Biosynthetic Origin of the 1-Oxygen of Umbelliferone in the Root Tissue of Sweet Potato

Bun-ichi Shimizu*, Kosuke Kai, Michiko Tamai, Hikaru Yamaguchi, Masaharu Mizutani, and Kanzo Sakata

Institute for Chemical Research, Gokasho Uji, Kyoto, 611-0011, Japan. Fax: +81-774-38-3229. E-mail: bsimz@scl.kyoto-u.ac.jp

* Author for correspondence and reprint requests

Z. Naturforsch. **63 c**, 687–690 (2008); received April 10/May 13, 2008

Oxidation of *p*-coumarate at the *ortho*-position is a key step to form umbelliferone. A tracer analysis using $^{18}O_2$ was performed in order to take information about the formation of umbelliferone in the root tissue of sweet potato. Mass fragmentation experiments revealed incorporation of an ^{18}O atom into the 1-position of umbelliferone. This result indicates that the lactone of umbelliferone is formed *via ortho*-hydroxylation of the *p*-coumarate unit using O_2.

Key words: ortho-Hydroxylation, Umbelliferone, Sweet Potato

Introduction

Coumarins are widely distributed in the plant kingdom (Keating and O'Kennedy, 1997; Zobel, 1997). They are thought to be involved in plant defense due to the induction following various stress events (Hamerski and Matern, 1988; Kitamura *et al.*, 1998; Matern, 1991; Shimizu *et al.*, 2005) and their antimicrobial activities (Carpinella *et al.*, 2005; Valle *et al.*, 1997). Formation of the lactone ring of the 2*H*-1-benzopyran-2-one core structure has not yet been fully explained, whereas coumarins in plants originate from the phenylpropanoid pathway (Brown, 1962). During formation of the lactone ring of coumarins, *ortho*-oxidation of cinnamates is the key step because it catalyzes an irreversible reaction and is the branch point from lignin biosynthesis. Occurrence of 2-glucosyloxycinnamic acid derivatives (Takaishi, 1968; Zhang *et al.,* 2006) suggests that *ortho*-hydroxylation of the ring of cinnamates is prior to ring-closure during coumarin formation (pathway A in Fig. 1); however, it is also reported that the ring oxygen atom of the coumarin structure of novobiocin from *Streptomyces viveus* originated from the carbonyl group *via* the direct attack by carboxylate to the *ortho*-position of the ring (pathway B in Fig. 1) (Bunton *et al.,* 1963).

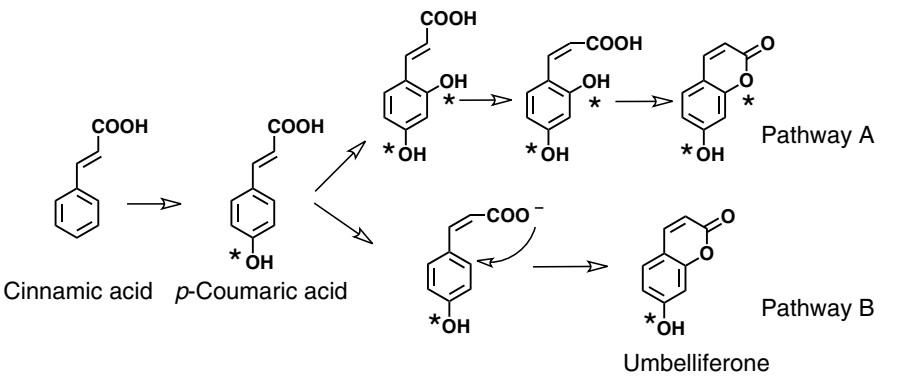

Fig. 1. Formation of the lactone ring of umbelliferone. Cinnamic acid is subjected to *para*-hydroxylation by cytochrome P450 to produce *p*-coumaric acid. Two pathways from *p*-coumaric acid to umbelliferone are postulated: pathway A is by *ortho*-hydroxylation, and pathway B by attack of carboxylate to the *ortho*-position. Asterisks mean the presumed positions of the labeled oxygen (^{18}O) in the respective pathways.

0939–5075/2008/0900–0687 $ 06.00

In order to take clues of *ortho*-oxidation involved in coumarin formation in plants, a tracer analysis of umbelliferone formation in the root tissues of sweet potato (*Ipomoea batatas*) using $^{18}O_2$ was performed.

Experimental

After preincubation under N_2 atmosphere in the darkness at 25 °C for 4 d, the root tuber of sweet potato (*I. batatas* cv. Kokei No. 14) was cut into discs (9 mm i. d. × 3 mm thickness) using a razor and a cork bowler. After storage in the darkness at 25 °C for 24 h under 100% humidity in N_2 atmosphere, the discs were treated with 20 μl of 1 mg/ml chitosan (Wako Pure Chemical, Tokyo, Japan). The discs were incubated in the darkness at 25 °C under 100% humidity in normal air or artificial air containing $^{18}O_2$ (Isotec, Miamisburg, OH, USA; $N_2/^{18}O_2/CO_2$ 4:1:0.01, v/v/v). After treatments with artificial air, the discs were extracted with 2 ml of methanol containing 10 μM 4-methylumbelliferone as an internal standard. The extract was subjected to HPLC analysis on a reversed-phased column, YMC-Pack Pro C18 (4.6 × 75 mm, 5 μm; YMC Inc., Tokyo, Japan). Elution of the column was performed by initial elution with a 3:17 methanol/water mixture with 0.1% acetic acid for 2 min and a linear gradient of 3:17 to 9:11 methanol/water mixtures containing 0.1% acetic acid at 40 °C; flow rate was 0.75 ml/min (fluorescence monitoring at 340 nm excitation and 420 nm emission).

Results and Discussion

Treatment with chitosan elicited umbelliferone accumulation (Fig. 2). A larger and more rapid induction of umbelliferone (retention time, 16.0 min) was found in the chitosan-treated discs after 5 h compared to the control. Accumulation of umbelliferone in the chitosan-treated discs exhibited the maximum level after 24 h. Electrospray ionization mass spectrometry (ESI-MS) on an API-3000 LC/MS/MS system (Applied Biosystems, Foster City, CA, USA) after HPLC separation described above was performed to examine the incorporation of O_2 into umbelliferone in the root tissues treated with chitosan under artificial air containing $^{18}O_2$ for 24 h. The mass spectrum of umbelliferone gave the protonated molecular ions of umbelliferone $[M+H]^+$ at *m/z* 163 (100%), 165 (33) and 167 (27) in the positive ion mode. The

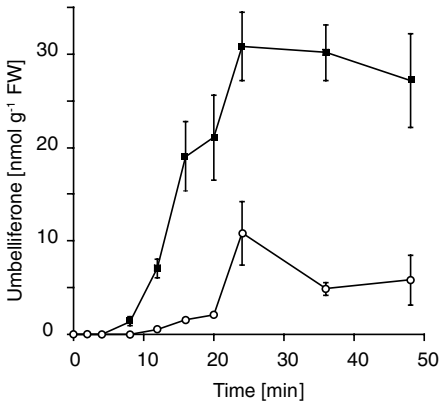

Fig. 2. Time-course of umbelliferone accumulation in the root tissue of sweet potato after the treatments. The umbelliferone levels in the root tissues of mock inoculation (open circles) and chitosan treatment (solid squares) are shown with standard error (n = 3).

protonated molecular ion at *m/z* 163 corresponds to non-labeled umbelliferone. No protonated molecular ions at *m/z* 165 and 167 were found under normal air without $^{18}O_2$ after treatment with chitosan solution (1 mg/ml) in H_2O or $H_2{}^{18}O$ (data not shown). The protonated molecular ions at *m/z* 165 and 167 indicate that one and two ^{18}O atoms were incorporated into umbelliferone, respectively. To investigate the positions of the labeled oxygen atom, MS/MS fragmentation experiments of the protonated molecular ions of umbelliferone in the positive ion mode were performed. The fragments were assigned according to Concannon *et al.* (2000). The ion at *m/z* 163 fragmented with the loss of CO and CO_2 to give a signal at *m/z* 135 and 119, respectively (Fig. 3A). The ion at *m/z* 167 fragmented to give the signals at *m/z* 139 with the loss of CO and at *m/z* 121 with the loss of $C^{18}OO$ (Fig. 3B). The ion at *m/z* 165 also fragmented with the loss of $C^{18}OO$ or CO_2 to give the signals at *m/z* 119 or 121, respectively, and with the loss of CO to give the signal at *m/z* 137 (Fig. 3C). These results indicate that one or two ^{18}O atoms are incorporated from $^{18}O_2$ into the 7- and/or 1-position of umbelliferone.

The oxygen atom of the 7-position is introduced by cytochrome P450, of which the enzyme family uses molecular oxygen during its catalysis (Mizutani *et al.,* 1997; Ehlting *et al.,* 2006). Incorporation of ^{18}O into the 1-position of umbelliferone strongly suggests that pathway A is operating, in which *ortho*-hydroxylation of a *p*-coumarate unit

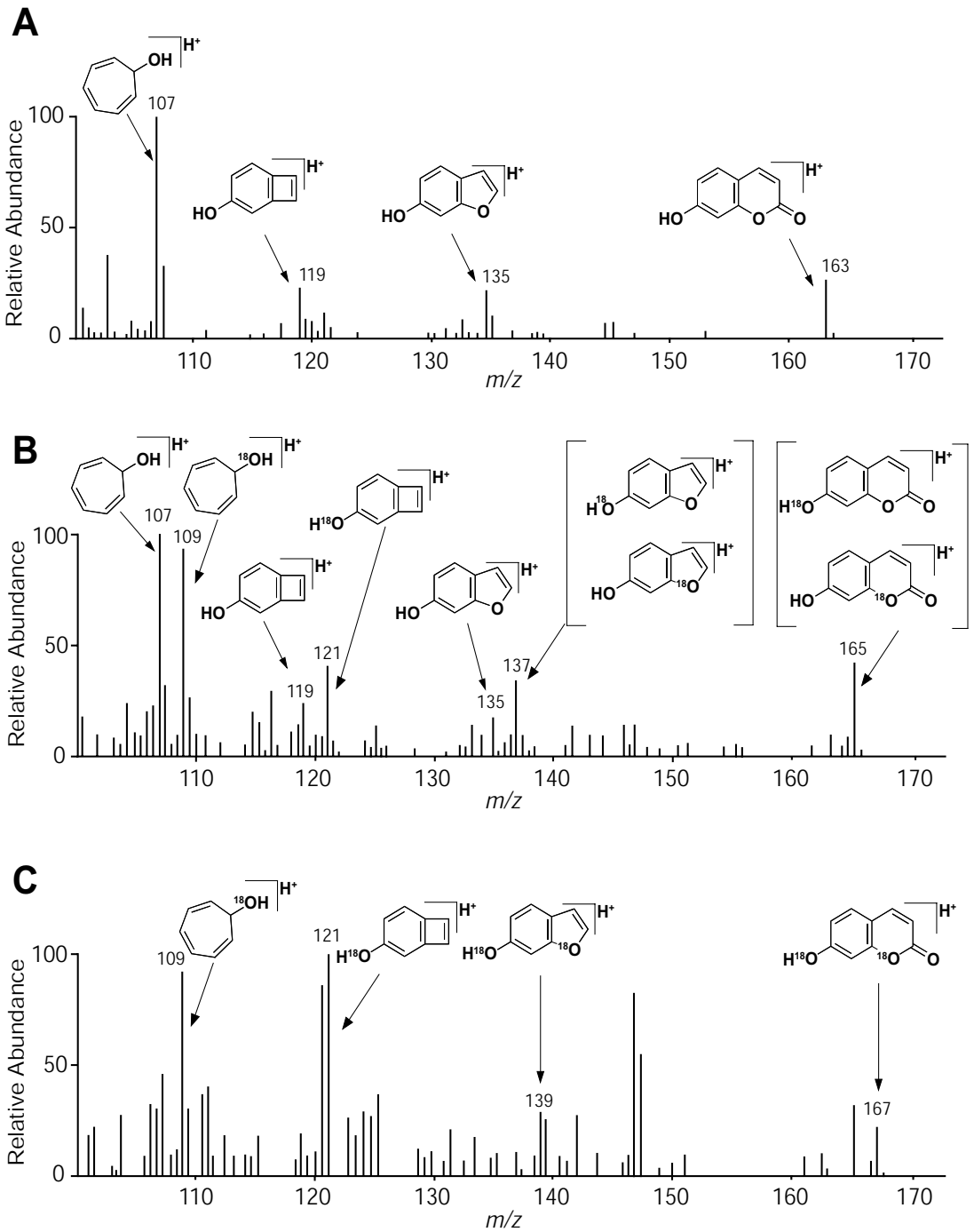

Fig. 3. ESI-MS/MS fragmentation patterns of the protonated molecular ions of umbelliferone at (A) *m/z* 163, (B) *m/z* 165, and (C) *m/z* 167.

proceeds during formation of umbelliferone. It is also suggested that the enzyme involved in the *ortho*-hydroxylation of *p*-coumarate uses O_2 for oxidation of its substrate. Added to cytochrome P450s, 2-oxoglutarate-dependent dioxygenases (Bugg, 2003) and flavin-monooxygenases (Berkel *et al.,* 2006) also use O_2 during their catalysis. They can be the candidate enzyme families of the *ortho*-hydroxylase involved in the umbelliferone formation.

Berkel W. J. H., Kamerbeek N. M., and Fraaije M. W. (2006), Flavoprotein monooxygenases, a diverse class of oxidative biocatalysts. J. Biotechnol. **124**, 670–689.

Brown S. A. (1962), Biosynthesis of coumarin and herniarin in lavender. Science **137**, 977–978.

Bugg T. D. H. (2003), Dioxygenase enzymes: catalytic mechanisms and chemical models. Tetrahedron **59**, 7075–7101.

Bunton C. A., Kenner G. W., Robinson M. J. T., and Webster B. R. (1963), Experiments related to the biosynthesis of novobiocin and other coumarins. Tetrahedron **19**, 1001–1010.

Carpinella M. C., Ferrayoli C. G., and Palacios S. M. (2005), Antifungal synergistic effect of scopoletin, a hydroxycoumarin isolated from *Melia azedarach* L. fruits. J. Agric. Food Chem. **53**, 2922–2927.

Concannon S., Ramachandran V. N., and Smyth W. F. (2000), A study of the electrospray ionization of selected coumarin derivatives and their subsequent fragmentation using an ion trap mass spectrometer. Rapid Commun. Mass Spectrom. **14**, 1157–1166.

Ehlting J., Hamberger B., Million-Rousseau R., and Werck-Reichhart D. (2006), Cytochromes P450 in phenolic metabolism. Phytochem. Rev. **5**, 239–270.

Hamerski D. and Matern U. (1988), Elicitor-induced biosynthesis of psoralens in *Ammi majus* L. suspension cultures. Microsomal conversion of demethylsuberosin into (+)-marmesin and psoralen. Eur. J. Biochem. **171**, 369–375.

Keating G. J. and O'Kennedy R. (1997), The chemistry and occurrence of coumarins. In: Coumarins: Biology, Applications and Mode of Action (O'Kennedy R. and Thornes R. D., eds.). John Wiley & Sons, Chichester, pp. 23–66.

Kitamura Y., Ikenaga T., Ooe Y., Hiraoka N., and Mizukami H. (1998), Induction of furanocoumarin biosynthesis in *Glehnia littoralis* cell suspension cultures by elicitor treatment. Phytochemistry **48**, 113–117.

Matern U. (1991), Coumarins and other phenylpropanoid compounds in the defense response of plant cells. Planta Med. **57**, S15–20.

Mizutani M., Ota D., and Sato R. (1997), Isolation of a cDNA and a genomic clone encoding cinnamate 4-hydroxylase from *Arabidopsis* and its expression manner *in planta.* Plant Physiol. **113**, 755–763.

Shimizu B., Miyagawa H., Ueno T., Sakata K., Watanabe K., and Ogawa K. (2005), Morning glory systemically accumulates scopoletin and scopolin after interaction with *Fusarium oxysporum*. Z. Naturforsch. **60c**, 83–90.

Takaishi K. (1968), Studies of the coumarin components in the leaves of cherry-tree. Yakugaku Zasshi **88**, 1467–1471 (in Japanese).

Valle T., López J. L., Hernández J. M., and Corchete P. (1997), Antifungal activity of scopoletin and its differential accumulation in *Ulmus pumila* and *Ulmus campestris* cell suspension cultures infected with *Ophiostoma ulmi* spores. Plant Sci. **125**, 97–101.

Zhang G., Zhang F., Yang L., Zhu E., Wang Z., Xu L., and Hu Z. (2006), Simultaneous analysis of *trans*- and *cis*-isomers of 2-glucosyloxycinnamic acids and coumarin derivatives in *Dendrobium thyrsiflorum* by high-performance liquid chromatography (HPLC)-photodiode array detection (DAD)-electrospray ionization (ESI)-tandem mass spectrometry (MS). Anal. Chim. Acta **571**, 17–24.

Zobel A. M. (1997), Coumarins in fruit and vegetables. In: Phytochemistry of Fruits and Vegetables (Tomas-Barberan F. A. A. and Robins R. J., eds.). Clarendon Press, Oxford, pp. 173–203.