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Spectral Similarities and Kinetic Differences of Two Tomato Plant Peroxidase Isoenzymes

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ABSTRACT

The kinetic and spectral properties of peroxidases A and B from the dwarf tomato plant were compared. The absolute absorption spectra were essentially the same for peroxidases A and B and their derivatives. Peroxidases A and B had different pH optima with guaiacol as the hydrogen donor but essentially the same optimum when pyrogallol was the substrate. The substrate concentrations required for optimum activity were different not only for the different substrates but also for each isoenzyme. When pyrogallol was used as the substrate, peroxidases A and B were 80% active when assayed under conditions optimal for the other isoenzyme. When guaiacol was used as the substrate, peroxidase A was completely inactive when assayed under conditions optimal for peroxidase B. In this case the pH was not optimum and the H_2O_2 concentration was inhibitory. Similarly, peroxidase B retained only 9% of its peroxidase activity toward guaiacol when assayed under conditions optimum for peroxidase A. In this case the pH was not optimum and the H_2O_2 was limiting. A possible role for peroxidase isoenzymes is discussed.

The reasons for the existence of peroxidase isoenzymes have not yet been established. As a step toward providing a rationale for their physiological significance, we have characterized differences in 2 of the 12 discrete peroxidase isoenzymes previously identified in the extreme dwarf tomato shoot (3). Large differences in activity occur depending on the hydrogen donor substrate and the reaction conditions. The 2 peroxidases, isoenzymes A and B, were separated and purified in sufficient quantities to permit spectral and kinetic studies. Purification was based on the fact that these 2 major water-soluble peroxidases in the extreme dwarf tomato plant migrate at different rates toward the anode during electrophoresis at pH 8.8 (3, 4). Studies were limited to the use of pyrogallol and guaiacol as the hydrogen donor substrates.

MATERIALS AND METHODS[2]

Peroxidase isoenzymes A and B from the extreme dwarf tomato plant were separated and purified as previously described, utilizing starch gel electrophoresis (3). All spectral and kinetic data were obtained from peroxidase isoenzymes separated by that procedure. Subsequently, it was found that peroxidases A and B could be obtained in larger quantities with greater ease by preparative continuous flow, carrier-free electrophoresis, utilizing sucrose to increase the density of the separating buffer (4).

The RZ values, absorbancy ratio 405 nm/280 nm (20), for peroxidase isoenzymes A and B were 1.6 and 1.0, respectively, at pH 5.5.

Spectral Determinations. The absolute absorption spectra of the peroxidase isoenzyme preparations and their derivatives were recorded, using a Bausch and Lomb Spectronic 600 spectrophotometer equipped with a Sargent model SRL recorder at a scan rate of 250 nm/min.

The peroxidase solutions were dialyzed against 0.025 M phosphate buffer at pH 5.5. Aliquots of 0.6 ml of the peroxidase solutions were placed in microcell cuvettes having a 1-cm light path and a capacity of 1 ml. Native or oxidized peroxidase consisted of the untreated peroxidase solutions. Reduced peroxidase was prepared by adding a few crystals of sodium dithionite to the peroxidase solutions. Reduced CO peroxidase was prepared by bubbling CO through the reduced peroxidase solutions for 60 sec. Alkaline peroxidase was prepared by adjusting the native peroxidase solutions to pH 10 with NaOH.

The reduced pyridine hemochromogens of the peroxidases were prepared using the technique of Morrison and Horie (16) with slight modification. Aliquots of 0.05 ml of 1 N NaOH and 0.2 ml of pyridine were added to 0.6 ml of the peroxidase solutions. A few crystals of dithionite were added to complete the reduction, and the absorbancy was then determined.

Kinetic Determinations. Baker reagent grade 30% H_2O_2 was freshly diluted, and peroxide concentration was determined by Kingzett's iodide method (11). Pyrogallol solutions were prepared fresh daily with Baker reagent grade pyrogallol. Guaiacol was obtained from the Aldrich Chemical Company, Inc., and was distilled in an 8 mm (internal diameter) × 61 cm long vacuum-jacketed, platinum spinning band column. The cut boiling at 94 to 96° (20 mm mercury) was used. At this head temperature the pot temperature was 104°. The crystalline guaiacol so obtained had a melting point of 28 to 29° and a refractive index of 1.5435.

The peroxidations of pyrogallol and guaiacol were followed at 25° in a Beckman DK-2A ratio recording spectrophotometer at 430 and 470 nm, respectively (2). The reactions were carried out in a total volume of 2.4 ml, and reaction rates were determined when they were linear with time. Under optimal conditions, all reactions proceeded at a rate of 0.2 to 0.3 OD/min.

The pH optima of peroxidases A and B were determined with acetate buffers from pH 3.5 to 5.5, phosphate buffers from pH 5.5 to 8.0, and tris-HCl buffers from 8.0 to 9.0. Protein was determined by the method of Lowry et al. (13), with bovine serum albumin as the standard.

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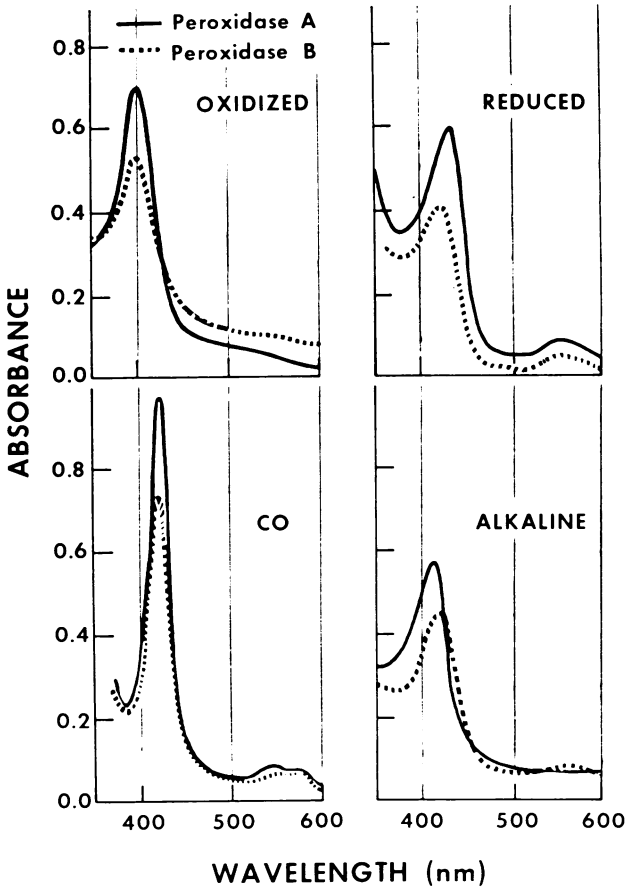

FIG. 1. Absolute absorption spectra of peroxidases A and B in the oxidized, reduced, CO-reduced, and alkaline states.

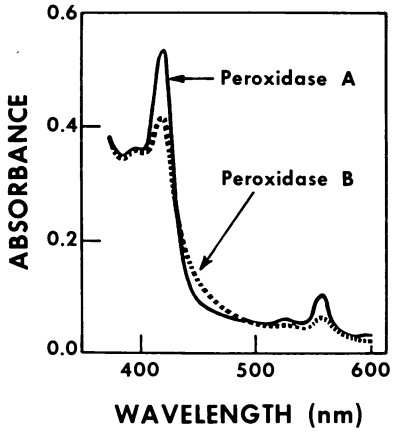

FIG. 2. Absorption spectra of the pyridine hemochromogens for peroxidases A and B.

RESULTS

Figure 1 shows the absolute absorption spectra of peroxidases A and B in the oxidized, reduced, CO-reduced, and alkaline states. Both isoenzymes had an absorption maximum in the Soret region at 405 nm in the oxidized or native form. Absorption maxima at 435 and 556 nm were evident for peroxidases A and B in the reduced state while both had absorption maxima at 424, 547, and 573 nm in the CO-reduced state. In the alkaline state, peroxidase A had an absorption maximum at 412 nm and peroxidase B at 418 nm. The absorption spectra of the pyridine hemochromogens for peroxidases A and B are shown in Fig. 2. Absorption maxima occur at 397, 420, 527, and 558 nm. As can be seen, peroxidases A and B and their derivatives were almost identical to each other in their absorption spectra and very similar to the absorption spectra of other peroxidases and hemin (6, 9, 14, 22).

The pH optima for peroxidases A and B with pyrogallol as substrate were very similar, but when guaiacol was used as the

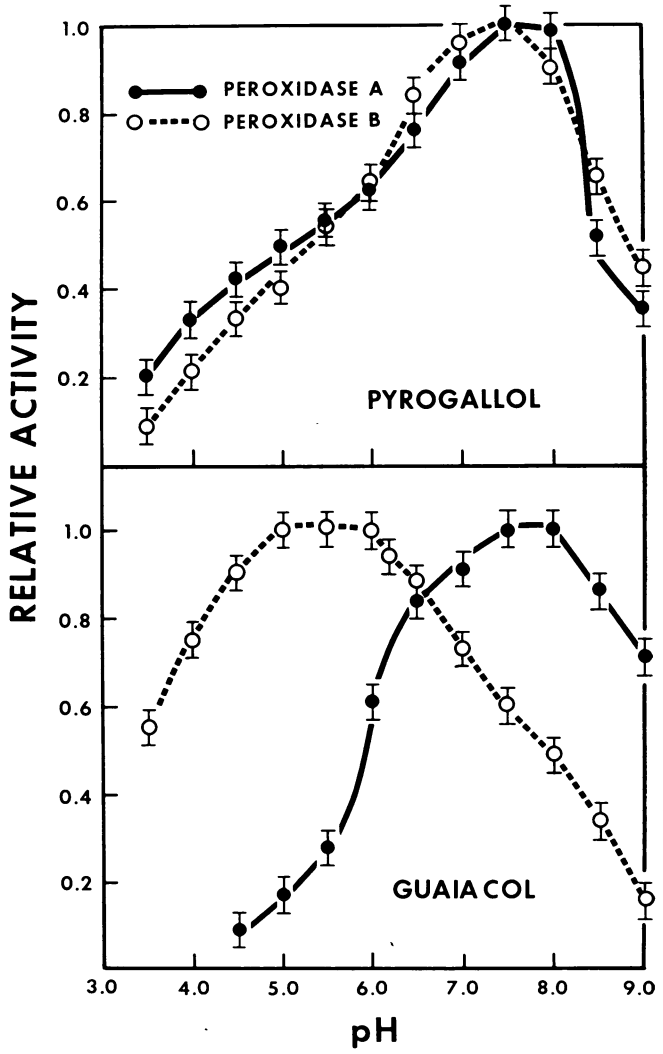

FIG. 3. pH optima for peroxidases A and B with pyrogallol and guaiacol as substrates. See Table I for assay conditions.

Table I. *Optimal Conditions for Peroxidases A and B with Pyrogallol and Guaiacol as Substrates*

The incubation mixture had a total volume of 2.4 ml.

	Pyrogallol		Guaiacol	
	Peroxidase A	Peroxidase B	Peroxidase A	Peroxidase B
Pyrogallol (μmoles)	5	5	...	...
Guaiacol (μmoles)	...	...	100	40
H_2O_2 (μmoles)	2	5	1	10
Protein (μg)	0.33	0.29	0.22	0.09
100 μmoles of buffer (pH)	7.75	7.5	7.75	5.5

Table II. *Specific Activities of Peroxidases A and B with Pyrogallol and Guaiacol as Substrates*

The assay conditions are described in Table I.

Peroxidase	Specific Activity		Pyrogallol to Guaiacol Ratio
	Pyrogallol	Guaiacol	
	μmoles substrate utilized[1]/mg protein × min		
A	1600	500	3.2
B	3400	1300	2.6

[1] Maehly and Chance (15).

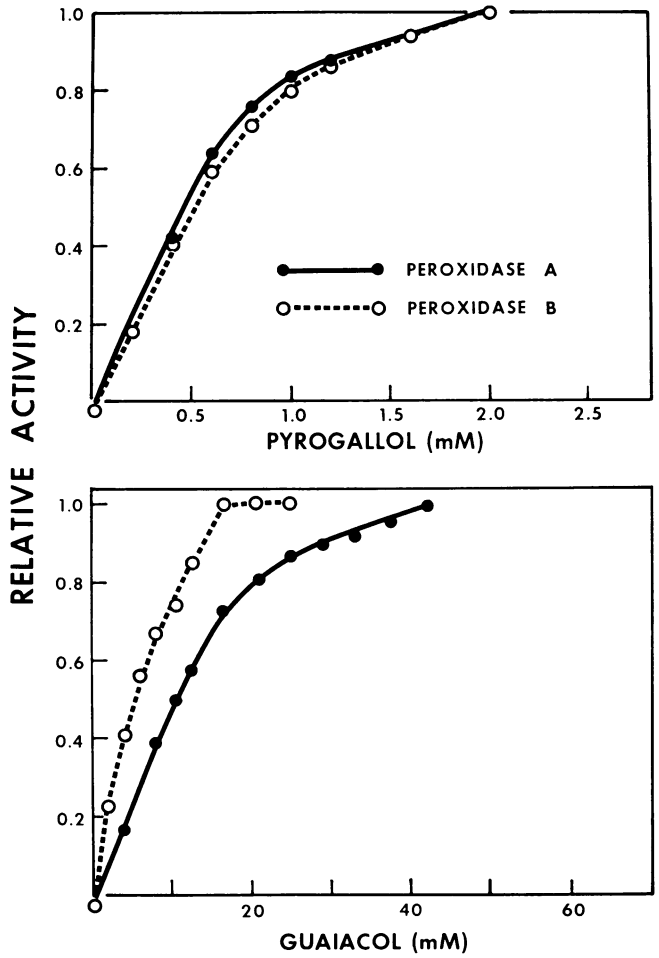

FIG. 4. Effect of substrate (hydrogen donor) concentration on peroxidase isoenzyme activity. Conditions for the assays are given in Table I.

substrate the optima were very different (Fig. 3). The pH optimum for peroxidase A was the same for both substrates, but that for peroxidase B was about 2 pH units lower with guaiacol than with pyrogallol as the substrate.

Peroxidases A and B had different substrate (hydrogen donor) and H_2O_2 concentration requirements for maximal activity. Table I lists the conditions necessary for maximal activity for peroxidases A and B with pyrogallol and guaiacol as substrates. All subsequent data were obtained from assays carried out under the conditions listed in Table I.

The specific activities of peroxidases A and B with pyrogallol and guaiacol as substrates are shown in Table II. The ratios of pyrogallol to guaiacol utilized with both peroxidases were very similar and indicated a slight degree of substrate specificity. Peroxidases A and B were about three times more "efficient" when pyrogallol was used as the substrate.

The effect of substrate (hydrogen donor) concentration on peroxidase isoenzyme activity is shown in Fig. 4. Both peroxidases A and B attained maximum activity with pyrogallol as the substrate at a concentration of 2 mM. When guaiacol was used as the substrate, peroxidases A and B had different substrate concentration requirements for maximum activity. Peroxidase A required 42 mM guaiacol for maximum activity while peroxidase B required a concentration of 17 mM guaiacol.

As indicated in Table I, peroxidases A and B required different H_2O_2 concentrations for maximal activity. Figure 5 shows the effect of various concentrations of H_2O_2 on peroxidase A and B activities with pyrogallol and guaiacol as hydrogen donors. With pyrogallcl as the substrate, peroxidases A and B showed maximal activities at similar H_2O_2 concentrations. As the H_2O_2 concentration was increased above that for maximal activity, peroxidases A and B were gradually inhibited but still retained about

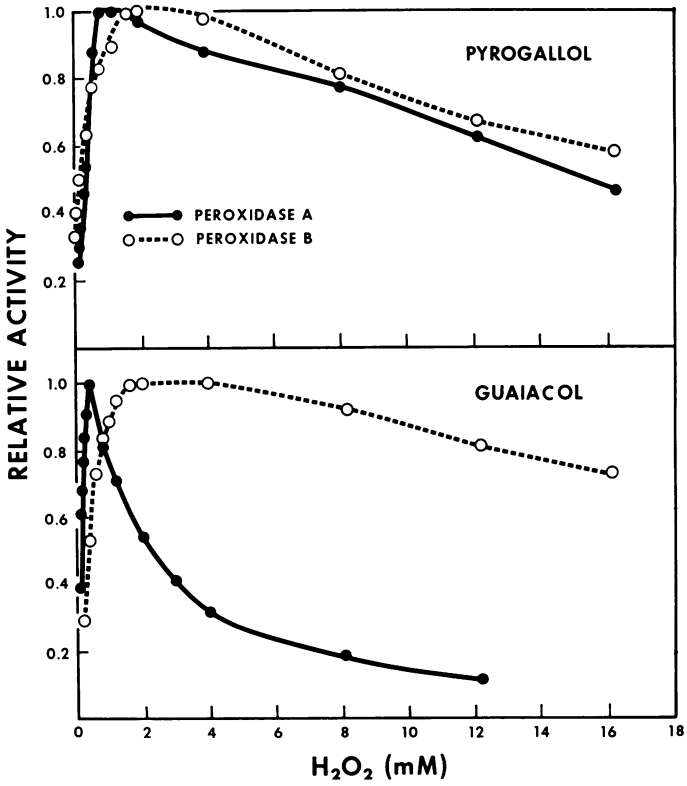

FIG. 5. Effect of H_2O_2 concentration on peroxidases A and B with pyrogallol and guaiacol as hydrogen donors. Conditions for the assays are given in Table I.

Table III. *Activities of Peroxidases A and B with Pyrogallol and Guaiacol When Assayed under Conditions Optimal for Peroxidases B and A, Respectively*

The optimal assay conditions are given in Table I.

Conditions Optimal for Peroxidase	Pyrogallol		Guaiacol	
	Relative activity of peroxidase		Relative activity of peroxidase	
	A	B	A	B
A	1.0	0.8	1.0	0.09
B	0.8	1.0	0	1.0

50% of their activity at H_2O_2 concentrations of 16 mM. When guaiacol was used as the substrate peroxidase B had about the same H_2O_2 requirement for maximal activity as with pyrogallol as substrate but was only 30% inhibited at an H_2O_2 concentration of 16 mM. Peroxidase A, however, was quite different in that maximum activity occurred at an H_2O_2 concentration that was less than half that required for peroxidase B. In addition, as the H_2O_2 concentration was increased above the amount required for maximal activity, peroxidase A was sharply inhibited. At H_2O_2 concentrations optimal for peroxidase B activity, peroxidase A was inhibited 50 to 70%. Peroxidase A retained only about 10% of its activity at an H_2O_2 concentration of 12 mM while peroxidase B was still 80% active.

Since peroxidases A and B had different H_2O_2 requirements and pH optima with the different substrates for maximal activity, it was of interest to see how active one peroxidase would be under the optimal conditions of the other peroxidase. Peroxidase A, therefore, was assayed under conditions that were optimal for peroxidase B and peroxidase B was assayed under conditions that were optimal for peroxidase A with the two substrates. The results are shown in Table III. When pyrogallol was used as the substrate, peroxidases A and B were still 80% active when assayed under conditions optimal for peroxidases B and A, respectively. This would be expected since the pH optima and H_2O_2 requirements were similar. When guaiacol was used as the substrate, peroxidase A was completely inactive when assayed under conditions optimal for peroxidase B. In this case the pH was not optimal and the H_2O_2 concentration was inhibitory. Similarly, peroxidase B retained only 9% of its peroxidase activity toward guaiacol when assayed under conditions optimal for peroxidase A. In this case, the pH was not optimal and the H_2O_2 was limiting.

DISCUSSION

In a previous publication (5), it was shown by starch gel electrophoresis that various tissues and organs of the tomato plant contained different complements of peroxidase isoenzymes. When the starch gels were stained for peroxidase activity with 0.1 M pyrogallol or guaiacol and 5 mM H_2O_2 at pH 5.5, peroxidase A would not stain when guaiacol was used as the hydrogen donor. It was therefore suggested that this might be due to substrate specificity. The present results show that the lack of peroxidase A activity in the starch gel with guaiacol was primarily due to an inhibition of the enzyme due to the pH and H_2O_2 concentration. Peroxidase B was 100% active under the same conditions. Differential staining of peroxidase isoenzymes with varying H_2O_2 concentrations has also been observed for bean peroxidase isoenzymes separated by disk electrophoresis (17). Similarly, peroxidase isoenzymes from wheat demonstrated varying activities toward *p*-phenylenediamine and guaiacol after starch gel electrophoresis (18).

The tomato peroxidase isoenzymes A and B were both capable of oxidizing pyrogallol and guaiacol in the presence of H_2O_2. Peroxidases A and B had essentially the same substrate requirements for maximal activity when pyrogallol was used as the substrate. However, when guaiacol was used as the hydrogen donor, peroxidases A and B had different hydrogen ion and H_2O_2 requirements for maximal activity. This is not unusual since other peroxidase isoenzymes from various plant sources have been shown to differ in their substrate requirements (8–10, 12, 17–19). Peroxidase A, however, was completely inhibited at pH 5.5 under the optimal substrate conditions for peroxidase B, while peroxidase B was less than 10% active at pH 7.75 under the optimal substrate conditions for peroxidase A. This suggests a possible role for peroxidase isoenzymes in that one isoenzyme is completely inactive under conditions optimal for another isoenzyme with a given substrate. It is not unreasonable to speculate that specific peroxidase isoenzymes are synthesized by specific tissues or organs in accordance with or response to the cellular environment and availability of specific substrates. Peroxidase B, for example, could operate very well under acidic conditions with a guaiacol-type substrate. The tomato fruit provides an acidic environment (1, 21) and, of the 12 peroxidases found in the tomato plant, peroxidase B is the only one found in the fruit (5). Peroxidase A is found in the epidermis, xylem, phloem fibers, and collenchyma cells of the tomato shoot (7), but the biochemical environment and the identity and availability of the natural substrate(s) are not known.

The fact that the various tissues and organs contain different complements of peroxidase isoenzymes and the large difference in activity found for the two separated isoenzymes imply a purpose rather than an accident of synthesis that yields merely a difference in electrophoretic behavior.

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