Separation of Capsaicin from Phenylpropanoid Compounds by High-Performance Liquid Chromatography To Determine the Biosynthetic Status of Cells and Tissues of *Capsicum frutescens* Mill. in Vivo and in Vitro

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A HPLC procedure was developed to separate vanillylamine, L-phenylalanine, caffeic acid, coumaric acid, ferulic acid, cinnamic acid, capsaicin, and dihydrocapsaicin. Using the HPLC method, concentrations of intermediates were determined in placenta, pericarp, cell cultures, immobilized cells, and immobilized placenta. The profile of intermediates showed higher capability of biotransformation in immobilized placenta over immobilized cells. Vanillylamine was not a limiting intermediate for capsaicin production. Three-week-old placenta separated from fruit had a higher quantity of capsaicin than did pericarp.

INTRODUCTION

Capsaicin, which is a component of green pepper fruits (Govindarajan et al., 1977), is derived from phenylpropanoid compounds. Cell cultures of *Capsicum annuum* and *Capsicum frutescens* produce capsaicin and leach out to the medium (Sudhakar Johnson et al., 1991). Production of capsaicin is enhanced several fold upon immobilization of cells due to channeling of precursors for capsaicin biosynthetic pathway (Lindsey and Yeoman, 1984). Placental tissue, which is the site of the synthesis of capsaicin (Iwai et al., 1979), can also be immobilized to produce capsaicin in vitro (Sudhakar Johnson et al., 1990).

The objective of this study was to depict the biochemical differences of high-capsaicin-producing placental tissues and low-producing callus. To achieve this objective, capsaicin, dihydrocapsaicin, and phenylpropanoid compounds were analyzed in placental tissue, callus, and pericarp. As a result, we report a suitable HPLC system for distinct separation of intermediates of the capsaicinoid pathway.

MATERIALS AND METHODS

Culture Conditions. Callus cultures of *C. frutescens* were initiated and maintained as reported earlier (Sudhakar Johnson et al., 1990). Placental tissue was isolated from the fruits of greenhouse-grown plants. Placental and callus tissues were immobilized separately in calcium alginate as described earlier (Sudhakar Johnson et al., 1990). One gram each of the immobilized cells and placental tissue was cultured in 40 mL of Murashige and Skoog's (1962) (MS) liquid medium supplemented with 3% sucrose, 2 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.5 mg L^{-1} kinetin (kn). The cultures were agitated on a rotary shaker at 90 rpm under continuous light (2000 lux) at 25 ± 2 °C.

Plant Material. *Isolation of Placental Tissue and Pericarp for Biochemical Analysis.* Fresh greenhouse-grown fruits of *C. frutescens* (3 weeks old) were collected, and placental tissue was separated. One gram of placental tissue was taken after all of the adhering seeds were removed. Pericarp from the above fruits was isolated after placental tissue was removed. The placental tissue and pericarp were solvent extracted as given below.

Extraction Procedure. Five hundred milligrams of fresh tissue or cells and placenta in alginate matrix was homogenized separately with 80% ethanol and centrifuged at 1000 rpm for 15 min, the supernatant was flash evaporated, and the resultant residue was dissolved in 500 μL of 80% ethanol. The procedure was repeated in triplicate to facilitate maximum extraction. Capsaicin from culture medium was extracted into ethyl acetate (3 × 40 mL), and the upper portions were collected. Ethyl acetate portions were flash evaporated to dryness and dissolved in 1.0 mL of 80% ethanol. The extractions were filtered through a 0.45-μm Millipore filter prior to injection on the HPLC.

HPLC Protocol. Chromatography was performed using a Shimadzu LC-6A liquid chromatograph with a Shimadzu C18 reversed-phase column (15 cm long × 4.5 mm i.d.), particle size 5 μm. A guard column containing the same packing was used to protect the analytical column. Resolution of vanillylamine, L-phenylalanine, caffeic acid, coumaric acid, ferulic acid, cinnamic acid, capsaicin, and dihydrocapsaicin was obtained at room temperature with a linear gradient of 0–100% acetonitrile (1 mL min^{-1}) in water (pH 3.0) for 35 min. The 100% acetonitrile condition was maintained for 2 min. The eluents were monitored by a UV–vis spectrophotometric detector at 236 nm.

Table I. HPLC Profile of Standard Phenylpropanoid Compounds, Vanillylamine, Capsaicin, and Dihydrocapsaicin

compound	retention time,[a] min	% acetonitrile at which the separation was achieved
vanillylamine	3.60 (±0.15)	10.40
L-phenylalanine	7.80 (±0.07)	22.89
caffeic acid	12.11 (±0.12)	34.41
coumaric acid	13.52 (±0.12)	38.80
ferulic acid	13.96 (±0.14)	40.55
cinnamic acid	17.67 (±0.04)	50.56
capsaicin	23.24 (±0.15)	66.20
dihydrocapsaicin	24.60 (±0.11)	72.20

[a] Data represent an average of five replicates (±SD).

RESULTS AND DISCUSSION

The HPLC procedure reported by Graham (1991) describes the separation of 38 compounds which include L-phenylalanine, caffeic acid, coumaric acid, and cinnamic acid. Graham's HPLC procedure was modified to give the separation of vanillylamine, capsaicin, and dihydrocapsaicin; retention time and percentage of acetonitrile at which the separation was achieved are given in Table I and Figure 1. This led us to quantify native concentrations of eight metabolites of the capsaicin biosynthetic pathway in cells, placenta, and pericarp (Tables II and III and Figure 2). The profile of the intermediates of capsaicin biosynthesis was studied to understand the biosynthetic status of cells and placental tissue and was correlated to its biosynthetic potential to produce capsaicin.

A time course study of the capsaicin-producing capability of immobilized cells and placental tissue (Figure 3)

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Table II. Profile of Intermediates of Capsaicin Biosynthetic Pathway in Immobilized Cells, Placental Tissue, and Callus[a]

compound	callus,[b] μg/g of fresh wt	immobilized cells[c] beads	immobilized cells[c] medium	immobilized cells[c] total, μg/g of fresh wt	immobilized placenta[d] beads	immobilized placenta[d] medium	immobilized placenta[d] total,[e] μg/g of fresh wt
vanillylamine	27 (±2.08)	87 (±3.69)	80 (±3.0)	167 (±6.69)	1298 (±43.76)	428 (±10)	172 (±53.76)
L-phenylalanine	150.07 (±5.77)	306 (±17.5)	ND[f]	306 (±17.5)	ND	ND	ND
caffeic acid	29.65 (±2.0)	ND	ND	ND	ND	ND	ND
coumaric acid	42.85 (±3.05)	ND	16.1 (±0.57)	16.1 (±0.57)	ND	ND	ND
ferulic acid	ND	ND	ND	ND	ND	124 (±6.97)	124 (±6.97)
cinnamic acid	ND	68 (±5.78)	44.9 (±3.3)	112.9 (±9.08)	ND	ND	ND
capsaicin	75.23 (±5.03)	5.45 (±0.67)	65 (±5.5)	70.45 (±6.17)	714 (±27.9)	1420 (±76.5)	2134 (±104.4)
dihydrocapsaicin	40 (±3.6)	15.35 (±0.61)	59 (±4.34)	74.35 (±4.95)	280 (±21.9)	1406 (±70.2)	1686 (±91.9)

[a] Data represent an average of five replicates (±SD). [b] 14-day-old callus cultured on MS agar medium. [c] Callus cells were immobilized in 2.5% calcium alginate. Beads: 1 g of cells immobilized in 2.5% calcium alginate. Culture medium: 40 mL of MS medium. [d] 1 g of placental tissue immobilized in 2.5% calcium alginate. [e] Total indicates beads consisting of 1 g of immobilized callus or placenta plus 40 mL of medium in which it is cultured. [f] ND, not detected.

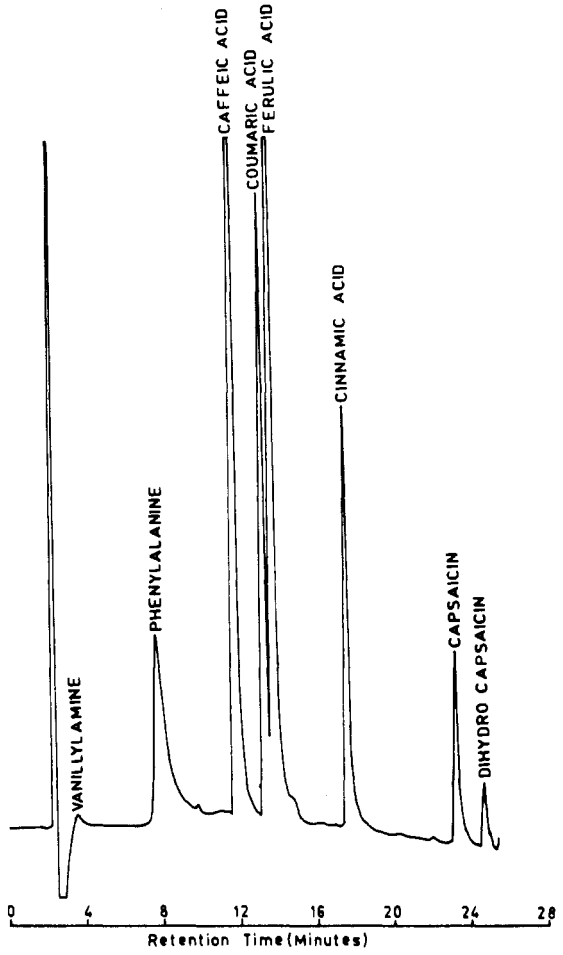

Figure 1. HPLC profile of standard phenylpropanoid compounds and vanillylamine, capsaicin, and dihydrocapsaicin. Chromatography was obtained using Shimadzu LC-6A with C_{18} reversed-phase column (15 cm × 4.5 mm i.d.) with a linear gradient of 0–100% acetonitrile (1 mL/min) in pH 3.0 water for 35 min; 100% was maintained for 2 min at the 35th min. Detection was at 236 nm.

indicated that immobilized placental tissue produced a higher amount of capsaicin than immobilized cells. The study of conversion of intermediates of capsaicin production in immobilized cells and immobilized placental tissue (Tables II and III) revealed higher contents of L-phenylalanine, coumaric acid, and cinnamic acid in immobilized cells, in contrast to immobilized placenta. Moreover, caffeic acid and ferulic acid were not detected in cultured immobilized cells. In contrast, immobilized placenta gave an over 10-fold higher level of vanillylamine and 124 μg of ferulic acid. The contents of capsaicin and dihydrocapsaicin were 30- and 22.6-fold higher in placenta

Table III. Profile of Intermediates of Capsaicin Biosynthetic Pathway in Pericarp and Placental Tissue[a]

compound	pericarp, μg/fruit	placental tissue, μg/g of fresh wt
vanillylamine	ND[b]	100 (±6.16)
L-phenylalanine	ND	910.76 (±30.5)
caffeic acid	ND	5.12 (±0.59)
coumaric acid	ND	1.36 (±0.21)
ferulic acid	ND	85 (±6.6)
cinnamic acid	ND	22.6 (±2.1)
capsaicin	502 (±21.28)	2161.8 (±106.23)
dihydrocapsaicin	620 (±25.65)	1584.92 (±87.1)

[a] Data represent an average of five replicates (±SD). [b] ND, not detected.

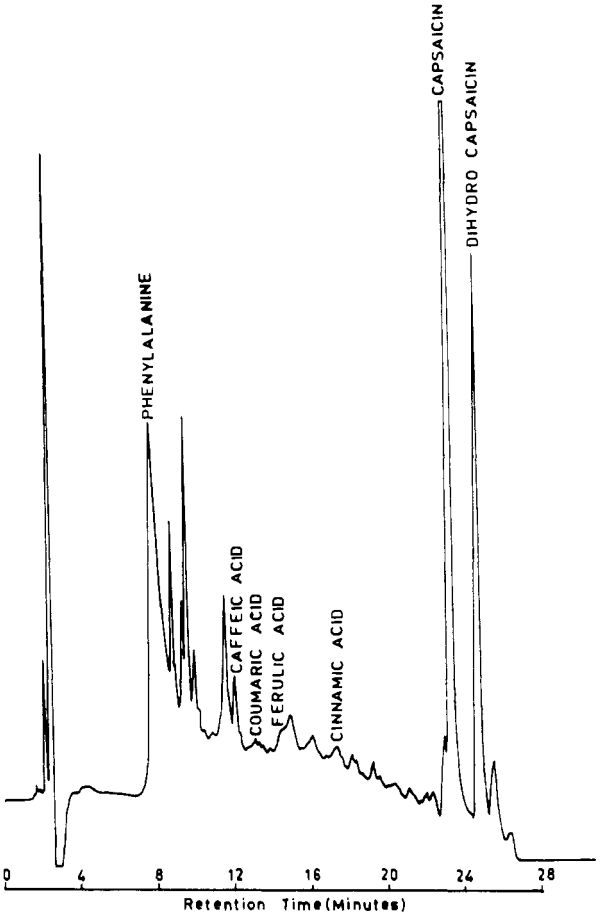

Figure 2. HPLC profile of 3-week-old placental tissue isolated from fresh fruits of *C. frutescens* and extracted in 80% ethanol. HPLC protocol was as in Figure 1.

than in immobilized cells. These results suggest that placental tissue has a high conversion of L-phenylalanine,

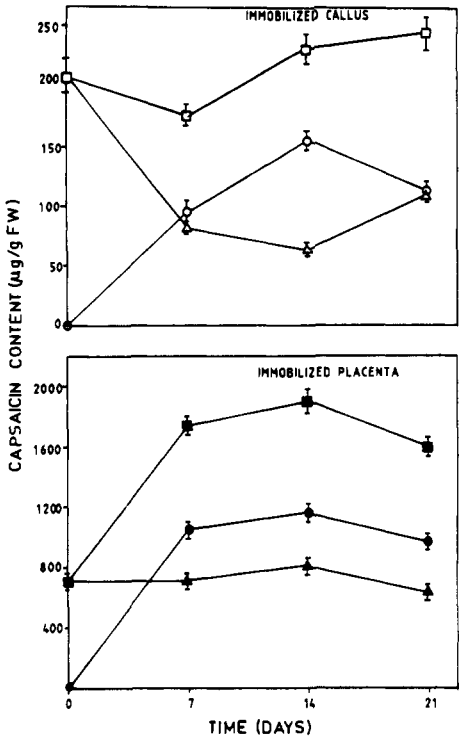

Figure 3. Time course study of capsaicin production in immobilized callus and placental tissues of *C. frutescens*. (△) Capsaicin content in 1 g of immobilized callus; (○) capsaicin content in 40 mL of medium; (□) total capsaicin per culture, i.e., beads + medium; (▲) capsaicin content in 1 g of immobilized placental tissues; (●) capsaicin content in 40 mL of medium; (■) total capsaicin per culture, i. e., beads + medium. Data represent an average of five replicates. Bars indicate ± SD.

caffeic acid, coumaric acid, and cinnamic acid to capsaicin moieties as reflected in higher amounts of capsaicinoids and other intermediates. However, high vanillylamine content in immobilized placenta shows that it may not limit the capsaicinoid production. The immobilized cells also show the presence of vanillylamine, which shows that both systems are active in producing this intermediate. In general, the immobilized placenta would have an efficient bioconversion process for biotransforming the intermediates of the capsaicinoid pathway, thereby resulting in higher production of capsaicin and dihydrocapsaicin.

The HPLC procedure also facilitated the analysis of placental tissue of fruits and pericarp to distinguish the distribution of the various intermediates of capsaicin and capsaicinoids. It was evident that the intermediates of capsaicinoids were not detected in pericarp tissues (Table III). Moreover, the capsaicinoid profiles were also higher in placenta than in pericarp in 3-week-old fruits as also reported by Iwai et al. (1979). These data suggest that eventually capsaicinoids may be translocated from the placenta to the pericarp with the advancement of fruit growth.

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Registry No. Vanillylamine, 1196-92-5; L-phenylalanine, 63-91-2; caffeic acid, 331-39-5; coumaric acid, 25429-38-3; ferulic acid, 1135-24-6; cinnamic acid, 621-82-9; capsaicin, 404-86-4; dihydrocapsaicin, 19408-84-5.