5,5′-Dicapsaicin, 4′-*O*-5-Dicapsaicin Ether, and Dehydrogenation Polymers with High Molecular Weights Are the Main Products of the Oxidation of Capsaicin by Peroxidase from Hot Pepper

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The nature of the products of capsaicin oxidation by hot pepper peroxidase was studied by gel permeation chromatography. Three main types of oxidation products were identified. Peak **1**, with an average molecular weight of 603 ± 12 and corresponding to dimers of capsaicin, was resolved by gas chromatography−mass spectrometry into the dimers 5,5′-dicapsaicin (**1a**) and 4′-*O*-5-dicapsaicin ether (**1b**). Peak **2** appears to be a polymeric product with a molecular weight of 4500 and probably corresponds to polymeric products with a mean degree of 15. Capsaicin−protein copolymers (peak **3**) also appear to be products of capsaicin oxidation, probably arising from protein and capsaicin copolymerization. These results suggest that *Capsicum* peroxidase oxidizes capsaicin to yield 5,5′-dicapsaicin and 4′-*O*-5-dicapsaicin ether, together a mixture of highly polymerized dehydrogenation products, all of them therefore of a lignin-like nature.

Keywords: *Capsaicin; capsaicin oxidation products; hot pepper; peroxidase*

INTRODUCTION

Capsaicin, the major pungent compound of hot pepper fruits (*Capsicum annuum* var. Annuum), is an amide derivative of vanillylamine and 8-methylnontrans-6-enoic acid (Leete and Louden, 1968; Bennett and Kirby, 1968). The vanillylamine moiety of capsaicin is biosynthetically derived from L-phenylalanine, while the branched fatty acid moiety is derived from valine (Leete and Louden, 1968; Bennett and Kirby, 1968; Iwai et al., 1979).

While considerable progress has been made in understanding the biosynthesis of capsaicin, the enzymology of the last steps of capsaicin metabolism and degradation is still not completely known. Feeding experiments with capsaicin precursors suggest that capsaicin biosynthesis in hot pepper fruits competes with an active accumulation of lignin-like substances in cell walls, which are probably derived from phenylpropanoid precursors and from capsaicin itself (Hall et al., 1987; Sukrasno and Yeoman, 1993). The reactions driving this competing sink are apparently mediated by peroxidase (EC 1.11.1.7) (Bernal et al., 1993a, 1995).

Arguments in favor of the participation of peroxidase in this metabolic pathway are based on the exclusive localization of peroxidase in placental epidermal cells from hot pepper fruits (Bernal et al., 1994b), and in the colocalization of peroxidase and capsaicin in vacuoles and cell walls of hot pepper cells (Bernal et al., 1993b), this evidence being further supported by the strong capsaicin-oxidizing activity of hot pepper peroxidase (Bernal et al., 1994a).

With this in mind, the present study was undertaken to investigate the products of capsaicin oxidation by peroxidases from *Capsicum* fruits. The results shown below illustrate that these products are 5,5′-dicapsaicin and 4′-*O*-5-dicapsaicin ether, together with a mixture of highly polymerized dehydrogenation products, all of them therefore of a lignin-like nature. A radical−radical coupling reaction mechanism to explain the appearance of these oxidation products is proposed.

MATERIALS AND METHODS

Plant Material. *C. annuum* var. Annuum fruits were obtained from a local market and stored at 4 °C until use.

Peroxidase Fraction. *Capsicum* fruits (40 g formula weight) were homogenized with a mortar and pestle in the presence of acetone at −20 °C. The homogenate was immediately filtered through one layer of filter paper at 4 °C in a Buchner funnel and the residue thoroughly washed with acetone at −20 °C until all pigments were removed. The protein precipitate was resuspended in 100 mL of 50 mM Tris [tris(hydroxymethyl)aminomethane]-HCl buffer (pH 7.5), containing 1 M KCl, and incubated with agitation for 1 h at 4 °C. The pepper protein solution thus obtained was clarified by centrifugation at 10000*g* for 30 min. The supernatant was used for the further purification of pepper peroxidase.

Purification of pepper peroxidase was performed by chromatography on phenyl-Sepharose CL-4B (Sottomayor et al., 1996). For this, the supernatant of the above centrifugation was concentrated with Aquacid I. $(NH_4)_2SO_4$ was added up to 1 M, and the protein was applied to a phenyl-Sepharose CL-4B column (50 × 1.5 cm). Bound proteins were eluted with a decreasing linear gradient of $(NH_4)_2SO_4$ [from 1.0 to 0.0 M $(NH_4)_2SO_4$ at a concentration change rate of 25 mM min^{-1} and with a flow rate of 2 mL min^{-1}] in 50 mM Tris-HCl (pH 7.5). Fractions of 8 mL were collected. All the fractions with peroxidase activity were pooled, concentrated with Aquacid I, and dialyzed overnight against 0.1 M Tris-acetate (pH 6.0). All procedures were carried out at 4 °C.

Spectrophotometric Assays. The spectrophotometric assay of capsaicin oxidation by *Capsicum* peroxidase was performed at 25 °C in a reaction medium containing 1 mM capsaicin (Sigma Chemical Co.) (from a 10 mM stock in methanol of HPLC grade), 0.1 mM H_2O_2, and 0.1 M Tris-acetate buffer (pH 6.0) (Bernal et al., 1993a). The reaction was initiated by the addition of enzyme. Capsaicin oxidation was monitored by increases in absorbance at 262 nm in a Uvikon 940 spectrophotometer (Kontron Instruments, Madrid, Spain), the oxidation rate being expressed in nanomoles per

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S0021-8561(95)00826-0 CCC: $12.00

second, for which a ϵ_{262} of 5.3×10^3 M^{-1} cm^{-1} was used for the oxidation products (Bernal et al., 1993a). One nanokatal of peroxidase was defined as the amount of protein that oxidized 1 nmol of substrate per second.

Oxidation of Capsaicin by Peroxidase. The oxidation of capsaicin by peroxidase was performed in a closed vessel containing 400 nkat of pepper peroxidase (corresponding to a catalytic activity against capsaicin of 0.4 μmol of capsaicin oxidized per second) in 25 mL of 0.1 M Tris-acetate buffer (pH 6.0). Capsaicin and H_2O_2 were added slowly up to a final concentration of 0.6 and 1.0 mM, respectively. After incubation for 24 h at 25 °C, the reaction was stopped by the addition of 25 mL of dimethylformamide (DMF). A control was carried out in the absence of H_2O_2.

Time Course of Peroxidase Inactivation during Capsaicin Oxidation. The peroxidase activity remaining in the reaction vessel was determined by removing samples at 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, and 24 h, peroxidase activity being determined with capsaicin as substrate (see above).

Fractionation of Capsaicin Oxidation Products by Gel Permeation Chromatography. The preliminary characterization of capsaicin oxidation products was performed by gel permeation chromatography using Sephadex LH-20 (Pharmacia) and DMF as eluent (Weymouth et al., 1993). For this, 5 mL of reaction medium, to which previously was added DMF up to 50% (v/v) (see above), was loaded on a Sephadex LH-20 column (1 × 50 cm) equilibrated with DMF. Fractions of 1.0 mL were collected. The UV absorbance (262 nm) of the eluate was monitored to detect the presence of capsaicin oxidation products.

Determination of Molecular Weights of Capsaicin Oxidation Products. For this, the Sephadex LH-20 column was calibrated using polystyrene standards with MWs of 12 860, 4075, 2500, and 687 (Aldrich Chemical Co.) and dihydrocapsaicin (MW = 307.4) (Sigma Chemical Co.). The apparent MWs of the capsaicin oxidation products were determined by comparison with the distribution coefficients (K_d) calculated for each of the standards.

Gas Chromatography−Mass Spectrometry (GC−MS) of Capsaicin Oxidation Products. GC−MS was performed on a 5993 Hewlett-Packard model coupled to a 5995 gas chromatograph/mass spectrophotometer and to a 2648A Graphics terminal, using a OW Chrompak 50 m × 0.20 mm inside diameter column, He pressure of 0.4 MPa, and a GC temperature program of 90−280 °C at 10 °C min^{-1}, with a 5 min hold at 280 °C, as previously described (Bernal et al., 1993a). TMS derivatives of capsaicin oxidation products for GC were prepared by reaction with Sigma-Sil-A (Sigma Chemical Co.) according to the procedure described by the manufacturer.

RESULTS AND DISCUSSION

Oxidation of Capsaicin by Pepper Peroxidase. The oxidation of capsaicin by a partially purified peroxidase from hot pepper was carried out in a closed vessel containing the enzyme (in excess), to which capsaicin and H_2O_2 were added. The reaction mixture was left for 24 h at 25 °C. This type of peroxidase-mediated dehydrogenation of phenolics (carried out by a one-off addition of reactants) is known as "bulk polymer" and is different from the "end-wise polymer" type (gradual addition of reactants), in which the degree of polymer formation is somewhat minor (Lai and Sarkanen, 1975; Weymouth et al., 1993).

In order to check whether the enzyme activity was limiting during the oxidative process, aliquots of the reaction medium were taken at several time intervals and peroxidase activity was measured. The results shown in Figure 1 illustrate that about 75% of the enzyme remains active after 24 h of incubation. In fact, only about 25% of the enzyme was inactivated in the course of the reaction, and this was mainly due to thermal inactivation, since similar results were found in the control carried out in the absence of H_2O_2 (Figure 1).

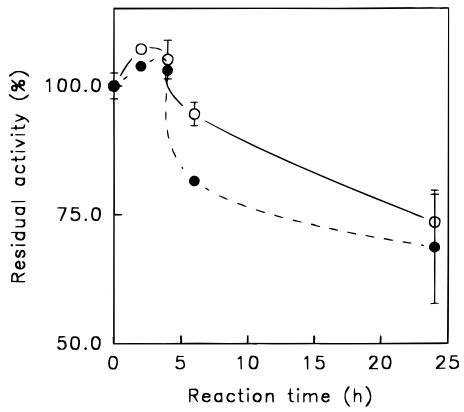


Figure 1. Time course of peroxidase inactivation during the oxidation of capsaicin by hot pepper peroxidase in the presence (●) and in the absence (○) of hydrogen peroxide.

These results are surprising since it is well-known that the oxidation of coniferyl alcohol (a phenolic compound structurally anologous to capsaicin) by peroxidase is accompanied by enzyme inactivation (Weymouth et al., 1993; Ferrer and Ros Barceló, 1994). In fact, in the course of coniferyl alcohol oxidation, total enzyme inactivation takes place in a few hours (Weymouth et al., 1993; Ferrer and Ros Barceló, 1994). Recently, it has been reported that peroxidase inactivation during the oxidation of phenols is mainly due to the inability of phenols, or phenoxy radicals, to reduce compound **III** (a highly oxidized species of peroxidase regarded as a key intermediate in the catalytic cycle of the enzyme) to the ferric enzyme (Chung and Aust, 1995). If this is so, it may be suspected that capsaicin, like veratryl alcohol and many other phenols (Chung and Aust, 1995), is capable of reducing compound **III**, thus avoiding peroxidase inactivation.

Apparent Molecular Weights of Capsaicin Oxidation Products. Reaction media, to which was added DMF up to 50% (v/v), were fractionated by gel permeation chromatography on Sephadex LH-20 using DMF as eluent. Previously, the column was calibrated with a series of polystyrene standards (M_w = 12860−687) and dihydrocapsaicin (M_w = 307), and the distribution coefficients (K_d) for each standard were calculated. When the K_d values were plotted versus the molecular weights, a strong correlation between K_d values and molecular weight was apparent ($\log M_w = 3.66 - 3.76K_d$; $r^2 = 0.9890$) (Figure 2).

Once the column was calibrated, the oxidation products of capsaicin were chromatographed, being detected in eluates by their absorbance at 262 nm (Figure 3). Three peaks were detected for capsaicin oxidation products (peaks 1−3, Figure 3). Peak 1 (K_d = 0.234), corresponding to compounds with an average molecular weight (see Figure 2) of 603 ± 12, apparently corresponds to dimers of capsaicin (M_w = 608), while peak 2 (K_d = 0.005) appears to be a polymeric product with a molecular weight of 4500. This dehydrogenation polymer probably corresponds to polymeric products with a mean polymerization degree of 15. Peak 2 (Figure 3) trails a plateau which suggests that other polymerization products with a polymerization degree of less than 15 may also be present.

Together with these oxidation products, material absorbing at 262 nm was also found in the first seven fractions (0−7 mL, peak 3, Figure 3). However, this

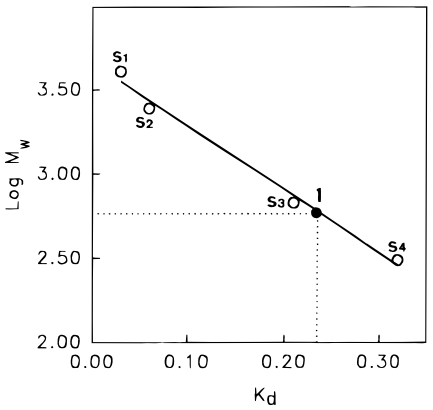


Figure 2. Molecular weight determination of capsaicin oxidation products (**1**, corresponding to peak 1 in Figure 3) from the plotting of the log M versus K_d values using a series of polystyrene standards of 4075 (S_1), 2500 (S_2), and 687 (S_3) and dihydrocapsaicin (S_4; 307) as molecular markers.

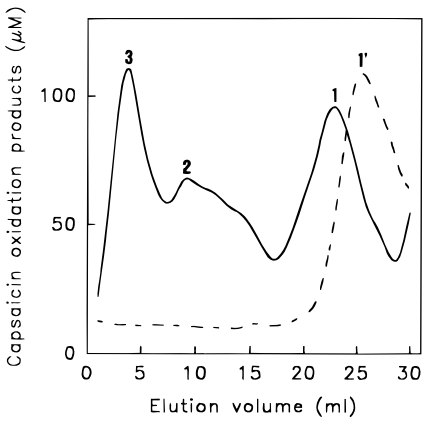


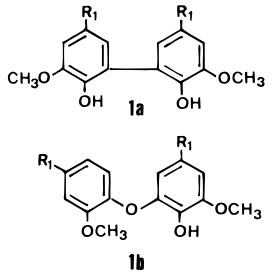
Figure 3. Gel permeation chromatography on Sephadex LH-20 of reaction media containing capsaicin and hot pepper peroxidase in the presence (solid line) and in the absence (dashed line) of hydrogen peroxide.

material, as can be deduced from its cloudy (colloidal) nature, appears to represent products of protein and capsaicin copolymerization, such copolymers frequently growing in reaction media during peroxidase-mediated phenolic oxidations (Evans and Himmelsbach, 1991). All these products (dimers, dehydrogenation polymers, and capsaicin−protein copolymers) were due to a peroxidase-mediated capsaicin oxidation since, in the absence of H_2O_2, only the peak corresponding to capsaicin (Figure 3, peak 1′) was found.

GC−MS Analysis of Capsaicin Oxidation Products with Low Molecular Weights. From the results shown in Figure 3, it became apparent that dimers of capsaicin are the main products of the peroxidase-mediated capsaicin oxidation. For this reason, and in order to elucidate their structure, the 18−28th fractions from the Sephadex LH-20 chromatogram were pooled and brought to dryness, and the TMS derivatives were prepared. Analysis of the TMS derivatives by GC−MS showed the presence of two main compounds. The TMS derivative of compound **1a** eluted with a retention time of 7.23 min, while the TMS derivative of compound **1b** eluted at 10.57 min.

The MS spectrum of the TMS derivative of compound **1a** showed mass fragments at m/z 753, 376, 266, and 248, with satellites at (m/z + 1) and (m/z + 2). These decomposition fragments appear to be conclusive for assigning to compound **1a** the structure of 5,5′-dicapsaicin (**1a** in Chart 1, M_w = 608). In fact, the ion at

Chart 1. Structure of 5,5′-Dicapsaicin (1a) and 4′-*O*-5-Dicapsaicin Ether (1b) Products of the Oxidative Coupling of Capsaicin by Hot Pepper Peroxidase



Scheme 1. Mass Fragmentation Pattern of the 4-*O*,4′-*O*-DiTMS Derivative of 5,5′-Dicapsaicin

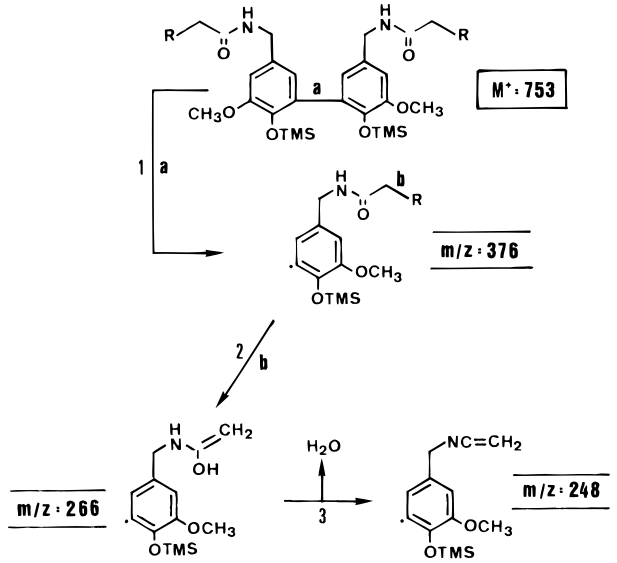


Table 1. *m/z* Ratio of the Main Fragment and the Average Molecular Weight (aM_w), Abundance (A), Elemental Composition (ec), and Theoretical Molecular Weight (tM_w) of the Decomposition Fragments of the TMS Derivative of 5,5′-Dicapsaicin

m/z	aM_w	A (%)	ec	tM_w
753 (M^+)	−	<2	$C_{42}H_{68}O_6N_2Si_2$	753.186
376 (M^+/2)	−	<2	$C_{21}H_{34}O_3NSi$	376.593
266	266.358	100	$C_{13}H_{20}O_3NSi$	266.393

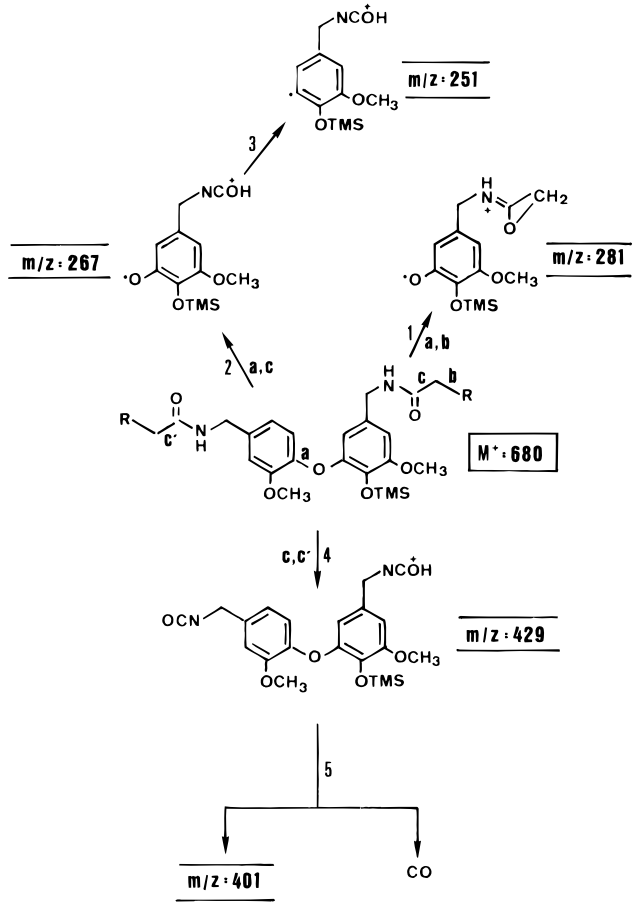
m/z 753 appears to be the molecular ion (M^+) of the 4-*O*,4′-*O*-diTMS derivative of 5,5′-dicapsaicin (m/z 753.186). Furthermore, the mass fragment at m/z 376 appears to result from the symmetric homolytic breakdown of M^+ at 5,5′ (see Scheme 1, arrow 1, breakdown in a). Theoretically, this symmetric breakdown results in two fragments with identical molecular weights (theoretical M_w = 376.593, Table 1). The abundance of these fragments is low (<2%, Table 1), as also occurs with the M^+ for the TMS derivative of capsaicin itself (<2%).

Likewise, the presence of mass fragments at m/z 266 and 248 supports this structure. Thus, the mass fragment at m/z 266 would have its origin in the β-elimination of the alkyl side chain (breakdown in b, arrow 2, Scheme 1) of the mass fragment m/z 376, a fragmentation pattern typical of alkyl amides. The mass fragment at m/z 248 apparently has its origin in mass fragment m/z 266 through the loss of H_2O (arrow 3, Scheme 1). This pattern of decomposition (β-elimination of olefin at the carbonyl side followed by the loss of water) is also typical of the TMS derivative of capsaicin. Furthermore, the calculation of the average M_w for each

Table 2. *m/z* Ratio of the Main Fragment and the Average Molecular Weight (aM_w), Abundance (*A*), Elemental Composition (ec), and Theoretical Molecular Weight (tM_w) of the Decomposition Fragments of the TMS Derivative of 4′-*O*-5-Dicapsaicin Ether

m/z	aM_w	*A* (%)	ec	tM_w
680 (M^+)	–	<2	$C_{39}H_{59}O_6N_2Si$	679.996
429	429.599	100	$C_{21}H_{24}O_6N_2Si$	429.528
401	401.498	22	$C_{20}H_{24}O_5N_2Si$	401.517
355	355.500	87	–	–
325	325.385	12	–	–
281	281.485	100	$C_{13}H_{19}O_4NSi$	281.385
267	267.498	52	$C_{12}H_{17}O_4NSi$	267.358
251	251.188	35	$C_{12}H_{17}O_3NSi$	251.358

Scheme 2. Mass Fragmentation Pattern of the 4-*O*-TMS Derivative of 4′-*O*-5-Dicapsaicin Ether

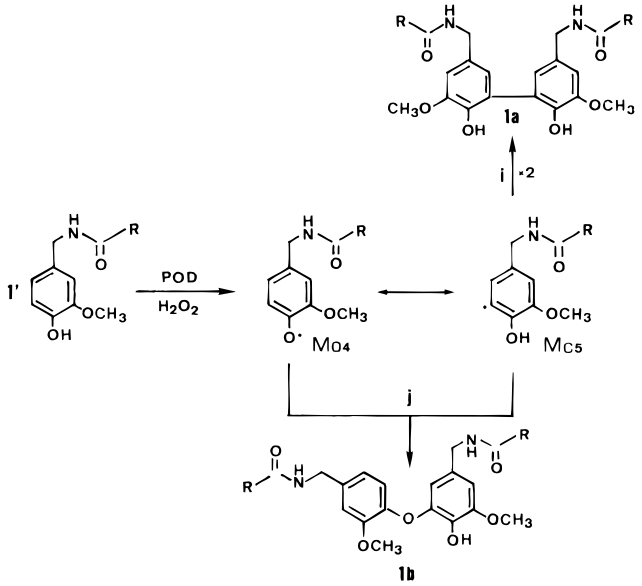


fragment from the abundance of *m/z*, (*m/z* + 1), and (*m/z* + 2) is in accordance with the theoretical M_w calculated from its suspected structure (Table 1).

The MS spectrum of the TMS derivative of compound **1b** shows mass fragments at *m/z* 680, 429, 401, 355, 325, 281, 267, and 251. These decomposition fragments also appear to be conclusive for assigning to compound **1b** the structure of 4′-*O*-5-dicapsaicin ether (**1b** in Chart 1, $M_w = 608$). In fact, the ion at *m/z* 680 appears to be the molecular ion (M^+) of the 4-*O*-TMS derivative of 4′-*O*-5-dicapsaicin ether (*m/z* 679.996, Table 2). Furthermore, the mass fragments at *m/z* 325 (325.385, 12%) and 355 (355.500, 87%) appear to result from the asymmetric breakdown of $(M + H)^+$ (325.385 + 355.500 = 680.885).

The mass fragment at *m/z* 281 probably arises from the asymmetric breakdown of the *O*-diphenyl ether bond (breakdown in a, arrow 1, Scheme 2) followed by β-elimination of the side chain (breakdown in b, arrow 1, Scheme 2), whereas mass fragments at *m/z* 267 and

Scheme 3. Proposed Reaction Scheme for the Formation of 5,5′-Dicapsaicin (1a) and 4′-*O*-5-Dicapsaicin Ether (1b) through a Radical–Radical Coupling Reaction during the Oxidation of Capsaicin (1′) by Hot Pepper Peroxidase



251 probably arise from the asymmetric breakdown of the *O*-diphenyl ether bond (breakdown in a, arrow 2, Scheme 2) followed by α-elimination of the side chain (breakdown in c, arrow 2, Scheme 2). Mass fragments at *m/z* 429 and 401 probably arise by α-elimination of the side chain as olefin (breakdown in c,c′, arrow 4, Scheme 2), followed by the loss of CO (arrow 5, Scheme 2). This fragmentation pattern is typical of capsaicin and, in general, of amides as well.

Furthermore, in those cases where the structures were tentatively identified, the calculation of the average M_w for each fragment from the abundance of *m/z*, (*m/z* + 1), and (*m/z* + 2) is in accordance with the theoretical M_w calculated from the suspected structure (Table 2).

A possible reaction scheme for the formation of **1a** (5,5′-dicapsaicin) and **1b** (4′-*O*-5-dicapsaicin ether) is proposed in Scheme 3 on the basis of the theory of radical phenolic coupling (Hapiot et al., 1994). The proposed reaction mechanism proceeds via an initial one-electron oxidation of capsaicin (**1′**) by peroxidase to form the 4-*O*-phenoxyl free radical (M_{O4}, in Scheme 3), which, in turn, produces a series of mesomeric radicals, the most stable (in the case of capsaicin) being where the unpaired electron is at C5 (M_{C5}, Scheme 3). These radicals spontaneously dimerize to form several possible structures; the coupling of two C5 phenolic radicals would give 5,5′-dicapsaicin (reaction i, Scheme 3), while the coupling of C5 phenolic radical with a 4-*O*-phenoxyl free radical would give 4′-*O*-5-dicapsaicin ether (reaction j, Scheme 3). These dimeric structures can further act as a nucleus, in which the highly polymerized dehydrogenation products also formed by the action of *Capsicum* peroxidase on capsaicin grow.

In conclusion, these results suggest that *Capsicum* peroxidase oxidizes capsaicin to yield 5,5′-dicapsaicin and 4′-*O*-5-dicapsaicin ether, together with a mixture of highly polymerized dehydrogenation products, all of them therefore of a lignin-like nature. Furthermore, these results lend weight to the biochemical evidence for supporting the existence of an oxidative competitive sink for phenylpropanoid intermediates of capsaicin

biosynthesis, which probably competes with capsaicin itself to yield lignin-like substances in the cell wall of *C. annuum* var. Annuum.

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Received for review December 19, 1995. Revised manuscript received July 30, 1996. Accepted July 30, 1996.[⊗] This work was supported by Grants CICYT ALI 573/93 and XUGA 10301/A/95. M.A.B. holds a fellowship from the Xunta de Galicia.

JF950826Y

[⊗] Abstract published in *Advance ACS Abstracts,* September 1, 1996.