Capsaicin Oxidation by Peroxidase from *Capsicum annuum* (Var. *annuum*) Fruits

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Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) oxidation by peroxidase (EC 1.11.1.7) from *Capsicum annuum* var. *annuum* fruits is studied. Capsaicin (Sigma) contains two components which are oxidized indistinctively by pepper peroxidase when capsaicin disappearance is monitored by HPLC. Spectrophotometric studies illustrated that capsaicin oxidation by pepper peroxidase showed maximal spectral changes at 262 nm. At this wavelength, $\epsilon_{262} = 5.3 \times 10^3\ M^{-1}\ cm^{-1}$ was calculated for the only absorbent oxidation product. The dependence of the capsaicin oxidation rate by pepper peroxidase on capsaicin and H_2O_2 concentrations reveals Michaelis–Menten-type kinetics with inhibition at high substrate concentrations and an acidic optimal pH. From these studies, it can be concluded that capsaicin is oxidized by pepper peroxidase and that a possible participation of peroxidase in capsaicin metabolism in the pepper fruit should be taken into account.

INTRODUCTION

Capsaicin and its analogues, called capsaicinoids, are the pungent compounds of the *Capsicum* fruit. Capsaicin, the major pungent compound of hot pepper fruits, is an amide derivative of vanillylamine and 8-methylnon-*trans*-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., 1979a).

Like other plant secondary metabolites (i.e., alkaloids), capsaicin is accumulated and later suffers a rapid turnover and degradation during cell development. This turnover is particularly intense in both *Capsicum* fruits (Iwai et al., 1979a; Suzuki et al., 1980) and cell suspension cultures (Salgado-Garciglia and Ochoa-Alejo, 1990; Johnson et al., 1990). In fruits, capsaicin is synthesized in the placenta (Iwai et al., 1979a; Fujiwake et al., 1982a). The key enzyme of capsaicin biosynthesis (capsaicinoid synthetase) is located in the tonoplast of epidermal cells (Fujiwake et al., 1982b), probably bound to the cytoplasmic face (Fujiwake et al., 1980b). Once synthesized, capsaicin is accumulated in the vacuoles of placental epidermal cells (Fujiwake et al., 1980a; Suzuki et al., 1980) until it is metabolized to unknown products. Thus, turnover and degradation are seen as the final steps of capsaicin metabolism.

While considerable progress has been made on the biosynthesis of capsaicin, the enzymology of the last steps in capsaicin metabolism and degradation is still incomplete. Peroxidase (EC 1.11.1.7) may be directly related with capsaicin metabolism since the vanillyl moiety of capsaicin is easily oxidized by this enzyme (Zapata et al., 1992). Peroxidases are widely distributed throughout the plant kingdom and are involved in a series of biosynthetic and degradative functions, in particular, phenolic (Berlin and Barz, 1975) and alkaloid (Perrey et al., 1989; Blom et al., 1991) metabolism. A peroxidase role in capsaicin metabolism may be hypothesized from its localization in vacuoles of the fleshy fruits' epidermal tissues (Ros Barceló and Muñoz, 1992).

Because of the vacuolar localization of both alkaloid-oxidizing peroxidases (Perrey et al., 1989; Blom et al., 1991; Ferrer et al., 1992) and capsaicin (Fujiwake et al., 1980a; Suzuki et al., 1980), the present study was undertaken to investigate the ability of peroxidases from *Capsicum* fruits to catalyze the oxidation of capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), the pungent compound of the hot pepper.

MATERIALS AND METHODS

Chemicals. Capsaicin, dihydrocapsaicin, bis(trimethylsilyl)-trifluoroacetamide containing 1% trimethylchlorosilane and horseradish peroxidase (HRP, type II) were purchased from Sigma Chemical Co. (Madrid, Spain). All of the other 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 −4 °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 50 mM Tris [tris(hydroxymethyl)aminomethane]-HCl buffer (pH 7.5) and incubated, with agitation, for 1 h at 4 °C. The pepper protein solution thus obtained was clarified by centrifugation at 3000*g* for 5 min. The supernatant, although a crude soluble protein fraction, was used routinely without further purification in all subsequent enzymatic studies.

Spectrophotometric Assays. Unless otherwise noted, the spectrophotometric assays for *Capsicum* peroxidase were performed at 25 °C in a reaction medium containing 1 mM capsaicin (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). The reaction was initiated by the addition of 4.3 pkat of peroxidase. For horseradish peroxidase assay, the reaction medium contained 0.6 mM capsaicin, 0.25 mM H_2O_2, and 16 pkat of enzyme in 0.1 M Tris–acetate buffer (pH 7.0). One picokatal of peroxidase was defined as the amount of protein that oxidized 1 pmol/s of 4-methoxy-α-naphthol, assayed according to the method of Ferrer et al. (1990). Both for *Capsicum* and for horseradish peroxidase, capsaicin oxidation was monitored by increases in absorbance at

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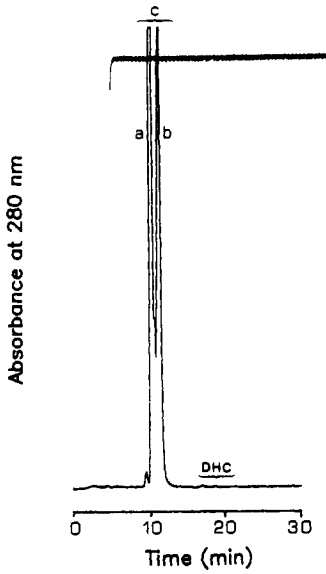

Figure 1. 1. HPLC chromatogram of Sigma capsaicin (C) separated by C_{18} reversed-phase chromatography in components a and b. Dihydrocapsaicin (DHC) was not detected in the chromatograms.

262 nm in an Uvikon 940 spectrophotometer (Kontron Intruments, Madrid, Spain), the oxidation rate being expressed in nanomoles per second, for which a $\epsilon_{262} = 5.3 \times 10^3$ M^{-1} cm^{-1} was calculated for the oxidation product (see Results).

High-Performance Liquid Chromatography (HPLC) of Capsaicin. Reversed-phase HPLC was carried out on a 15 cm × 0.4 cm i.d. Spherisorb ODS (C_{18}) (5 μm) column (Tracer Analitica S.A., Barcelona, Spain) using a Waters (Waters Associates, Millipore, Milford, MA) system comprising a Model 510 pump, a Model U6K sample injector, a Model 730 system controller, and a Model 481 UV detector operated at 280 nm (0.1 AUFS). The mobile phase was methanol/water (60:40) (Krajewska and Powers, 1986) at a flow rate of 1.0 mL/min. To follow the time course of capsaicin disappearance in reaction media by HPLC, the reaction media were prepared as described above and samples were passed over a C_{18} Sep-Pak cartridge (Waters Associates) preconditioned with 5 mL of methanol and 5 mL of water. The loaded cartridge was washed with 5 mL of water, and capsaicin was eluted with methanol.

Gas Chromatography–Mass Spectrometry (GC–MS) of Capsaicin. GC–MS was performed on a Hewlett-Packard Model 5993 coupled to a 5995 gas chromatograph–mass spectrometer and to a 2648A graphics terminal, using a OW Chrompak 50 m × 0.20 mm i.d. column, He pressure of 0.4 MPa, and a GC temperature program of 90–280 °C at 10 °C/min, with a 5-min hold at 280 °C. The run time was 60 min for an injection port temperature of 250 °C and an electron multiplier voltage of 1600 V.

TMS derivative of capsaicin for GC was prepared by addition of 50 μL of pyridine and 100 μL of bis(trimethylsilyl)trifluoroacetamide (BSTFA), containing 1% trimethylchlorosilane (TMCS) to 5 mg of capsaicin, and heating for 30 min at 100 °C.

RESULTS AND DISCUSSION

GC–MS of the TMS derivative of Sigma capsaicin gave one homogeneous peak, with a mass fragmentation pattern whose most representative ions were at m/z = 377 (M^+) and 209 ($[M - RNH]^+$). These are in agreement with the fragmentation pattern reported in the literature (Masada et al., 1971; Lee et al., 1976; Iwai et al., 1979b). However, HPLC analysis of Sigma capsaicin revealed the presence of two components: component a at R_t = 10.68 min and component b at R_t = 11.65 min (Figure 1). Similar results on the heterogeneity of commercial preparations of capsaicin by HPLC have been reported by Lee et al. (1976). Those authors concluded that commercial preparations of capsaicin also contained minor amounts of dihydrocapsaicin (8-methyl-*N*-vanillylnonanamide). However, this was not the case of Sigma capsaicin, since dihydrocapsaicin eluted in this HPLC system with a R_t = 18.57 min.

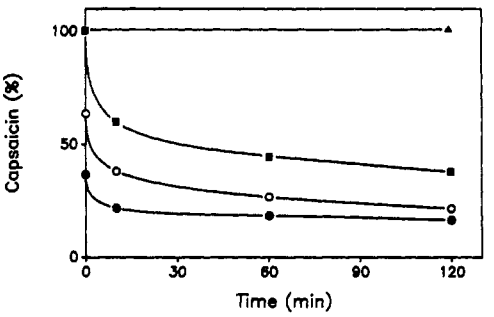

Figure 2. Time course of the disappearance of capsaicin (■) as percentage of the initial concentration (0.6 mM) in a reaction medium containing pepper peroxidase followed by HPLC and of capsaicin components a (○) and b (●). The time course of the level of capsaicin in a reaction medium in the absence of H_2O_2 (▲) is also shown.

The time course of capsaicin oxidation by *Capsicum* peroxidase was monitored by HPLC. Results shown in Figure 2 show that components a and b of commercial capsaicin were simultaneously oxidized by pepper peroxidase. Thus, this enzyme preparation does not discriminate between the two components of commercial capsaicin (Figure 2). The disappearance of capsaicin from the reaction media was strictly dependent on the addition of H_2O_2 (Figure 2).

Components a and b of this commercial preparation of capsaicin were not distinguishable spectrophotometrically during the oxidation of capsaicin by horseradish peroxidase. Thus, during the spectrophotometrically followed reaction, only one stable oxidation product seems to be formed. This was demonstrated by the application of the graphic analysis procedure of Coleman et al. (1970) to a set of consecutive UV spectra of a reaction medium containing horseradish peroxidase and H_2O_2 (Figure 3). The coincidence in the origin of the coordinate axis of the three straight trace families corresponding to each wavelength value (Figure 3) shows the occurrence of only two species kinetically correlated in the reaction mixtures: the substrate and a stable reaction product. On the basis of the well-established reaction mechanism for phenolic oxidation by peroxidase in the presence of H_2O_2 (Fry, 1986), the plausible oxidation product of capsaicin should be its corresponding dimer.

Similarly to the oxidation of capsaicin by horseradish peroxidase, the oxidation of capsaicin by *Capsicum* peroxidase, followed spectrophotometrically, was also strictly dependent on the presence of H_2O_2. This was easily monitored by the study of the spectral changes in the reaction media with time (Figure 4). Spectral changes in the absence of H_2O_2 did not take place. Consecutive spectra of reaction media containing capsaicin and H_2O_2 showed maximal spectral changes at 262 nm (Figure 4).

The apparent extinction coefficient for the oxidation product of capsaicin was calculated spectrophotometrically from the increases in A_{262} at t = 0 to constant A_{262} values which were obtained at up to 20 min after the reaction was begun by the addition of enzyme. For this, a low concentration range (0.01–0.05 mM) of capsaicin in the reaction medium was used. From the plotting of the final A_{262} vs the initial concentration of capsaicin (data not shown), $\epsilon_{262} = 5.3 \times 10^3$ M^{-1} cm^{-1} was calculated for the oxidation product.

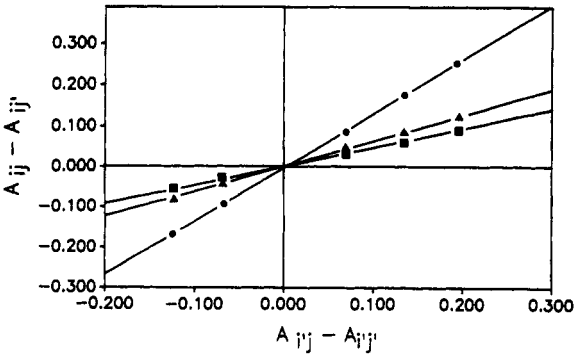

Figure 3. Graphic analysis of the consecutive spectra of a reaction medium containing 0.6 mM capsaicin, 0.25 mM H_2O_2, 16 pkat of horseradish peroxidase, and 0.1 M Tris–acetate buffer (pH 7.0), according to the method of Coleman et al. (1970). A scan speed of 500 nm/min was used at 1.0-min intervals. In this analysis, A_{ij} is the absorbance at wavelength i obtained during tracing j, so that A_{12} is the absorbance at 250 nm during the second tracing of the absorption spectrum. The selected wavelengths were i_1 = 250 nm (●), i_2 = 280 nm (■), and i_3 = 300 nm (▲); i'= 260 nm; j'=3 (third tracing). Hence, the ordinate value ($A_{250,2}$–$A_{250,3}$) is the difference of absorbances, at 250 nm, between the second and third tracings, and the abscissa value ($A_{260,2}$–$A_{260,3}$) is the difference of absorbances, at 260 nm, between the second and third tracings, taking the value $A_{260,3}$ as reference in this graphical analysis. The test of two species without restriction was applied.

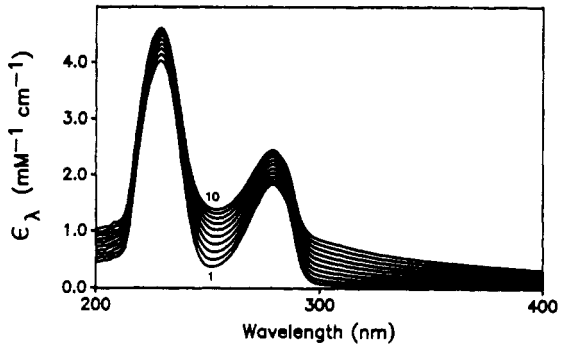

Figure 4. Spectrophotometric recording of a reaction medium containing capsaicin and pepper peroxidase (see Materials and Methods). A scan speed of 500 nm/min was used at 1-min intervals. (Line 1) 30 s after the addition of enzyme.

As observed with horseradish peroxidase, the dependence of the oxidation rate of capsaicin by *Capsicum* peroxidase on capsaicin and H_2O_2 concentrations shows a kinetic behavior of the Michaelis–Menten type at low substrate concentrations, with inhibition at high substrate concentrations (data not shown). Owing to the inhibition observed at high substrate concentrations and the fact that valid K_M values cannot be defined for oxidations catalyzed by peroxidases, since these reactions show no sign of reversibility or complex enzyme–substrate formation (Dunford and Stillman, 1976), K_M values were not calculated for the peroxidase-catalyzed oxidation of capsaicin. Even so, an optimal concentration of 0.1 mM for H_2O_2 and an optimal concentration of 1.0 mM for capsaicin were found, the latter being near the solubility limit of capsaicin in aqueous solutions.

The effect of pH on capsaicin oxidation by *Capsicum* peroxidase is shown in Figure 5. As can be observed from this figure, the optimal pH of the oxidative reaction was in the acidic range, showing a good adaptation of the enzymatic reaction to the acidic pH normally found in vacuoles (Boller, 1982).

In conclusion, *Capsicum* peroxidase oxidizes capsaicin. Due to the vacuolar localization of alkaloid-oxidizing

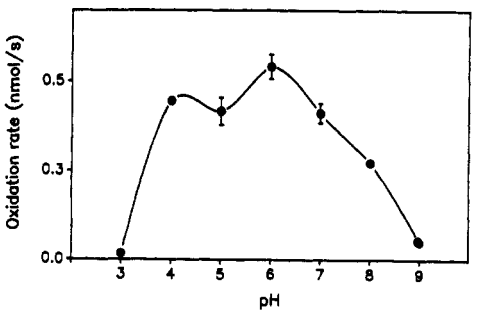

Figure 5. Influence of pH on capsaicin oxidation by pepper peroxidase (see Materials and Methods) in reaction media containing 0.1 M Tris–acetate buffer of variable pH. Bars show SE (n = 3).

peroxidases (Perrey et al., 1989; Blom et al., 1991; Ferrer et al., 1992) and capsaicin (Fujiwake et al., 1980a; Suzuki et al., 1980), the possible participation of peroxidase in capsaicin catabolism should be taken into account. This is supported by the fact that this enzymatic system shows maximal activity in the acidic pH range normally found in vacuoles.

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