BIOSYNTHESIS OF VANILLIN VIA FERULIC ACID IN VANILLA PLANIFOLIA

O. NEGISHI¹ and Y. Negishi²

¹ Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan
² Institute of Nutrition Sciences, Kagawa Nutrition University, Sakado, Saitama 350-0288, Japan

Abstract

Several ¹⁴C-labelled precursors were fed to vanilla bean disks and their conversions to glucovanillin examined. The results showed radioactivities of 11%, 15%, 29% and 24% were incorporated into glucovanillin within 24 hr from ¹⁴C-labelled phenylalanine, 4-coumaric acid, ferulic acid and methionine, respectively. In the process of incorporation of methionine into glucovanillin, ¹⁴CH₃ of methionine was also trapped by unlabelled ferulic acid. However, ¹⁴C-labelled 4-hydroxybenzaldehyde and 4-hydroxybenzyl alcohol were not converted to glucovanillin. These results suggest that the synthetic pathway in Vanilla planifolia for vanillin is 4-coumaric acid → ferulic acid → vanillin → glucovanillin.

Introduction

Vanillin is accumulated as a glucoside in vanilla beans (Vanilla planifolia). The good aroma of vanilla is generated by “curing”, a fermentation process in which glucovanillin (vanillin glucoside) is hydrolyzed by glucosidase in vanilla bean. On the formation of vanillin in vanilla bean, Anwar (1) proposed that vanillin is synthesized from coniferin, a precursor of lignin, by shortening of the C₃ side chain and hydrolysis of glucoside, whereas Zenk (2) proposed that vanillin is formed from ferulic acid with shortening of the side chain by β-oxidation. Tokoro et al. (3) proposed a pathway to form glucovanillin from the glucoside of the 4-hydroxybenzyl alcohol after analysis of glucosides in green vanilla beans. Furthermore, Yazaki et al. (4) reported the existence of a non-oxidative reaction for shortening of the phenylpropanoid side-chain and the formation of 4-hydroxybenzaldehyde from 4-coumaric acid (C₆-C₃ compound) in cell free extract of Lithospermum erythrorhizon cell cultures. On the other hand, Podstolski et al. (5) also reported an enzyme involved in the reaction that converts 4-coumaric acid to 4-hydroxybenzaldehyde in tissue culture of vanilla. However, the complete biosynthetic pathway of vanillin from phenylpropanoids has not been demonstrated yet. In order to clarify the biosynthetic pathway for vanillin, we carried out pulse-chase experiments with ¹⁴C-labelled compounds in green vanilla beans.

Experimental

Radioisotopes. [U-¹⁴C]-L-Phenylalanine (Phe, 360 mCi/mmol), [U-¹⁴C]-L-tyrosine (360 mCi/mmol) and [methyl-¹⁴C]-L-methionine (Met, 54 mCi/mmol) were purchased
from Moravek Biochemicals, Inc., Brea, CA. [14C]-Methyl iodide (7.6 mCi/mmol) was a product of DuPont, Boston, MA.

**Syntheses of 14C-precursors.** [U-14C]-4-Coumaric acid was prepared by the deamination of [U-14C]-L-tyrosine with phenylalanine ammonia lyase (6). [O-methyl-14C]-Ferulic acid was synthesized by the condensation between malonic acid and vanillin that was prepared by the methylation of 3,4-dihydroxybenzaldehyde with 14CH3I via the modification of 4-position by benzyl group (6, 7). [U-14C]-4-Hydroxybenzaldehyde was prepared by the ozonolysis of [U-14C]-4-coumaric acid with an ozone generator (DMO-10BDF, Ishimori Co. Ltd., Tokyo, Japan) (6). Furthermore, [U-14C]-4-hydroxybenzyl alcohol was obtained by the reduction of [U-14C]-4-hydroxybenzaldehyde with NaBH4 (6). [U-14C]-4-Coumaric acid (50 mCi/mmol), [O-methyl-14C]-ferulic acid (7.6 mCi/mmol), [U-14C]-4-hydroxybenzaldehyde (50 mCi/mmol), [U-14C]-4-hydroxybenzyl alcohol (50 mCi/mmol), [U-14C]-L-Phe (50 mCi/mmol), [methyl-14C]-L-Met (54 mCi/mmol) were used as 14C-precursors in the feeding experiments.

**Feeding experiments.** Green vanilla beans 6 months after pollination from Indonesia were washed with 0.02% chloramphenicol and cut into 2 cm length pieces (2 g). Subsequently, the 2 cm piece of green bean was further sliced into 2 mm lengths to form 10 disks, these disks were separately placed on a Petri dish. Each disk was fed 10 μl of 14C-labelled compound (0.2 μCi) and incubated at 26°C under light conditions. After 1, 3, 6, 12 and 24 hr, sets of 10 disks each were frozen and stored at -80°C until the following extraction.

**Extraction and separation of 14C-labelled metabolites.** Extraction of 14C-labelled metabolites from ten disks of vanilla beans was carried out by homogenizing them with MeOH. After the separation of 14C-labelled aglycones with ether from the MeOH extract, the remaining water solution was passed through an Amberlite XAD-2 column. The column was washed with water, followed by the elution of 14C-labelled glucosides with MeOH. The solutions containing aglycones and glucosides were concentrated and lyophilized. Furthermore, 50% MeOH solutions of the lyophilized powders were used to analyse the metabolites by HPLC (TSK-gel ODS 80Ts, 5 μm, 250 × 4.6 mm ID, Tosoh Corporation, Japan) (8). The flow rate was 0.6 ml/min and eluate was monitored at 280 nm. Furthermore, eluates were collected for every 1 min and their radioactivities were measured with a scintillation counter.

**Results**

**Feeding experiments with several 14C-compounds.** 14C-4-Coumaric acid (15%) and 14C-ferulic acid (29%) were incorporated into glucovanillin within 24 hours (Figures 1-a, b). This suggests that ferulic acid is the precursor nearer to vanillin than 4-coumaric acid. During the early time of the feeding experiments the higher incorporations of 14C into unknown compounds were observed. These unknown compounds from 4-coumaric acid and ferulic acid were shown to be their glucose esters (data not shown). 14C-Labels from 4-hydroxybenzaldehyde distributed to 4-hydroxybenzyl alcohol and their glucosides (Figure 1-c). In addition, conversion of 4-hydroxybenzyl alcohol to its glucoside seems to be easy (Figure 1-d). However, 14C-labelled 4-hydroxybenzaldehyde and 4-hydroxybenzyl alcohol were not converted to glucovanillin. 14C from Phe was incorporated into 4-coumaric acid, ferulic acid, their glucose esters, vanillin and glucovanillin (11% within 24 hr) (Figure 1-e). In the 14C-Met-feeding experiment to which unlabelled ferulic acid was added (Figure 1-g), the rate of conversion from Met to glucovanillin slowed (16% within 24 hr) when
compared to the control (24% within 24 hr) (Figure 1-f). The incorporation of much $^{14}\text{C}$ into ferulic acid was observed at 3 hr after feeding and into feruloyl glucose during the incubation times from 3 to 24 hrs. These results suggest ferulic acid is directly converted to vanillin, not via feruloyl glucose which decreased slowly. Slow conversion rates for glucose esters are also shown in (Figures 1-a, b and e).

**Figure 1.** Feeding experiments with several $^{14}\text{C}$-compounds. $^{14}\text{C}$-4-Coumaric acid (a), $^{14}\text{C}$- ferulic acid (b), $^{14}\text{C}$-4-hydroxybenzaldehyde (c), $^{14}\text{C}$-4-hydroxybezyl alcohol (d), $^{14}\text{C}$-Phe (e), $^{14}\text{C}$-Met (f) and $^{14}\text{C}$-Met + cold ferulic acid (g) were fed to vanilla green bean disks. In the experiment of (g), 0.4 μmol of ferulic acid was added toward 2 μCi of Met. ○, vanillin; ●, glucovanillin; ○, vanillyl alcohol glucoside; □, 4-coumaric acid; ■, unknown-1 or 4-coumaroyl glucose (trans); ☽, unknown-2 or 4-coumaroyl glucose (cis); ◆, ferulic acid; ●, unknown-3 or feruloyl glucose (trans); ◊, unknown-4 or feruloyl glucose (cis); △, 4-hydroxybenzaldehyde; ◇, 4-hydroxybezyl alcohol glucoside; ▽, 4-hydroxybezyl alcohol; ▽, 4-hydroxybezyl alcohol glucoside; ☼, glucosides A & B (3); ★, unknown-5; ❍, unknown-6; and ■ in (e) and ◆ in (f) and (g) show total 4-coumaroyl and feruloyl glucose, respectively. The decreases of $^{14}\text{C}$-precursors in (e)-(g) were not determined.

**Biosynthetic pathway for vanillin.** Based on these results we propose that vanillin is synthesized via ferulic acid from phenylpropanoids (Scheme 1). Pathway from ferulic acid to vanillin can be supported by the existence of enzyme catalyzing the reaction in the cell-free extract. We have already detected the enzyme activity and the partial purification and characterization of the key enzyme are in progress.
Scheme 1. Proposed biosynthetic pathway for vanillin and related compounds from phenylpropanoids, and formation of their glucosides and glucose esters in Vanilla planifolia. (a) 4-coumaric acid; (b) 4-hydroxybenzaldehyde; (c) 4-hydroxybenzyl alcohol; (d) ferulic acid; (e) vanillin; and (a)' (b)' (c)' (d)' (e)' show the respective glucose esters or glucosides. Glc A&B are esters of tartaric acid and 2 molecules of (c)' (3).

References