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PURIFICATION, CHARACTERIZATION AND KINETIC PROPERTIES OF PEPPER FRUIT ACIDIC PEROXIDASE

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Key Word Index—*Capsicum annuum*; Solanaceae; pepper fruit; purification; peroxidase.

Abstract—A soluble acidic peroxidase (EC 1.11.1.7) was purified about 300-fold from the pericarp of pepper (*Capsicum annuum* L.) fruits by ammonium sulphate fractionation followed by chromatography in columns of Sephadex G-100, Q-Sepharose and Superose 12 PC 3.2/30. The purified enzyme has a pI of 3.8 and a M_r, determined by gel filtration, of 50 k. The enzyme was stable in a pH range from pH 6 to 9 and was resistant to high temperature. The ability of the acidic peroxidase to oxidize capsaicin was studied. The oxidation follows the accepted model for peroxidase oxidations, in which compound I (CoI) and compound II (CoII) appear to be the main intermediates in the catalytic cycle. Kinetic constants for H_2O_2 [K_1 (CoI formation constant) = 41 μM^{-1} sec^{-1}] and for capsaicin [K_3 (CoII reduction constant) = 3.5 μM^{-1} sec^{-1}] suggest that the acidic peroxidase has a higher H_2O_2 reactivity than other peroxidases, and that capsaicin is a good substrate for CoII reduction. © 1997 Elsevier Science Ltd

INTRODUCTION

Peroxidase (EC 1.11.1.7) is an enzyme that catalyses the oxidation of a large number of aromatic structures at the expense of H_2O_2. This kind of enzyme has been identified in all higher plants studied. Two main groups of isoperoxidases have been distinguished, acidic and basic, with a pI ranging from *ca* 3.5 to 9.5 [1]. The precise role of individual isoforms of peroxidases remains unclear owing to the lack of information on the localization of the enzymes and the availability of their specific substrates *in vivo*. However, basic peroxidase has been found to be most effective in IAA catabolism, and acidic isoforms are believed to be associated with lignification [2, 3].

Changes in peroxidase levels and isoform distribution are known to accompany higher plant processes, although there are few cases where the isoforms involved have been purified and characterized. Peroxidases are implicated in a number of higher plant processes such as the senescence of fruits and vegetables [4].

Peroxidases have recently been considered to play a role in the metabolism of alkaloids. In hot peppers, like other plant alkaloids, capsaicinoids accumulate and later undergo a rapid turnover and degradation during fruit development. While considerable progress has been made on the biosynthesis of capsaicinoids, the enzymology of the last steps in capsaicinoid metabolism and degradation is still incomplete. Basic peroxidase of high pI may be directly related to capsaicinoid metabolism since both capsaicin, dihydrocapsaicin and their phenolic precursors are easily oxidized by this enzyme [5–7]. Nevertheless, the participation of acidic peroxidase in capsaicinoid metabolism is still unknown.

The aim of the present work was to purify and characterize the catalytic properties of this acidic peroxidase isoenzyme during the oxidation of capsaicin. Since this isoenzyme is the principal component of peroxidase polymorphism in the first stage in the pepper fruit, this material was chosen for its purification.

RESULTS AND DISCUSSION

Subcellular fractionation

In order to determine the location of most of the peroxidase activity we did a subcellular fractionation on the pericarp of pepper fruit, discarding the placenta due to its low activity. The greatest fraction is the soluble one (96.8%) similar to the one found in *Capsicum* leaves (89%) [8], tomato fruit (85%) [9] and *Pisum sativum* root (79%) [10]. But in other cases, such as strawberry fruits [11], the fraction associated with membranes is always 100–1000 times greater than the soluble one.

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Table 1. Purification of acidic peroxidase from pepper fruits. Data correspond to average of four typical purifications

Purification step	Total activity (pkat)	Total protein (mg)	Specific activity (pkat mg^{-1} protein)	Purification (-fold)	Yield (%)
Crude extract	227	111	2.04	1	100
$(NH_4^+)_2SO_4$	193	42	4.53	2.2	85.0
Sephadex G-100	83.5	7.4	11.3	5.5	36.7
Q-Sepharose	25.4	0.21	123	60	11.2
Superose 12 PC 3.2/30	2.8	0.005	608	298	1.2

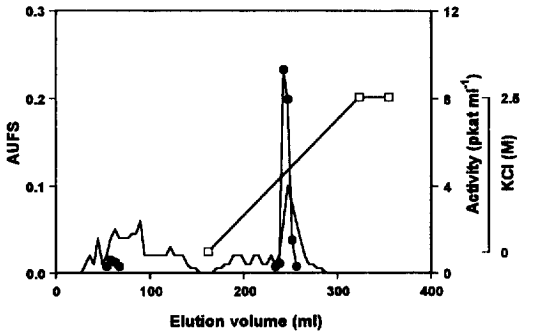

Fig. 1. Peroxidase elution profile on Q-Sepharose anion exchange medium: (—) *A* 280 nm; (-●-) PRX and (-□-) KCL. The column was eluted with a 0 to 2.5 M KCl linear gradient.

Purification

The purification of acidic peroxidase from *Capsicum* fruit is summarized in Table 1, which shows that the purification achieved was close to 300-fold. The Q-Sepharose chromatographic step (Fig. 1) turned out to be one of the most important steps in the purification. The peroxidase activity eluted in two major peaks: the first one, with low activity, corresponds to basic peroxidase; and the second one corresponds to acidic peroxidases. This peak, which appears after having applied the KCl gradient, has much more activity, and a high KCl concentration (1.2 M) was necessary for its elution. The purification factor obtained in this step was 60.4. The last step with the Smart micropurification System provided a purification factor of 298 (Fig. 2), greater or similar to the one obtained by other authors using molecular exclusion and ionic exchange chromatography [11–13].

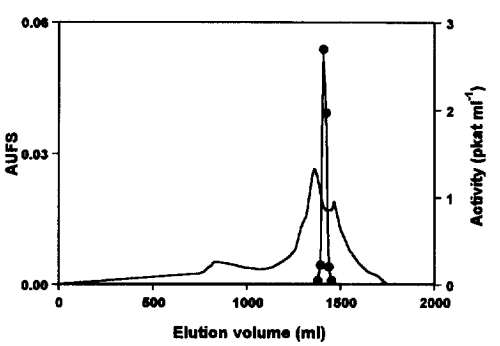

Fig. 2. Peroxidase elution profile on Superose 12 PC 3.2/30: (—) *A* 280 nm and (-●-) PRX.

Enzyme characterization

Figure 3 shows the different steps in the purification process analysed by electrofocusing (IEF). Lane a (crude extract), and lane b (isolated from Sephadex G-100) show acidic and basic peroxidases. In lane d the purified acidic peroxidase appears with a 3.8 pI, similar to that reported in tobacco [14]. Like other fruits [15], the pepper shows several isoenzymes with very different isoelectric points, with acidic peroxidase being predominant in this stage of development.

The native M_r of the acidic peroxidase was estimated to be 50 k by analytical gel filtration. This value is similar to those of *Acer pseudoplatanus* acidic peroxidase (42 k) [16]; peanut isoenzymes (42 k) [17] and tomato isoperoxidase (46 k) [18]. This is consistent with the range between 40 and 50 k which is reported

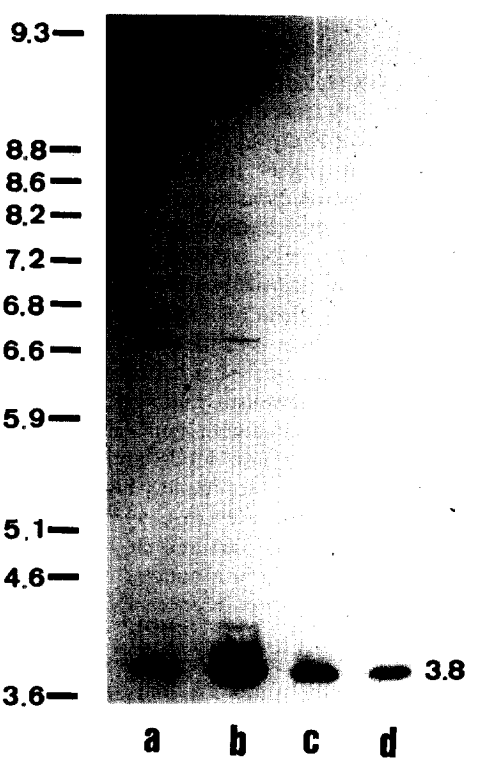

Fig. 3. IEF on polyacrylamide gel 3.5–9.5 pH range of samples obtained in several purification stages: (lane a) crude extract; (lane b) isolate from Sephadex-G100; (lane c) isolate from Q-Sepharose; (lane d) isolate from Superose 12 PC 3.2/30.

in the peroxidases, although other M_rs have been obtained outside this interval.

After incubation at different pH values, the enzyme was stable for 45 min in a range from pH 6 to 9. At pH 5 the enzyme lost activity only after an incubation of 30 min. Moreover, pH values under 5 caused a marked inactivation, and the enzyme stability was lost at pH 3 after 5 min incubation. This effect, which has been attributed to the loss of the heme group at low pH by Burnette [19], has also been reported in other fruits [11].

At 50° the enzyme is very stable and its activity only decreases by 25%. At 60° the stability is lower, because after 15 min of incubation it only maintains a 25% of the total activity, and at higher temperatures the decrease is much more drastic: 5 min incubation is enough to decrease the initial activity by over 90% at 80°, and totally inactivates the enzyme at 100°.

Both peroxidases and lipoxygenases have a high thermostability, attributed to the presence of sugars in their structure [20]. However, this great thermostability cannot be extended to all peroxidase isoenzymes, due to the existence of isoenzymes with different resistance to temperature [15, 21]. The acidic peroxidase of pepper fruit presents, in comparison with others, exhibits a great stability against temperature.

No significant differences were detected with or without $CaCl_2$ in the different assays. These results are in agreement with those reported by other authors [13, 22] who indicated that only cationic peroxidases are susceptible to activation by Ca^{2+} ions.

Kinetic properties

The capsaicin oxidation by *Capsicum* acidic peroxidase was monitored by measuring the increases in A at 262 nm over time. Using 4MN oxidation as a reference, we observed that the capsaicin was more efficiently oxidized by the purified peroxidase isoenzyme than by the total soluble unpurified peroxidase (Table 2).

The dependence of the H_2O_2 reduction rate was fitted for each concentration of capsaicin according to the generally accepted mechanism for peroxidase reaction [22]:

Table 2. Differences in oxidation efficiency between the acidic isoperoxidase and the total soluble unpurified peroxidase

	Fraction Soluble	Acidic
4MN*	6.6	0.46
CAP*	14.4	1.33
CAP/4MN Ratio	2.2	2.9

* 4MN and CAP mean enzymatic activity using 4MN and capsaicin, respectively, as substrate. Values for enzymatic activity are expressed in pkat per g of fr. wt.

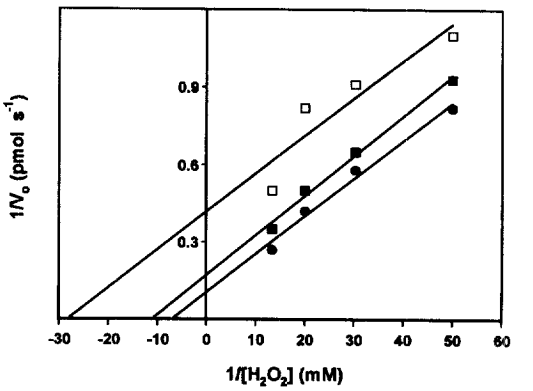

Fig. 4. Reciprocal plots with H_2O_2 as the variable substrate. Capsaicin concentrations: (-□-) 0.4 mM; (-■-) 0.3 mM; (-●-) 0.2 mM.

$$P + H_2O_2 \xrightarrow{K_1} CoI + H_2O$$

$$CoI + AH \xrightarrow{K_2} CoII + A\bullet$$

$$CoII + AH \xrightarrow{K_3} P + A\bullet$$

Where AH is capsaicin and CoI and CoII are the key intermediates in the peroxidase cycle, compound I and compound II.

Assuming that $K_2 > K_3$, as occurs for most peroxidases [23], the steady-state equation rate may be written as:

$$V = \frac{2[E]K_3[\text{Cap}][H_2O_2]}{(K_3/K_1)[\text{Cap}] + [H_2O_2]}$$

The concentration of the enzyme $[E]$ was obtained in relation to known concentrations of HRP. The initial rates of oxidation of capsaicin were determined in the presence of H_2O_2 at various concentrations of capsaicin ranging between 0.2 and 0.4 mM. When reciprocal values of the initial rate of oxidation of capsaicin were plotted against those of H_2O_2 concentration, parallel straight lines were obtained (Fig. 4). Double reciprocal plots allow us to calculate A and B values for each capsaicin concentration, where $A = 2[E]K_3[AH]$ and $B = [K_3/K_1][AH]$. Plotting A *vs* B values results in a straight line (Fig. 5), and from its slope $= 2[E]K_1$, the rate constant K_1 was calculated to be 41 μM^{-1} sec^{-1}.

Similarly, K_3 can be calculated from the slope of parameter A plotted *vs* the concentration of the reducing substrate B, where $A = 2[E]K_1[H_2O_2]$ and $B = [K_1/K_3][H_2O_2]$. Double reciprocal plots (Fig. 6), and plots of parameter A *vs* B values (Fig. 7) resulting in a straight line with a slope $= 2[E]K_3$. In this case the rate constant K_3 was 3.5 μM^{-1} sec^{-1}.

The K_1 value obtained from steady-state kinetic data (41 μM^{-1} sec^{-1}), compared with the K_1 values of barley (1.7, 2.5 and 6.7 μM^{-1} sec^{-1}) [24] and grapevine (1.7 μM^{-1} sec^{-1}) [25] peroxidase, suggests that this isoenzyme reacts more quickly than the other peroxidases in the presence of H_2O_2.

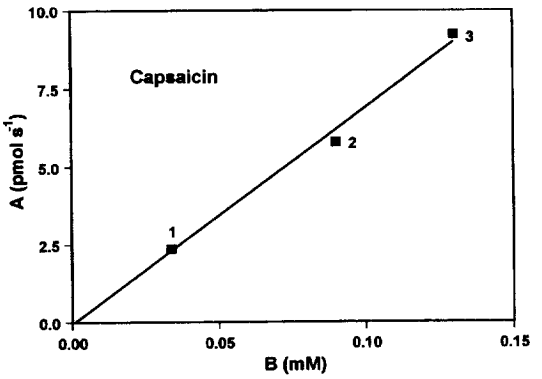

Fig. 5. Plots of parameters *A vs B* obtained by varying H_2O_2 for three capsaicin concentrations: [0.2 mM (1), 0.3 mM (2) and 0.4 mM (3)].

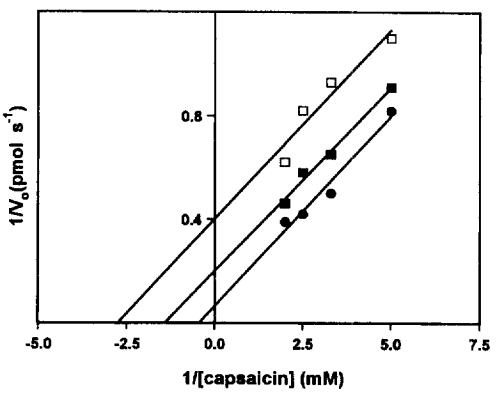

Fig. 6. Reciprocal plots with capsaicin as the variable substrate. H_2O_2 concentrations: (-□-) 0.05 mM; (-■-) 0.033 mM; (-●-) 0.025 mM.

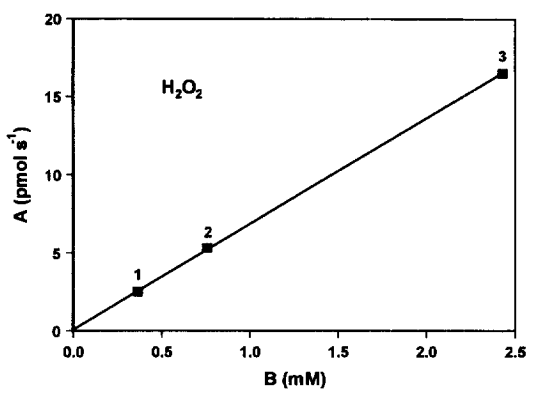

Fig. 7. Plots of parameters *A vs B* obtained by varying capsaicin for three H_2O_2 concentrations: [0.025 mM (1), 0.033 mM (2) and 0.05 mM (3)].

In comparison with the peroxidases previously cited, the K_3 values obtained for pepper fruit peroxidase (3.5 μM^{-1} sec^{-1}) are similar to those obtained by the other authors. Barley peroxidases assayed using different phenolic substrates such as ferulic acid, caffeic acid and conyferyl alcohol show K_3 values between 2.4 and 3.2 μM^{-1} sec^{-1} [24], and grapevine peroxidase for the *trans*-resveratrol shows a K_3 value of 11.9 μM^{-1} sec^{-1} [25]. This reveals that the capsaicin is a good substrate for CoII reduction, similar to the substrates cited above.

We can conclude that fruit pepper acidic peroxidase oxidizes the capsaicin efficiently. Because K_M values are not valid for calculating the peroxidase-catalysed oxidation of capsaicin, since this reaction shows no sign of reversibility, we have applied the recently accepted pattern [23]. By applying this mechanism, we obtained kinetic constants that allowed us to compare acidic pepper peroxidase with other peroxidases.

EXPERIMENTAL

Plant material. Capsicum annuum L. (*var. annuum*) fruits were obtained from a local market and stored at −30°. Calyx and peduncle were discarded before experiments.

Subcellular fractionation. Pericarp and placenta from pepper fruits were homogenized separately at 4° in the presence of 0.25 M sucrose, 1 mM $Mg(OAc)_2$, and 50 mM Tris–HCl, pH 7.2. Soluble, membrane and cell-wall bound frs were prepd as described in ref. [8].

Enzyme extraction and purification. All steps were performed at 4°. The pepper pericarp (40 g) was homogenized in a Waring Blendor in the presence of 80 ml buffer I with the following composition: 0.1 M Tris, 1 mM EDTA, 2 mM DTT, pH 7.5, supplemented with 8 g of PVPP. After filtration through four layers of nylon gauze, the homogenate was centrifuged at 1000 *g* for 15 min. The supernatant was recentrifuged at 27000 *g* for 20 min. The resultant supernatant was considered to be a soluble crude peroxidase fr. Solid $(NH_4)_2SO_4$ was added slowly by stirring to the crude soluble peroxidase fr. to 40% satn, and the resulting ppt. was removed by centrifugation at 10000 *g* for 10 min. The supernatant carrying most of the peroxidase activity was then brought to 85% satn. The ppt. collected by centrifugation at 10000 *g* for 10 min was redissolved in a minimum vol. of buffer II: (50 mM Tris–HCl, pH 7.5), and dialysed against the same buffer for 12 hr. 3 ml of dialysate extract was applied onto a Sephadex G-100 column (27.5 × 1.6 cm) equilibrated with buffer II. A 0.5 ml min^{-1} flow rate of the same buffer was used for elution. The frs were monitored for protein (A_{280}) and peroxidase activity according to the procedures described below. Samples with high sp. act. recovered from the Sephadex G-100 chromatography were applied to anionic-exchange chromatography on a Q-Sepharose column (16 × 2.3 cm) equilibrated with buffer II. Bound peroxidases were eluted with a linear KCl gradient (0–2.5 M) at a flow rate of 0.75 ml min^{-1}. The active frs were combined and dialysed against distilled ×2 H_2O. This extract was loaded onto a Superose 12 PC 3.2/30 column equilibrated with buffer II supplemented with NaCl 0.15 M. A sample was applied to the column and washed with the same buffer at 0.03 ml min^{-1} flow rate. The peroxidase active frs were combined and stored at −30° until analysis.

Peroxidase assay and protein determination. Peroxidase activity was assayed according to ref. [26]. In order to determine the kinetic constants, the oxidation was assayed in a reaction medium containing variable concns of capsaicin and H_2O_2, and 0.1 M Tris–HOAc buffer, pH 6.0. The reaction was initiated by the addition of 50 μl of sample. Oxidation of capsaicin was monitored by the variation in A at 262 nm using an $\varepsilon_{262} = 5.3 \times 103$ mol^{-1} cm^{-1} [5]. Protein content was determined as in ref. [27].

Enzyme pH and heat stability. The pH stability was determined by incubating the enzyme for 5, 15 and 30 min at pH between 3–9 in 50 mM Tris–acetate buffer, and measuring the remaining activity under standard conditions. Heat stability was studied by the incubation of enzyme at various temps between 50–100°, taking aliquots at different times and assaying them under standard conditions.

Molecular mass determination. The M_r of the peroxidase was determined in a Smart System (Pharmacia) using a Superose 12 PC 3.2/30 (3.2 × 300 mm) column. The mobile phase was buffer II containing 0.15 M NaCl, at a flow rate of 30 μl min^{-1}. A mixt. of cytochrome c (12 400), carbonic anhydrase (29 000), bovine serum albumin (67 000) and alcohol dehydrogenase (150 000) were used as the standard proteins.

Isoelectric focusing. IEF was performed on a Pharmacia Multiphor II using Ampholine PAGplate pH = 3.5–9.5 polyacrylamide gels. A kit of isoelectric point markers (IEF Mix 3.6–9.3 Sigma) was used for calculating the pI of the peroxidase. Peroxidase activity was detected in gels by incubating them for 15 min at 25° in 50 mM Tris–HCl buffer, pH 7.5, containing 1 mM 4MN followed by the addition of H_2O_2 to a final concn of 0.33 mM.

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