Oxidation of Capsaicin and Capsaicin Phenolic Precursors by the Basic Peroxidase Isoenzyme B_6 from Hot Pepper

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The ability of hot pepper peroxidase to oxidize the phenolic precursors of capsaicin biosynthesis [*p*-coumaric acid (**3**), caffeic acid (**4**), ferulic acid (**5**), vanillin (**6**), and vanillylamine (**7**)] was studied. The results showed that hot pepper peroxidase, and especially hot pepper peroxidase isoenzyme B_6 (Prx B_6), is capable of oxidizing the phenolic precursors of capsaicin (**8**), caffeic acid and ferulic acid being the best substrates. Vanillylamine was the only precursor that did not act as substrate for peroxidase-catalyzed oxidations. Since the basic peroxidase isoenzyme B_6 is located in cell walls, it seems plausible that this isoenzyme may be involved in the insolubilization of phenylpropanoid precursors *in muro*. These results lend weight to the biochemical evidence for supporting the existence of an oxidative competitive sink for phenylpropanoid intermediates of capsaicin biosynthesis, and which probably competes with capsaicin itself to yield lignin-like substances, in the cell wall of *Capsicum annuum* var. Annuum.

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

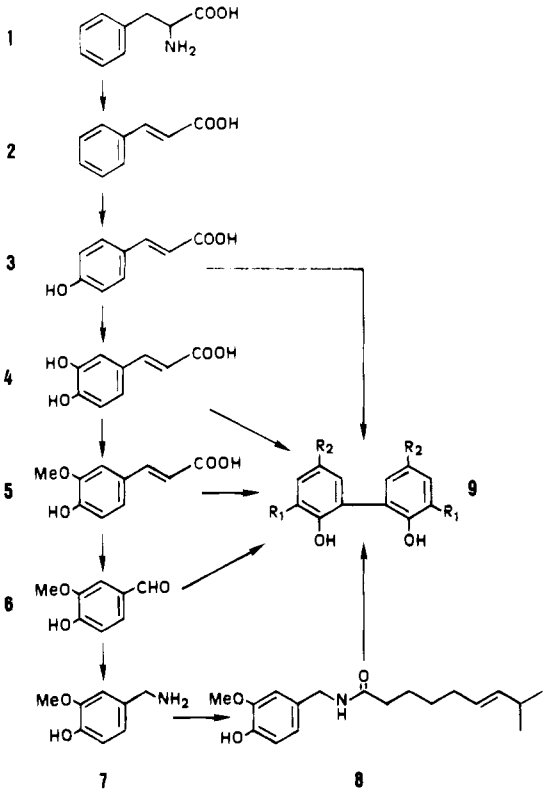
INTRODUCTION

Capsaicin (**8**), the major pungent compound of hot pepper fruits (*Capsicum annuum* var. Annuum), is an amide derivative of vanillylamine and *trans*-8-methyl-non-6-enoic acid (Leete and Louden, 1968; Bennett and Kirby, 1968). The vanillylamine moiety of capsaicin is biosynthetically derived from L-phenylalanine (**1**, Scheme 1), while the branched fatty acid moiety is derived from valine (Leete and Louden, 1968; Bennett and Kirby, 1968; Iwai et al., 1979).

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, a process which takes place at the onset of capsaicin accumulation (Sukrasno and Yeoman, 1993). Thus, the cell wall-bound phenolic fraction acts as a major competing sink for the phenylpropanoid precursors of capsaicin (Hall et al., 1987). Through this mechanism, it appears possible to maintain extremely low levels of the phenolic precursors of capsaicin, irrespective of the source of the tissue or its developmental stage (Hall et al., 1987).

In hot pepper fruits, capsaicin is synthesized in the placenta (Iwai et al., 1979; Fujiwake et al., 1982; Suzuki et al., 1981) and accumulated in vacuoles of placental epidermal cells (Fujiwake et al., 1980; Suzuki et al., 1980) until it is metabolized to unknown products, perhaps of a lignin-like nature (Bernal et al., 1993a). Peroxidase (EC 1.11.1.7) activity is involved in this catabolic reaction (Bernal et al., 1993a). Arguments in favor of a participation of peroxidase in capsaicin turnover and degradation are based on the exclusive localization of peroxidase in placental epidermal cells

Scheme 1. Pathway of Capsaicin (8) Biosynthesis in *Capsicum* Showing the Principal Phenolic Intermediates, and Probable Shunt of These Compounds to Lignin-like (9) Substances, through L-Phenylalanine (1), Cinnamic Acid (2), *p*-Coumaric Acid (3), Caffeic Acid (4), Ferulic Acid (5), Vanillin (6), and Vanillylamine (7)



from hot pepper fruits (Bernal et al., 1994b), and in the colocalization of peroxidase, especially the basic peroxidase isoenzyme B_6 (Bernal et al., 1993b), and capsaicin in vacuoles of hot pepper cells, this evidence being further supported by the strong capsaicin-oxidizing

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activity of this basic peroxidase isoenzyme (Bernal et al., 1994a).

Since the basic peroxidase isoenzyme B_6 is also located in cell walls (Bernal et al., 1993b), it seems plausible to think that this isoenzyme may also be involved in the insolubilization of phenylpropanoid precursors in the cell walls, since this reaction is frequently shown by peroxidase (Whitmore, 1976; Fry, 1984). Bearing this in mind, the present study was undertaken to investigate the ability of peroxidases from *Capsicum* fruits to catalyze the oxidation of capsaicin and of their precursors, in order to provide biochemical evidence of an oxidative competitive sink for phenylpropanoid intermediates of capsaicin biosynthesis in the cell wall of *Capsicum annuum* (var. Annuum).

MATERIALS AND METHODS

Chemicals. Caffeic acid (**4**), ferulic acid (**5**), capsaicin (**8**), and horseradish peroxidase (HRP, type II) were purchased from Sigma Chemical Co. (Madrid, Spain); *p*-coumaric acid (**3**), from Fluka Chemika-BioChemika (Buchs, Switzerland); vanillin (**6**), from Merck (Darmstadt, Germany). Vanillylamine (**7**) was obtained from Aldrich Chemical Co. (Madrid, Spain). The rest of the chemicals used in this study were of analytical grade.

Plant Material. *Capsicum annuum* (var. Annuum) fruits were obtained from a local market and stored at −30 °C until use.

Peroxidase Fraction. *Capsicum* fruits 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 Büchner funnel and the residue thoroughly washed with acetone at −20 °C until all pigments were removed. The protein precipitate was resuspended in 1.0 M KCl and 50 mM Tris (tris[hydroxymethyl]aminomethane)-HCl buffer (pH 7.5) and incubated, with stirring, for 1 h at 4 °C. The hot pepper protein solution obtained was clarified by centrifugation at 3000*g* for 5 min. The supernatant was either used directly or for further purification of peroxidase isoenzyme B_6.

Purification of the Basic Peroxidase Isoenzyme B_6 by Preparative Isoelectric Focusing. Purification of the basic peroxidase isoenzyme B_6 by preparative isoelectric focusing was carried out using a Rotofor preparative IEF cell (Bio-Rad Laboratories, Richmond, CA) (Bernal et al., 1994a). For this, briefly, protein (about 140 mg) from pepper homogenates was dialyzed overnight against 4 L of bidistilled deionized water. This protein fraction was supplemented with 10% (v/v) glycerol and 1.5% (v/v) Bio-Lyte Ampholytes (Bio-Rad) of 3−10 pH range and loaded into the Rotofor cell (Bernal et al., 1994a). Prerunning was carried out by filling the focusing chamber with 55 mL of distilled water and running at 5 W constant power for 5 min, using 0.1 M H_3PO_4 as electrolyte for the anode and 0.1 M NaOH as electrolyte for the cathode (Bernal et al., 1994a). For the purification of basic peroxidase isoenzyme B_6 in the Rotofor cell, optimal conditions were 800 V of constant voltage for 16 h at 4 °C for a starting current of 15−20 mA.

Once focusing was completed, the electrofocusing cell was fractioned into 20 aliquots ranging from acidic to basic pI proteins. Ampholytes and glycerol were removed from protein fractions by incubation in 1.0 M NaCl for 45 min at 4 °C and overnight dialysis against 5 mM Tris-HCl buffer (pH 7.5). Basic peroxidase isoenzyme B_6 was eluted in fractions 18−20 of the pH gradient (Bernal et al., 1994a).

Spectrophotometric Assays. Unless otherwise stated, the spectrophotometric assays for *Capsicum* peroxidase were performed at 25 °C in a reaction medium containing the phenolic (from a 10 mM stock in methanol of HPLC grade) and H_2O_2 at optimal (saturating) concentrations (see Table 1) in 0.1 M tris-acetate buffer (pH 5.0). The reaction was initiated by the addition of either 80 pkat of unpurified *Capsicum* peroxidase or 40 pkat of purified basic peroxidase isoenzyme B_6. To test the ability of horseradish peroxidase to oxidize the precursors, 3 nkat of enzyme were used. One kat of peroxidase was defined as the amount of protein that oxidized 1 mol/s of 4-methoxy-α-naphthol, assayed according to Ferrer et al. (1990). For all the enzyme fractions assayed, the oxidation of phenolics was monitored by observing increases or decreases in absorbance in the UV spectrum (see Table 2), the oxidation rates being expressed in nmol/s nkat. For this the ϵ_i were calculated for each oxidation product (see Table 2).

Table 1. Optimal Concentration of Phenolic Precursors of Capsaicin and H_2O_2 for the Oxidation of These Compounds by *Capsicum* Peroxidase[a]

	phenolic (mM)	H_2O_2 (mM)
p-coumaric acid	0.10	1.0
caffeic acid	0.15	4.0
ferulic acid	0.20	1.0
vanillin	1.00	1.0
capsaicin	0.60	0.1

[a] The spectrophotometric assays for *Capsicum* peroxidase were performed at 25 °C in a reaction medium containing the phenolic and H_2O_2 in 0.1 M tris-acetate buffer (pH 5.0). The reaction was initiated by the addition of 80 pkat of unpurified *Capsicum* peroxidase and followed for 1 min.

Table 2. Apparent Extinction Coefficients (ϵ, M^{-1} cm^{-1}), Wavelengths at Which Maximal Spectral Changes Take Place (λ, nm), and Nature of These Spectral Changes (Increases [i] or Decreases [d]), during the Oxidation of Phenolic Precursors of Capsaicin by *Capsicum* and Horseradish Peroxidase

	ϵ	λ	nature
p-coumaric acid	11925	287	d
caffeic acid	11209	312	d
ferulic acid	10992	310	d
vanillin	5482	249	i
vanillylamine[a]			
capsaicin	5300	262	i

[a] At the concentration used (0.1 mM) this compound did not show spectral changes after incubation with *Capsicum* or with horseradish peroxidase.

RESULTS AND DISCUSSION

Phenolic precursors of capsaicin biosynthesis in hot pepper fruits are shown in Scheme 1. Thus, *p*-coumaric acid (**3**), caffeic acid (**4**), ferulic acid (**5**), vanillin (**6**), and vanillylamine (**7**) appear to be the main phenolic precursors of capsaicin (**8**) biosynthesis in *Capsicum*. The ability of hot pepper peroxidase to oxidize these phenolic precursors was followed spectrophotometrically by monitoring the UV spectrum in reaction media in the presence and in the absence of H_2O_2.

For all the phenolics studied, except vanillylamine, incubation with hot pepper peroxidase and H_2O_2 resulted in changes in their UV spectrum with time (Table 2). Maximal spectral changes took place at 287 nm (for *p*-coumaric acid), 312 nm (for caffeic acid), 310 nm (for ferulic acid), and 249 nm (for vanillin). With the exception of caffeic acid, these spectral changes were strictly dependent on the presence of enzyme and H_2O_2 and therefore due to peroxidatic activities. An inability to oxidize vanillylamine was also shown by horseradish peroxidase, which, even a higher concentrations (3 nkat/mL), was incapable of oxidizing vanillylamine.

Prior to the kinetic characterization of hot pepper peroxidase for oxidizing these phenolic compounds, the apparent extinction coefficient for the oxidation product of each phenolic was calculated. This was carried out spectrophotometrically by measuring the increases/decreases in A_i at $t = 0$ (A_0) to constant A_i (A_∞); values were obtained at up to 20 min after the reaction was

Table 3. k_{cat}, K_M, and Efficacy in the Oxidation of Phenolic Precursors of Capsaicin by *Capsicum* Peroxidase

	k_{cat} (nmol/s nkat)	K_M (mM)	efficacy (k_{cat}/K_M)
p-coumaric acid			
phenol	1.696	0.065	26.1
H_2O_2	1.178	0.152	7.8
caffeic acid			
phenol	16.988	0.283	60.0
H_2O_2	7.215	0.412	17.5
ferulic acid			
phenol	14.600	0.184	79.3
H_2O_2	9.376	0.357	26.3
vanillin			
phenol	12.296	0.976	12.6
H_2O_2	7.826	0.092	85.1

begun by the addition of horseradish peroxidase. For this, a low concentration range (1−25 μM) of phenolics was used in the reaction medium. By plotting the difference ($A_0 - A_\infty$) vs the initial concentration of phenolic, an apparent ϵ_i for each phenolic oxidation product was calculated (Table 2).

The dependence of the oxidation rate of each phenolic by hot pepper peroxidase on phenolic and H_2O_2 concentrations shows a kinetic behavior of the Michaelis-Menten type at low substrate concentrations. Although valid K_M values cannot be defined for oxidations catalyzed by peroxidases, since these reactions show no sign of reversibility or of complex enzyme−substrate formation (Dunford and Stillman, 1976), apparent K_M values were calculated for the peroxidase-catalyzed oxidation of each phenolic precursor. This was achieved by using the SigmaPlot Scientific Graphing Software (Jandel Scientific) for a hyperbolic curve. The results are shown in Table 3 and illustrate that *p*-coumaric acid was the phenolic precursor with the lowest K_M for the enzyme, although caffeic acid and ferulic acid were the phenolics most efficiently oxidized by this hot pepper peroxidase fraction. With regard to H_2O_2, this was more efficiently consumed (higher k_{cat}/K_M value) in the peroxidase-catalyzed oxidation of vanillin (Table 3).

Hot peppers at fruit softening show a simple peroxidase isoenzyme pattern when analyzed by analytical isoelectric focusing. This shows the major presence of peroxidase isoenzyme B_6 (Bernal et al., 1994a). In order to characterize the ability of hot pepper peroxidase isoenzyme B_6 to oxidize capsaicin phenolic precursors, this isoenzyme was purified by preparative isoelectric focusing in glycerol-stabilized 3.0−10.0 pH gradients. Examination by analytical isoelectric focusing of the purified isoenzyme B_6 reveals that it migrates as the only isoperoxidase band during electrophoresis (Bernal et al., 1994a).

The ability of this basic peroxidase isoenzyme (Prx B_6) to oxidize capsaicin phenolic precursors was studied, and its properties were compared with that of unpurified peroxidase (Σ Prx_i), i.e., the enzyme resulting for the mixture of all the hot pepper peroxidase isoenzymes. The results are shown in Table 4 and illustrate that although specific activities of unpurified peroxidase fractions were higher for caffeic acid and ferulic acid, a property which is also shown by the basic peroxidase isoenzyme Prx B_6, capsaicin was the most efficiently oxidized compound by this purified peroxidase isoenzyme.

Finally, these results suggest that hot pepper peroxidase, particularly pepper peroxidase isoenzyme B_6 (Prx B_6), is capable of oxidizing the phenolic precursors of capsaicin, caffeic acid, and ferulic acid being the best

Table 4. Specific Activities (nmol of Phenolic Oxidized/s nkat) of Unpurified Peroxidase Fractions (Σ Prx_i) and of Basic Peroxidase Isoenzyme B_6 (Prx B_6) during the Oxidation of Phenolic Precursors of Capsaicin by *Capsicum* Peroxidase

	Σ Prx_i	Prx B_6
p-coumaric acid	1.027	1.466
caffeic acid	3.971	3.684
ferulic acid	3.292	3.340
vanillin	2.831	2.147
vanillylamine	0.000	0.000
capsaicin	2.577	4.015

substrates. Since the basic peroxidase isoenzyme B_6 is located in cell walls (Bernal et al., 1993b), it seems plausible that this isoenzyme may be involved in the insolubilization of phenylpropanoids precursors *in muro*. These results lend support to the biochemical evidence which points to the existence of an oxidative competitive sink for phenylpropanoids intermediates of capsaicin biosynthesis, and which probably competes with capsaicin itself, in the cell wall of *C. annuum* (var. Annuum).

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