JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Specific Synthesis of 5,5′-Dicapsaicin by Cell Suspension Cultures of *Capsicum annuum* Var. *annuum* (Chili Jalapeño Chigol) and Their Soluble and NaCl-Extracted Cell Wall Protein Fractions

VÍCTOR M. MARTÍNEZ-JUÁREZ,[†] NEFTALI OCHOA-ALEJO,[‡] EDMUNDO LOZOYA-GLORIA,[‡] MARÍA L. VILLARREAL-ORTEGA,[#] ARMANDO ARIZA-CASTOLO,[§] FERNANDO J. ESPARZA-GARCÍA,[†] AND GRACIANO CALVA-CALVA*,[†]

Departamento de Biotecnología y Bioingeniería y Departamento de Química, Centro de Investigación y de Estudios Avanzados del IPN, Avenia IPN 2508, C.P. 07360 México D.F., Mexico; Departamento de Ingeniería Genética de Plantas, Unidad de Biotecnología e Ingeniería Genética de Plantas, Centro de Investigación y de Estudios Avanzados del IPN, Kilómetro 9.6 Libramiento Norte Carretera Irapuato-León, Apartado Postal 629, 36500 Irapuato, Guanajuato, Mexico; and Centro de Investigación en Biotecnología, Universidad Autónoma del Estado de Morelos, Avenida Universidad 1001, Col. Chamilpa, C.P. 62210 Cuernavaca, Morelos, Mexico

HPLC-UV, ^{1}H NMR, ^{13}C NMR, and ^{1}H−^{1}H COSY analyses revealed that exogenous capsaicin was specifically converted into 5,5′-dicapsaicin by both cell suspension cultures of *Capsicum annuum* var. *annuum* (chili Jalapeño chigol) and their soluble and NaCl-extracted cell wall protein fractions under oxidative conditions. In cell suspension cultures 5,5′-dicapsaicin was found only in biomass of capsaicin-fed cultures. This compound has not been detected before either in fresh fruits or in in vitro cultures of *Capsicum.* The transformation of capsaicin by different protein fractions revealed that most of the enzymatic activity was located in the NaCl-extracted, or ionic cell wall bound, protein, and that it was strictly dependent on H_2O_2. These results might in part explain some previously described features of capsaicin production by in vitro cultures of *Capsicum.* The implications of the results regarding the catabolism of capsaicinoids are discussed.

KEYWORDS: Capsaicin; 5,5′-dicapsaicin; capsaicinoids; *Capsicum*; chili pepper; capsaicin catabolism; capsaicin degradation; 5,5′-dicapsaicin NMR

INTRODUCTION

Capsaicin is the major component of capsaicinoids, the pungent principles from hot chili pepper fruits (*Capsicum* spp.). Although considerable progress has been made in understanding the biosynthesis of capsaicinoids, the last steps of the metabolic pathway, especially turnover and degradation, are not well-known. Capsaicin is the amide of vanillylamine and 8-methyl-*trans*-6-nonenoic acid (*1*, *2*). The vanillylamine moiety of capsaicin is biosynthetically derived from L-phenylalanine, whereas the branched fatty acid moiety is derived from valine (*3*, *4*). The accumulation and stability of capsaicinoids, in both fruits (*5*−*9*) and cell suspension cultures (*5*, *7*, *10*) of *Capsicum*, have been associated with a particular development stage of fruits and cell cultures. Like most natural products, capsaicinoids accumulate in fruits and in vitro cell cultures and later undergo rapid declination during the fruit or cell culture senescence. The fate of capsaicinoids after declination is uncertain. For example, in previous work (*7*, *10*), feeding putative precursors and precursor analogues of capsaicin and capsaicin itself to both isolated placental epidermal strips and cell suspension cultures of *Capsicum* resulted in the transformation of the fed material into a capsaicin glycoside and other unidentified compounds. On the other hand, it has been suggested that peroxidases from both fruit (*11*−*13*) and cell suspension cultures making capsaicinoids (*14*) might participate in capsaicinoid turnover or catabolism. Although it has been reported that H_2O_2-dependent peroxidases from hot pepper fruits transformed capsaicin into 4-*O*-5′-dicapsaicin ether and 5,5′-dicapsaicin (*15*), these dimers have not been detected in hot *Capsicum* fruits or in in vitro cultures. Regularly, plant peroxidases hold a variety of physiological functions, in particular in secondary cell wall lignification, surface protection, and metabolism of hormone and

* Author to whom correspondence should be addressed [telephone (55) 5061 3800, ext. 4348; fax (55) 5061 3313; e-mail gcalva@mail.cinvestav.mx].
[†] Biotecnología y Bioingeniería, CINVESTAV-México.
[‡] Ingeniería Genética de Plantas, CINVESTAV-Irapuato.
[#] Centro de Biotecnología, UAEM.
[§] Química, CINVESTAV-México.

10.1021/jf035214p CCC: $27.50
Published on Web 01/29/2004

alkaloids (*11*, *16*, *17*), which might be related to their lack of substrate specificity in vitro (*18*). These previous results encouraged us to study, by feeding cell suspension cultures with capsaicin and precursor analogues, the capsaicinoid catabolism to gain some insight in the reactions postulated as part of both capsaicinoid biosynthesis and turnover or degradation. Here, results are presented that demonstrate how capsaicin is specifically transformed by both the soluble and the NaCl-extracted cell wall protein fraction from cell cultures of *Capsicum* fed with capsaicin. The implications of these results regarding the capsaicinoid catabolism are discussed.

MATERIALS AND METHODS

Plant Cell Cultures. Seeds of *Capsicum annuum* var. *annuum* (chili Jalapeño Chigol) were surface sterilized as detailed previously (*19*). Callus tissue was initiated from hypocotyl explants on Murashige and Skoog (MS) medium (*20*) supplemented with 12.5 μM 2,4-dichlorophenoxyacetic acid, 0.5 μM kinetin, 30 g/L sucrose, and 2 g/L Gelrite (Phytagel, Sigma Chemical Co.). Cell suspension cultures were prepared by transferring portions of 3 g of fresh and friable callus tissue in 50 mL of MS liquid medium contained in 250 mL Erlenmeyer flasks and supplemented as above but without Gelrite. These cell suspension cultures were incubated on an orbital shaker (1 in. orbit diameter) at 90−100 rpm, 25 ± 2 °C, under continuous illumination (5400 lx; Cool White fluorescent tubes) and maintained by subculture at intervals of 15−21 days.

Capsaicin Feeding Cultures. For capsaicin feeding studies a stock solution of capsaicin (66 mM) in ethanol was prepared and filter-sterilized through 0.22 μm pore sterile Acrodisc filters (Gelman Sciences Co., Northampton, U.K.). Capsaicin was added to 50 mL of fresh culture medium to give a final concentration of 66 μM immediately before inoculation with 3 g of fresh weight of cells from suspension cultures in the exponential phase of growth (15 days of culture). The appropriate concentration of capsaicin was defined on the basis of our previous results (*7*, *10*).

Extraction of Phenolics and Capsaicinoids. The cells and medium from a complete flask culture (50 mL/flask) were separated by vacuum filtration through Whatman no. 41 filter paper. The biomass was washed three times with cold water, ground under liquid N_2, and extracted for 1 h with methanol. The methanolic extract was filtered through Whatman no. 41 filter paper and concentrated to 300 μL for analysis of the capsaicinoid and phenolic compounds profile by HPLC. On the other hand, 20 mL of free cell medium was extracted three times with ethyl acetate 1:1 (v/v). The organic fractions were pooled and concentrated using a rotary evaporator and redissolved in 300 μL of methanol for HPLC analysis. All data represent averages of at least three independent cultures.

HPLC-UV Analysis of Phenolics and Capsaicinoids. The HPLC system, from Thermo Separation Products Inc., comprised a Spectra System P2000 binary pump, a Spectra System AS1000 autosampler fitted with a 20 μL loop, and a Spectra System UV−visible optical scanning detector set at high-speed mode. The detector signals were captured and processed by the PC1000 System Software (Thermo Separation Products Inc.) loaded in an IBM 300PL personal computer. This software included programs for system setup, control and monitoring of running samples, and qualitative and quantitative analyses (integration functions). Separation of capsaicinoids and phenolics in the samples was achieved using a Prodigy ODS2 5 μm column (250 mm × 4.6 mm i.d.) from Phenomenex (Cheshire, U.K.). Elution was performed using a gradient of 1 mM trifluoroacetic acid (TFA) and acetonitrile (ACN), as follows [time (min), 1 mM TFA (%)]: 0, 90; 5, 90; 10, 80; 25, 80; 35, 40; 55, 40; 60, 10; 65, 10; 70, 90; 80, 90. The flow rate was maintained at 1 mL/min, and the detector was fixed at 230, 260, or 280 nm. The absorption spectra of the peaks were recorded between 200 and 365 nm at a rate of 10 spectra per second. The integration was set at 280, 260, and/or 230 nm as required. For capsaicin transformation products, showing a spectral pattern similar to that of capsaicinoids, the concentration was estimated on the basis of spectral equivalence with capsaicin. Under these conditions, although different for each compound, the detection limit was ~65 pmol/injection, which for capsaicin corresponds to 20 ng and for cinnamic acid, 9 ng.

Preparation of the NaCl-Extracted Cell Wall Protein Fraction. This protein fraction, also referred to as the ionically bound cell wall protein, was extracted from fresh cell biomass of cell suspension cultures of *Capsicum* as described by Church and Galston (*21*), with some modifications. Fresh biomass from cell cultures was washed by repeated resuspension in 1:5 (w/v) 25 mM Tris-HCl buffer, pH 7.0, until no peroxidase (POX) activity versus 80 mM guaiacol/H_2O_2 in Tris-HCl buffer (50 mM, pH 7.0) was detected in the filtered buffer. To release the NaCl-extracting cell wall protein fraction, the washed biomass was centrifuged at 8000 rpm for 10 min and resuspended in 3 parts (w/v) of extraction buffer consisting of 50 mM Tris-HCl, pH 7.0, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 1 M NaCl, 5% glycerol, and 1 mM dithiothreitol (DTT). After 2 h of incubation at 4 °C, the mixture was centrifuged at 8000 rpm for 30 min, and the supernatant, designated the NaCl-extracted cell wall protein fraction, was collected. Protein content was estimated according to the method of Bradford (*22*) with bovine serum albumin (BSA) as standard protein. The regression equation for data from 0 to 12.5 μg of BSA/mL was $Y = 0.047X + 0.015$ ($n = 10$, $r^2 = 0.98$).

Assay for Transformation of Capsaicin by Protein Extracts from Cell Suspension Cultures. Capsaicin (600 μM final concentration) was used as substrate, and the assay was performed at 30 °C in 1 mL of reaction mixture containing 50 mM Tris-HCl, pH 7.0, and 19 mM H_2O_2. The reaction was started by the addition of 100 μg of protein and stopped after 75 min, unless otherwise indicated, by extraction twice with ethyl acetate (1:1, v/v). The organic extracts were combined and evaporated under a stream of oxygen-free N_2 and further processed and analyzed by HPLC-UV as described before for capsaicinoids and phenolic compounds.

Purification of the Capsaicin Transformation Product X. The separation and analysis of the capsaicin transformation product **X** was achieved by HPLC in 70 repeated runs as indicated above. After collection, the fractions were pooled (≈70 mL) and extracted with ethyl acetate (1:1, v/v). The solvent was dried under a stream of oxygen-free nitrogen and redissolved in 5 mL of methanol, and the purity of the fractions was verified by HPLC-UV. When the HPLC chromatogram showed more than one peak, the fraction was further refined using the same protocol. Afterward, identification and structural analysis of the capsaicin transformation compound (named **X**) from the capsaicin-feeding experiments were performed by a combination of spectral and chromatographic techniques.

NMR and COSY Analyses. Samples of the collected compound **X** (1 mL) and standard capsaicin were further analyzed by ^{1}H NMR, ^{13}C NMR, and ^{1}H−^{1}H COSY. The ^{1}H and ^{1}H−^{1}H COSY spectra were recorded at 300.13 MHz in a Bruker 300 AVANCE spectrometer equipped with a 5 mm multinuclear probe, using $CDCl_3$. The chemical shifts are referenced to internal $(CH_3)_4Si$ ($\delta^1H = 0$, $\delta^{13}C = 0$). The ^{1}H parameter was adjusted to have a resolution better than 0.2 Hz. The connectivity was established by 2D ^{1}H−^{1}H correlation spectroscopy, COSY-45, spectrum (spectral width = 2600 Hz, 1024 data points in F2 and 128 in F1, eight scans were used). The ^{13}C proton decoupling NMR spectrum was recorded at 67.94 MHz with a JEOL 270 GSX-DELTA spectrometer equipped with a 5 mm multinuclear probe using the $CDCl_3$ solution. The total detection time was 8 h.

GC-MS Analysis. The collected compound **X** and standard capsaicin were analyzed by GC-MS using a CG-TRIO-1S mass spectrometer (VG Masslab, Manchester, U.K.) with an EI mass selective detector (Hewlett-Packard) linked directly to an HP-5890 GC equipped with an OV column.

Statistical Analyses. To estimate the coefficients of the linear equations that best predicted the value for protein and metabolites in cell cultures, the routine for linear regression analysis of the statistical package SPSS was used (*23*).

RESULTS AND DISCUSSION

Growth of Cell Suspension Cultures in the Presence and Absence of Capsaicin. Cells of *C. annuum* var. *annuum* were cultured in 250 mL conical flasks containing 50 mL of medium

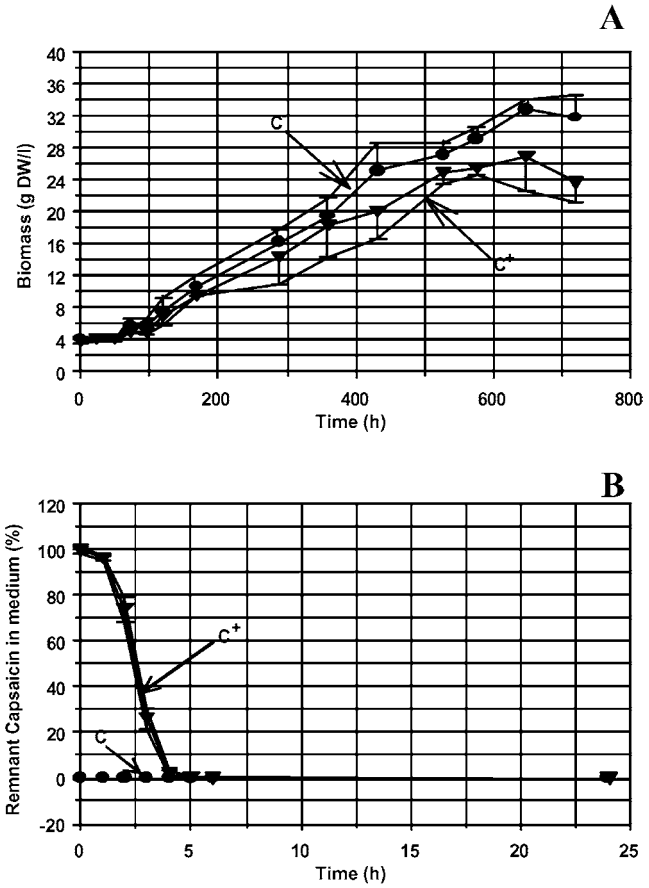


Figure 1. Time course of growth (**A**) and disappearance of capsaicin (**B**) from the medium of cell suspension cultures of *C. annuum* var. *annuum* fed (C^+) and not fed (C^-) with capsaicin 66 μM. Capsaicinoids were not detected at any time in cell cultures not fed with capsaicin. Error bars represent the standard deviation of three cultures.

culture supplied (C^+) and not supplied (C^-) with capsaicin at a final concentration of 66 μM. The presence of capsaicin resulted in a slight phytostatic effect in comparison with nontreated cultures (**Figure 1A**). Both cultures showed a lag phase of 3−4 days, when exogenous capsaicin disappeared from the culture medium (**Figure 1B**), followed by a similar exponential growth phase for 14−16 days before entering at the stationary phase. The exponential increase in dry weight per unit volume was sustained at a specific growth rate for both cultures, based on cell dry weight, in the range of 0.2−0.3 day^{-1}. However, maximum biomass productions, although achieved after 28 days for both cultures, were 25.5 ± 0.7 g of dry weight/L for capsaicin-treated (C^+) cultures and 29.1 ± 1.6 g of dry weight/L for non-capsaicin-treated (C^-) cultures. Similar values for the specific growth rate and growth pattern have been observed for other *Capsicum* varieties in several studies (*5*, *7*, *24*). The specific growth rate of cells during the exponential growth phase of batch cell cultures is usually constant and determined by the biochemical characteristic of the cells and the environmental and nutritional factors (*25*). Thus, although the similarity in the specific growth rate and growth pattern for both C^+ and C^- cultures represents a complex situation to identify how capsaicin affected the cell metabolism and therefore the final biomass production, the results confirmed that these cultures were competent to grow in the presence of exogenous capsaicin as previously reported (*7*, *10*).

Fate of Capsaicin. In a previous work we reported that exogenous capsaicin was converted into several capsaicin glycosides by cell suspension cultures of *Capsicum chinense*

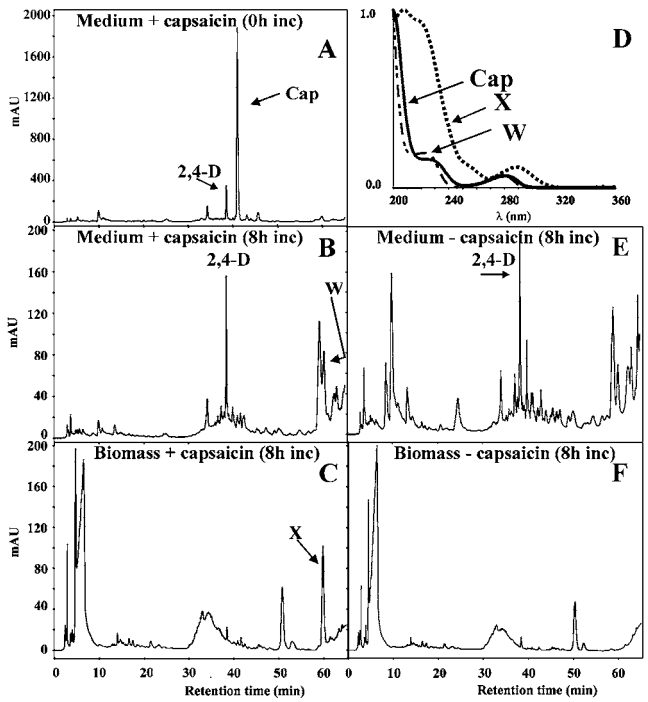


Figure 2. HPLC 230 nm chromatograms of phenolic extracts from the culture medium (**A**, **B**, **E**) and from biomass (**C**, **F**) of cell suspension cultures fed with 66 μM capsaicin (**A**, **B**, **C**), at time zero (**A**) and after 8 h of incubation (**B**, **C**), in relation to the 8 h capsaicin-untreated cell cultures (**E**, **F**). The spectral pattern of the major capsaicin-related compound (**X**) is shown in panel **D**. Data represent extracts from a whole flask chosen from three cultures. Cap, capsaicin; 2,4-D, 2,4-dichlorophenoxyacetic acid; W, unknown compound.

able to synthesize capsaicin (*10*). In contrast, regardless of the age of the cultures, in this study capsaicinoids were never detected in cultures not fed with capsaicin (**Figure 1**). Hence, because of the sensitivity of the analytical method, the capsaicin content in control cultures must have been <2 pmol/culture (0.28 ng/culture). In contrast, when exogenous capsaicin was supplied to these cultures, it disappeared very rapidly from the culture medium (**Figure 1B**). Usually, the maximum rate uptake of capsaicin occurred during the first 5 h. After 3−4 h, <40% of capsaicin remained in the medium, and no capsaicin was recovered from either the biomass or medium after 8 h of incubation. As reported for the accumulation pattern of other natural compounds produced by plant cell suspension cultures (*26*−*28*), the high rate of this disappearance at very early steps of culture might be related to a rapid uptake of capsaicin by the cells and/or to its conversion into other compounds. In fact, the HPLC-UV analysis for capsaicinoids and phenolics in extracts from biomass and culture medium of capsaicin-treated cultures (**Figure 2A**−**C**) revealed the presence of an unknown compound eluting at 59 min (peak X) in the methanolic extracts from biomass after 8 h of incubation (**Figure 2C**). This novel peak X was never detected in capsaicin-untreated cultures (**Figure 2E**,**F**), despite the age of the culture. Although the chromatograms for extracts from culture medium of both capsaicin-fed (C^+) (**Figure 2B**) and capsaicin unfed (C^-) (**Figure 2E**) cell cultures also showed peaks (W) at a similar retention time as **X** from biomass of the C^+ cultures, the spectral pattern was different from that for **X**. Because **X** eluted latter than capsaicin and its HPLC-UV spectrum closely resembled that from capsaicin but with a red shift of 10 nm (**Figure 2D**), it was predicted that capsaicin should have been transformed into this less polar novel compound. However, these results

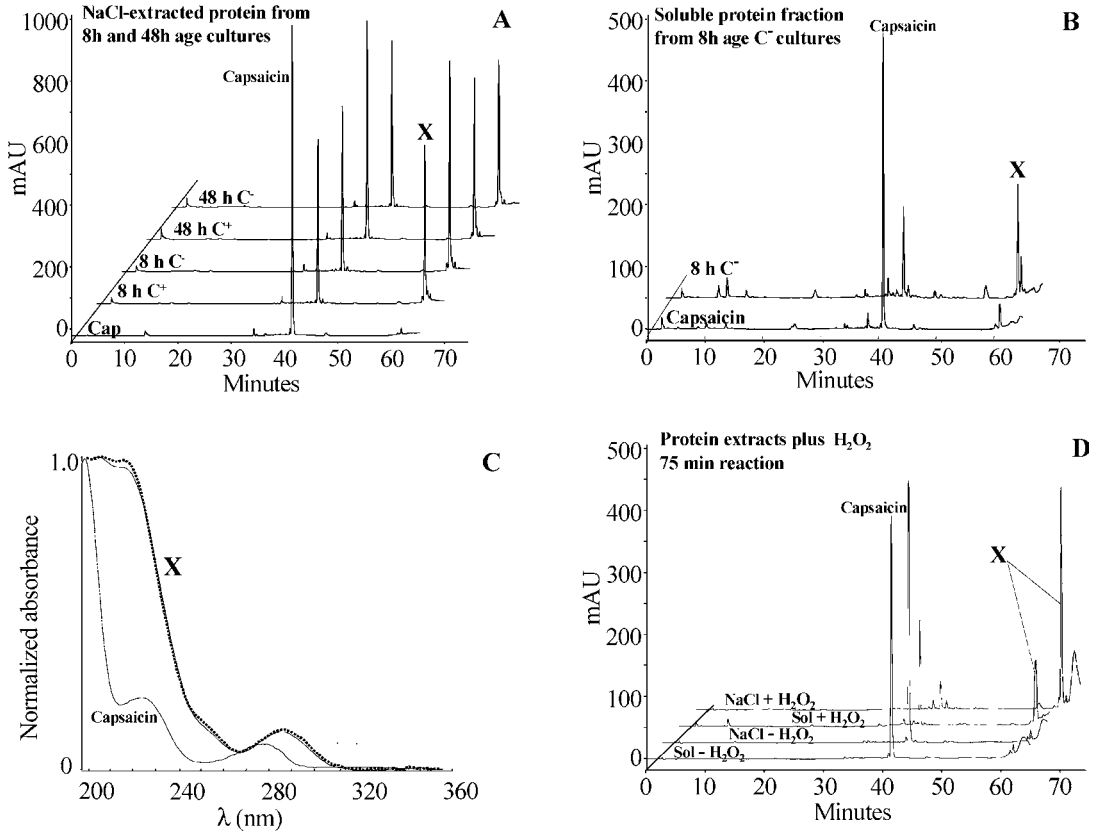


Figure 3. HPLC 230 nm chromatograms of extracts from enzymatic transformation reaction of capsaicin by the ionic cell wall-bounded or NaCl-extracted (**A**, **D**) and the soluble (**B**, **D**) protein fractions from capsaicin-untreated (C^-) and capsaicin-treated (C^+) cell suspension cultures after 8 and 48 h of growth under such conditions. The reaction mixtures were incubated for 75 min. Chromatograms in panel **D** show that the capsaicin transformation product **X** was not produced when H_2O_2 was lacking in the enzymatic reaction mixture. The spectral pattern of X (**C**) was identical with that shown in **Figure 2**.

contrast with our previous study in which exogenous capsaicin was conjugated by cell suspension cultures of *C. chinense* into a more polar glycoside (*10*) and with those of Lindsey (*29*) and Yeoman et al. (*30*), who found that cell suspension cultures of *Capsicum frutescens* apparently had no action on exogenous capsaicin. On the other hand, as pointed out before, several studies have suggested that capsaicinoids may be transformed into dimeric compounds by peroxidases from both fruits (*11*−*13*) and cell suspension cultures making capsaicinoids (*14*). Similar dimeric compounds of capsaicinoids have been isolated from air-dried powdered fruits of *Capsicum* (*31*). Also, 5,5′-dicapsaicin was putatively identified as the unique oxidation product of capsaicin when used as antioxidant of linoleic acid in the presence of the radical initiator 2,2′-azobis(isobutyronitrile) (*32*) and in the photochemical oxidation of capsaicin in aqueous solution (*33*). As observed for compound **X**, the UV spectral pattern of these reported dimeric compounds was similar to that of capsaicin (*11*, *12*), and they were also less polar than capsaicin (*15*, *32*). To our knowledge, none of these compounds has been detected either in fresh fruits or in in vitro cultures of *Capsicum*, even in capsaicin-feeding studies (*30*, *34*, *35*). Therefore, the structure of **X**, and whether this compound was produced from capsaicin by the action of protein extracts from cell suspension cultures under oxidative conditions, were further investigated.

Transformation of Capsaicin by Protein Extracts from Cell Suspension Cultures. The formation of compound **X** by the cell suspension cultures might have arisen by the action of a diversity of enzymes over the exogenous capsaicin. However, because several studies suggest that peroxidases might participate in the capsaicinoid catabolism in *Capsicum* fruits (*9*, *12*, *13*, *15*), the ability of protein extracts from biomass of these cell suspension cultures to transform capsaicin into compound **X** under oxidative conditions was investigated. The soluble protein and the NaCl-extracted protein from the cell wall, which contains the ionic cell wall-bound protein (*21*), from both capsaicin-treated and capsaicin-untreated cell suspension cultures grown for 8 and 48 h were tested (**Figure 3**). Surprisingly, the HPLC-UV analyses revealed that, in contrast with the reports from the literature (*15*), capsaicin was transformed exclusively into compound **X** previously detected in the biomass of cell suspension cultures fed with capsaicin (**Figure 2C**), by both the soluble and the NaCl-extracted protein (**Figure 3A**,**B**). The ability of the extracts of protein to transform capsaicin into **X** was independent of the age of the cultures and the pretreatment with capsaicin (**Figure 3A**). After 75 min of incubation, the percent of remaining capsaicin in the enzymatic reaction mixture containing the ionic cell wall protein fraction was 5 ± 1.8%, and the yield of the oxidation product **X**, evaluated on the basis of the HPLC capsaicin response factor at 230 nm, was 95 ± 5.8%.

As reported in the literature for the enzymatic oxidation of capsaicin (*15*) and dihydrocapsaicin (*12*) by *Capsicum* peroxidases, the transformation of capsaicin by the protein extracts was strictly dependent on the presence of H_2O_2 (**Figure 3D**). However, in such studies the enzymatic reaction products for capsaicin were 5,5′-dicapsaicin, 4′-*O*-5-dicapsaicin ether, and some dehydrogenated unknown polymers. Thus, although our results at this point were uncertain, it was predicted that the enzyme responsible for the conversion of capsaicin might be a

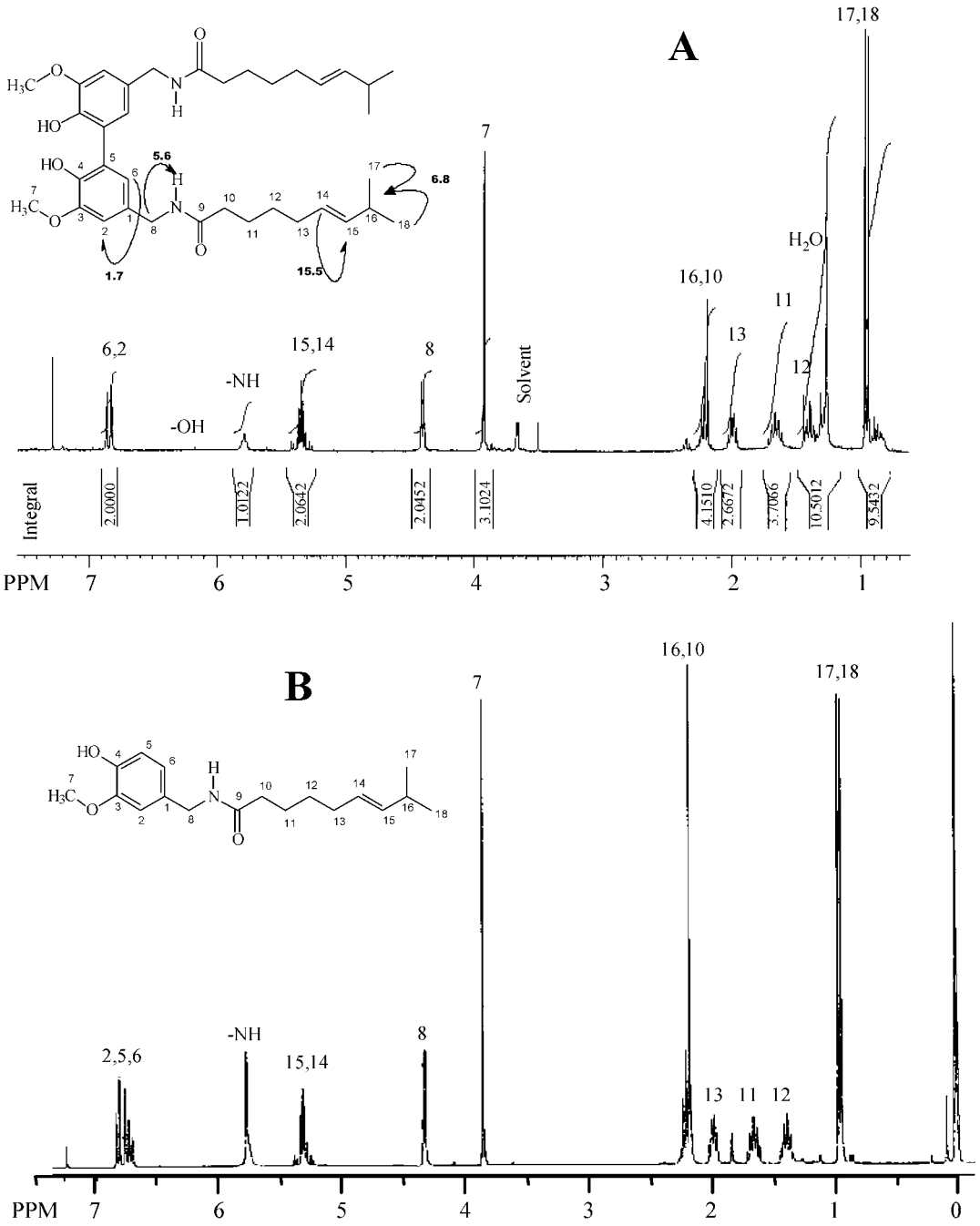


Figure 4. ^{1}H NMR spectra of the capsaicin enzymatic oxidation product **X** (**A**) and standard capsaicin (**B**). The signals at δ 3.5 and 3.6 in panel **A** correspond to the solvent used for sample preparation. The spectra were recorded at 300.13 MHz with a Bruker 300 AVANCE spectrometer equipped with a 5 mm multinuclear probe, using $CDCl_3$. The chemical shifts are referenced to internal $(CH_3)_4Si$ ($\delta^1H = 0$, $\delta^{13}C = 0$).

specific constitutive peroxidase present in the cultures that do not accumulate capsaicinoids. To explore this hypothesis and to understand the capsaicin enzymatic transformation reaction, a sufficient quantity of compound **X** (≈10 mg) from the HPLC column was collected and its structure investigated.

Identification of the Capsaicin Transformation Product. The capsaicin transformation product **X** was purified by HPLC and then analyzed by ^{1}H, ^{13}C NMR, ^{1}H−^{1}H COSY, and GC-MS. The ^{1}H NMR spectral pattern revealed similar resonances for **X** and capsaicin, except at the aromatic region, from δ 6.5 to 7.0 (**Figure 4**). The aromatic protons of the capsaicin spectrum showed three signals, a singlet and two doublets (**Figure 4B**), whereas the spectrum for compound **X** showed only two double resonances in the same region (**Figure 4A**). This observation suggests that only the aromatic ring was involved in the transformation of capsaicin into compound **X**. The extra peaks observed in the aliphatic region of the ^{1}H NMR spectrum were better analyzed by a ^{1}H−^{1}H COSY experiment to find the connectivities between carbons (**Figure 5**). The aromatic protons (2 and 6) provided an isolated spin system, but the COSY analysis of the remainder of the spectrum in **Figure 5** provided the peak assignment and confirms the structure suggested for compound **X** in **Table 1**. The results from the connectivities (**Table 1**) revealed that **X** has an alkyl moiety as capsaicin; that is, **X** should keep intact the alkyl moiety present in capsaicin. Thus, the extra peaks in the aliphatic region must come from impurities in the sample, probably from the solvents used during the purification process. Furthermore, the ^{1}H,^{1}H coupling constant $^4J_{HH} = 1.7$ Hz for the aromatic portion of **X** observed in the signals at δ 6.83 and 6.86 revealed

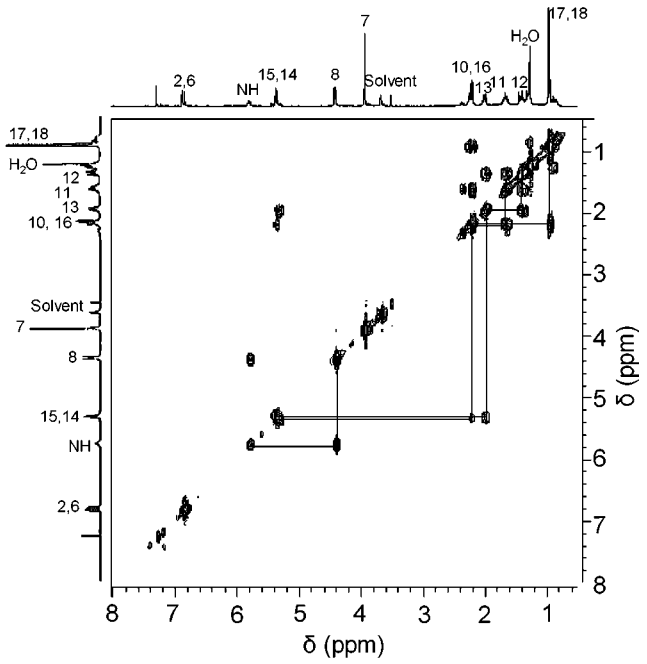
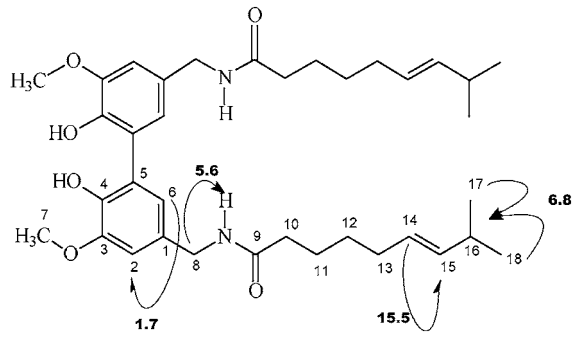


Figure 5. 300 MHz ^{1}H–^{1}H COSY spectrum of compound **X** with connectivities of the NH and aliphatic protons.

Table 1. ^{1}H and ^{13}C NMR Data of Compound **X** in $CDCl_3$



	δ ^{13}C	δ ^{1}H	$^nJ_{HH}$ (Hz)	connectivities by COSY
1	130.35			
2	110.27	6.83	1.7 (d)	H2–H6
3	147.91			
4	142.18			
5	123.98			
6	122.80	6.86	1.7 (d)	H2–H6
7	56.23	3.93	(s)	
8	43.63	4.40	5.6 (d)	H8–NH
9	172.94			
10	36.78	2.19	7.0 (t)	H10–H11
11	29.38	1.66	7.0 (tt)	H10–H11, H11–H12
12	29.77	1.37	7.0 (tt)	H11–H12, H12–H13
13	32.32	1.98	7.0 (dt)	H12–H13, H13–H14
14	126.54	5.34	6.8; 15.5 (dt)	H13–H14, H14–H15
15	138.14	5.37	15.5; 6.8 (dt)	H14–H15, H15–H16
16	31.04	2.21	6.8 (m)	H15–H16, H16–H17, H17–H18
17	22.72	0.94	6.8 (d)	H16–H17
18	22.72	0.94	6.8 (d)	H16–H18
OH		6.17	(s)	
NH		5.79	5.6 (t, br)	H8–NH

that these are in the meta position and coupled to each other, whereas the vinyl proton shown at $^3J_{HH} = 15.5$ Hz correspond to a trans position. These data support the structure of *N*,*N*′-(5,5′-bis-4-hydroxy-3-methoxybenzyl)di-8-methyl-non-(*E*)-6-enamida or 5,5′-dicapsaicin for **X** (**Table 1**). The ^{13}C NMR data from the spectrum (**Table 1**) were in good agreement with the proposed structure, and it was confirmed on the basis of the substituent chemical shift (*36*). The structure was also confirmed by analysis of the mass spectral analysis of **X** on the basis of the theoretical fragmentation pattern of 5,5′-dicapsaicin. The mass spectrum showed the molecular ion at *m*/*z* 608 and exhibited fragment ions at *m*/*z* (% abundance) 455 (7.2), 440 (2.9), 302 (3.2), 287.3 (100), 272 (46.7), 168 (13.9), 153 (8.6), and 136 (13.1). The MS data also showed a fragment of 168, indicating the presence of the fatty acid moiety bonded to the amine group. Therefore, these data confirm a molecular formula of $C_{36}H_{52}N_2O_6$ for **X**, which corresponds to two molecules of capsaicin minus two hydrogen atoms. These results agree with the data and structure for the dimer of capsaicin reported for the photochemical oxidation product of capsaicin in aqueous solution (*33*) and the autoxidation product of capsaicin initiated by 2,2-azobisisobutyronitrile (*32*). In the former reference (*33*), the assignment of the NMR signals was based on NOE experiments and 2D spectra (^{1}H−^{13}C COSY, ^{1}H−^{13}C long-range COSY, and ^{1}H−^{1}H COSY). Our results were in good agreement with that study and pointed out that the transformation product of capsaicin was 5,5′-dicapsacin.

The ^{13}C spectrum was recorded at 67.94 MHz in a single-pulse broadband proton-decoupling method with a JEOL 270 GSX Delta spectrometer. δ, chemical shift, is reported in parts per million. Multiplicities are in parentheses [(s) singlet; (d) doublet; (t) triplet; (m) multiple; and (br) broad signal].

Thus, as for the photochemical and autoxidation of capsaicin, but in contrast with the transformation of capsaicin by peroxidases (*15*), in the present study the oxidation product of capsaicin was exclusively 5,5′-dicapsaicin. To our knowledge, this is the first report of this dimeric compound being detected in in vitro cultures of *Capsicum.* Because the conversion of capsaicin was performed by both cell suspension cultures of *C. annuum* var. *annuum* and their soluble and NaCl-extracted protein, the challenge now is to isolate and characterize the enzyme activity in vitro.

The capsaicin transformation product, 5,5′-dicapsaicin, was detected exclusively in the biomass from cell suspension cultures fed with capsaicin. These results diverge from those found for cell suspension cultures of *C. chinense* that transformed exogenous capsaicin into several capsaicin glycosides, and, like capsaicinoids, these glycosides were strictly extracellular (*7*, *10*). In the present work the capsaicin glycoside was never detected in the cell cultures. However, the decline of the exogenous capsaicin observed in the cell suspension cultures used in this work agrees with several reports for the variation pattern of capsaicinoids in both in vitro cultures (*7*, *37*, *38*) and *Capsicum* fruits (*3*, *7*, *39*). Such a pattern supports the hypothesis that the metabolism of capsaicinoids does not end with the biosynthesis and accumulation as pointed out by several authors (*3*, *5*, *40*−*42*), but they are further transformed or degraded. This might not be surprising because several studies to elucidate the role of natural products in plants have shown that these kinds of compounds are not metabolically inert products but are subject to turnover and degradation processes in the producing plant or cell culture (*43*, *44*). Indeed, it has been suggested that practically any natural compound from both plants and in vitro plant cell cultures may undergo turnover or degradation (*45*), and their net accumulation should be the result of a balance between these processes, which are species-specific (*43*−*45*). Thus, the differences between the results of this work and those that report capsaicinoids as stable end products (*3*, *5*, *40*−*42*) might be due to the characteristic variability of the biological systems (*46*). For example, it has been reported that cinnamic

acid was rapidly incorporated into the insoluble fraction of the cell wall in variable proportions: 25% in cell suspension cultures of *Phaseolus vulgaris* (*47*) and 83% in cell cultures of *C. frutescens* (*29*). At this moment, the final fate of capsaicin in the cell cultures remains uncertain. Because 5,5′-dicapsaicin was found only in the biomass, the target might be the plasmalemma, the cell wall, or it might remain in the intermembranal space for further degradation. This could be in agreement with the observations that capsisomes, the storage vesicles of capsaicinoids in *Capsicum* fruits, might melt with the plasmalemma of epidermal cells of placental tissue and then the capsaicinoids would spread to the lamellated walls (*48*).

The transformation of capsaicin with different protein fractions under oxidative conditions revealed that, although most of the enzymatic activity was located in the ionic cell wall bound protein (**Figure 2**), the soluble protein fraction also had the ability to oxidize capsaicin. Similar observations have been reported for the oxidation of 4-methoxy-α-naphthol by protein extracts with different peroxidase activities from *Capsicum* fruits (*49*); however, in that paper the major activity was found in the soluble fraction. Thus, capsaicin catabolism might be a compartment-dependent process and different kinds of enzymes might be involved in each compartment (*49*). From this, because in plant cell cultures capsaicinoids are released to the medium (*5*, *30*, *50*, *51*), it could be difficult for the transformation of capsaicin into 5,5′-dicapsaicin to take place in the culture medium. On the other hand, the formation of 5,5′-dicapsaicin by the enzymatic oxidation of capsaicin confirms that the antioxidant mechanism of capsaicin partially involves a hydrogen donation from the phenolic moiety as proposed in the literature (*52*). This finding also supports the hypothesis that capsaicinoids may naturally act as chain-braking antioxidants of fatty acids in scavenging lipid peroxyl radicals in fruits.

The high rate at which *Capsicum* cell suspension cultures take up capsaicin might in part explain some previously described features of capsaicin production in cultures; on some occasions capsaicin may be detected, whereas on other occasions it may not be (*5*, *30*, *37*, *50*, *51*). The detection of capsaicin, or indeed of any capsaicinoid in the cultures, should depend on the balance between biosynthesis and transformation.

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Received for review October 18, 2003. Revised manuscript received December 22, 2003. Accepted December 23, 2003. This work was supported by the Consejo Nacional de Ciencia y Tecnología (CONACyT México: 211085-5-29307B). V.M.M.-J. acknowledges a doctoral fellowship from CONACyT.

JF035214P