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

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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 2H-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-glucosyl-oxycinnamic 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 niveus 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.
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 borer. 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 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$_2$O 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 O₂ for oxidation of its substrate. Added to cytochrome P450s, 2-oxoglutarate-dependent dioxygenases (Bugg, 2003) and flavin-monoxygenases (Berkel et al., 2006) also use O₂ during their catalysis. They can be the candidate enzyme families of the ortho-hydroxylase involved in the umbelliferone formation.


