethylallyl groups are found to be derived from the non-mevalonate pathway (Hano et al., 1994; Asada et al., 2000) which is operative in the formation of isoprenoids biosynthesized in the plastids such as monoterpenoids (Eisenreich et al., 1997; Adam et al., 1998; Turner et al., 1999), as well as β-carotene and prenyl chains of chlorophylls (Lichtenthaler et al., 1997; Disch et al., 1998). In contrast, in *Lithospermum erythrorhizon* cultured cells, geranyl pyrophosphate supplied from the mevalonate pathway in the endoplasmic reticulum/cytosol (Sommer et al., 1995; Li et al., 1998) was utilized for the prenylation of *p*-hydroxybenzoic acid which took place in the endoplasmic reticulum (Yamaga et al., 1993). In SFG biosynthesis, 2′-hydroxylation catalyzed by a cytochrome P450 monooxygenase occurred between two prenylations, and most of cytochrome P450 enzymes were associated with the endoplasmic reticulum (von Wachenfeldt and Johnson, 1995). Thus, in *Sophora* cells, the reaction site of the two prenylations in relation to the source of two dimethylallyl moieties might be different. For the complete elucidation of the lavandulyl moiety formation in *Sophora* cells, it will be necessary to clarify the subcellular localization of the three membrane-bound enzymes including 8-DMAN 2′-hydroxylase and two prenyltransferases as well as the origin of dimethylallyl diphosphate.

3. Experimental

3.1. Chemicals

(−)-(*2S*)-8-Dimethylallylnaringenin was enzymatically synthesized using naringenin 8-dimethylallyltransferase from *Sophora flavescens* cultured cells (Yamamoto et al., 2000a). 6-Dimethylallylnaringenin, 3′-dimethylallylnaringenin, 3′,6-di-(dimethylallyl)naringenin, 6″,6″-dimethyl-4″,5″-dihydropyrano-[2″,3″:6,7]naringenin and 6″,6″-dimethyl-4″,5″-dihydropyrano-[2″,3″:7,8]naringenin were chemically synthesized from naringenin and 2-methyl-3-buten-2-ol in the presence of boron trifluoride/etherate according to the method of Tahara et al. (1994). Apigenin, genistein, kaempferol, naringenin and quercetin were purchased from Funakoshi, Japan.

3.2. Plant material and culture method

The origin and subculturing of callus cultures (Yamamoto et al., 1991) and the establishment of cell suspension cultures (Yamamoto et al., 1996) of *Sophora flavescens* were described in the previous papers.

3.3. Enzyme preparation

For enzyme preparation, all procedures were carried out at 4 °C. Thirty grams of *S. flavescens* cells cultured for 8–10 days were homogenized by a Teflon homogenizer in 100 mM K-Pi buffer (pH 6.5, 60 ml) containing 10 mM

DTT and 3 g PVPP. The homogenate was centrifuged at 12,000×*g* for 20 min, and the supernatant was further ultracentrifuged at 100,000×*g* for 20 min. The pellet was washed twice with 100 mM Tris–HCl buffer (pH 8.5) containing 10 mM DTT and then resuspended in 5 ml of the same buffer.

3.4. Protein content

Protein content was determined according to the method of Bradford (1976).

3.5. Enzyme reaction

The incubation mixture contained 150 nmol 8-dimethylallylnaringenin dissolved in 15 μl EtOH, 150 nmol NADPH and 60 μl of the microsomal fraction (ca. 0.5 mg of microsomal protein) in a total vol. of 150 μl. The reaction was initiated by the addition of NADPH to the mixture and, after incubation for 60 min at 30 °C, was terminated by the addition of 30 μl of 6 M HCl. The reaction mixture was extracted with 150 μl of EtOAc containing 50 nmol 1-naphthaleneacetic acid as an internal standard. The EtOAc extract was evaporated, dissolved in 70 μl MeOH and analyzed by HPLC.

For the investigation of the effects of different gaseous environments, test tubes (10 ml) containing all the reaction components except NADPH were sealed with septum caps in ice-water bath, and 200 ml of N_2, CO/O_2 (9:1) mixture or air was slowly bubbled into the mixture, respectively. The reaction was initiated by the addition of 150 nmol NADPH in the 15 μl of the same buffer. In the System Biotron, incubation was carried out for 60 min at 30 °C in the dark (tubes covered with aluminum foil) and under 20,000 lx of fluorescent lights for examining the effect of the light on the inhibition by CO. The reaction was stopped, treated and analyzed as described above.

For the oxygen consuming experiments, 40 mM glucose, 50 units glucose oxidase (Nacalai Tesque, Japan) and 105 units catalase (Sigma, USA) were added to the enzyme solution containing 150 nmol 8-DMAN and, after preincubation for 30 min at 30 °C, the reactions were initiated by addition of 150 nmol NADPH.

3.6. HPLC analysis

The amount of leachianone G formed was determined by HPLC using Capcellpak C18 AG-120 column (4.6×250 mm, Shiseido, Japan) in an oven at 40 °C, with CH_3CN/H_2O linear gradient solvent system containing 1% AcOH, from 20% to 60% CH_3CN in 40 min, at a flow rate of 0.9 ml/min, monitoring the absorption at 294 nm or using a photodiode-array SPDM-6A system (Shimadzu, Japan). The quantities were calculated from the peak area at 294 nm recorded by Chromatopac C-R4A (Shimadzu, Japan).

3.7. Isolation and identification of the reaction product

The microsomal fraction obtained from 30 g of the fresh cells was incubated with 750 nmol 8-DMAN and 750 nmol NADPH in 100 mM Tris–HCl buffer (pH 8.5, total vol. 7.5 ml) at 30 °C for 2 h. The reaction was terminated by the addition of 1.5 ml of 6 M HCl, and the products were extracted with EtOAc (3×7.5 ml). The organic layers were combined and concd in vacuo. The residue was dissolved in MeOH and purified by HPLC using the following conditions: column, Hikarisil C18 (4.6×250 mm, Asahi Chemical Ind. Co., Ltd., Japan); solvent, CH_3CN/H_2O linear gradient solvent system, from 20% CH_3CN to 50% CH_3CN in 60 min; flow rate, 1 ml/min; oven temp., 40 °C; detection, 294 nm. The fraction around Rt 50 min was collected and evaporated in vacuo.

EI–MS. *m*/*z*; 356 (M^+), 338, 323, 295, 283, 270, 205, 192, 177, 165, 136.

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