

# Tomato Peroxidase

## PURIFICATION, CHARACTERIZATION, AND CATALYTIC PROPERTIES<sup>1</sup>

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### ABSTRACT

A major peroxidase has been found in the tomato pericarp (*Lycopersicon esculentum* var. Tropic) of the ripe and green fruit. A purification scheme yielding this enzyme approximately 85% pure has been developed. The tomato enzyme resembles horseradish peroxidase (HRP) in a standard peroxidase assay and in its ability to be reduced to ferropoxidase, to be converted to oxyferropoxidase (compound III), and to form peroxidase complexes with hydrogen peroxide (compounds I and II). In contrast to the HRP, the tomato peroxidase fails to catalyze the aerobic oxidation of indole-3-acetic acid in the presence of 2,4-dichlorophenol and manganese. The tomato peroxidase can be resolved into two nonidentical subunits in the presence of dithiothreitol while HRP remains as a single polypeptide chain after such treatment. Dithiothreitol is oxidized in the presence of tomato or horseradish peroxidase with the enzymes accumulating in their oxyferropoxidase forms during the oxidation reaction. Whereas HRP returns to its free ferric form at the end of the reaction, the tomato enzyme is converted into a form that absorbs at 442 nanometers.

A single peroxidase which has been shown to exist in tomato fruit extracts and to exhibit some IAA oxidase activity (5, 8) has been implicated both in the production of ethylene (12, 17, 18) and in the destruction of the plant growth hormone IAA (8). Although it is not unusual to relate the action of the enzyme peroxidase with the control of the above hormones, in the case of tomato fruit it is premature to assume such a relationship because of insufficient information about the physical and catalytic properties of this peroxidase and the lack of a purification method which would allow a quantitative estimation and a complete isozyme composition of the noncovalently bound peroxidases of the fruit (22, 23).

Spectral properties and pH optima of the tomato fruit peroxidase in catalyzing the oxidation of redogenic substrates in the presence of H<sub>2</sub>O<sub>2</sub> have been previously reported by Evans (6). The present report deals with additional properties of the purified enzyme including its capability to be converted to complexes of higher oxidation states (compounds I, II, III) which are thought to be important for the IAA oxidase activity of peroxidases (13, 26). The enzyme HRP,<sup>3</sup> a well studied peroxidase, was employed in order to compare some of its properties with similar properties of the tomato enzyme.

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<sup>3</sup> Abbreviations: HRP: horseradish peroxidase; DCP: 2,4-dichlorophenol.

### MATERIALS AND METHODS

**Extraction of Crude Peroxidases.** Fully grown green or ripe tomatoes (*Lycopersicon esculentum* var. Tropic) were picked from plants grown in a greenhouse during fall and spring. Tomato pericarp (640 g) was homogenized in a Waring Blendor in the presence of 1 liter of 0.1 M phosphate buffer (pH 6.5) supplemented with 20 g of insoluble PVP from Calbiochem and 10 g of sodium ascorbate. The homogenate was filtered through a triple layer of cheesecloth, and the residue was washed twice with 100 ml of 0.1 M phosphate buffer (pH 6.5). The filtrates were combined and centrifuged at 2,000 g for 10 min. The supernatant, to be referred to as crude soluble peroxidase fraction, was stored at 0 C for further use. The pellet and the solid material on the cheesecloth were suspended in 200 ml of 0.2 M sodium maleate—0.2 M calcium chloride adjusted to pH 6.5. The suspension was then sonicated for 5 min and centrifuged at 2,000g for 10 min. Under this treatment peroxidases that were ionically bound to the tomato pulp were solubilized (17). These peroxidases remained in solution after the supernatant was dialyzed twice against 4 liters of 5 mM phosphate buffer (pH 6.5) for 20 hr and will be referred to as crude ionically bound peroxidases. All steps of the above procedure took place at 4 C.

**Ammonium Sulfate Precipitation.** Ammonium sulfate precipitation was used as a purification step for the crude soluble peroxidase fraction but not for the crude ionically bound peroxidases. In the former case, solid ammonium sulfate was added slowly with stirring to the crude soluble peroxidase fraction to 40% saturation, and the resulting precipitate was removed by centrifugation. The supernatant carrying most of the peroxidase activity was then brought to 85% saturation. The precipitate, collected by centrifugation, was dissolved in 50 ml of distilled H<sub>2</sub>O and dialyzed twice against 4 liters of 5 mM phosphate buffer (pH 6.5) for 20 hr. Inactive precipitate was removed by centrifugation. All steps of the above procedure were carried out at 4 C.

**Ion Exchange Chromatography.** Ion exchange chromatography of the ammonium sulfate-fractionated soluble peroxidase and of the crude ionically bound peroxidase preparations was carried out separately on columns (2.5 × 40 cm) of QAE-Sephadex A-50 anion exchanger from Pharmacia, equilibrated with 5 mM phosphate buffer (pH 6.5). Material absorbed on the column was eluted with a linear gradient of 0 to 0.5 M in NaCl 5 mM phosphate buffer (pH 6.5) and rechromatographed on a similar column with a linear gradient of 0 to 0.4 M NaCl in 5 mM phosphate buffer (pH 6.5). All steps of the ion exchange chromatography were carried at 4 C.

**Alternative Method.** In an alternative method of purification usually applied to preparations from green tomato pericarp, the gradient elution of peroxidase from the first QAE-Sephadex was replaced by a single step elution with 0.27 M NaCl in 5 mM phosphate buffer (pH 6.5). Subsequent steps were the same.

**Bio-Gel P-150 Filtration.** Samples with high peroxidase activity

recovered from the second QAE-Sephadex column fractionation of the soluble peroxidases were pooled, dialyzed twice against 4 liters of distilled H<sub>2</sub>O for 10 hr and concentrated by placing the dialysis sac overnight on a small amount of aquacide powder (1–2 g) purchased from Calbiochem. During this procedure a white precipitate was formed and then removed by centrifugation without reduction of the total peroxidase activity of the supernatant. Three ml of the supernatant containing 4 to 6 × 10<sup>6</sup> units of peroxidase activity were subjected to gel filtration on a column (2 × 75 cm) of Bio-Gel P-150, 100 to 200 mesh from Bio-Rad Lab swollen in 10 mM phosphate buffer (pH 7.6). All steps of the above procedure were carried at 4 C.

**Peroxidase Assay.** The assay of peroxidase activity was a variation of Luck's method (14). Prior to each assay, 1.7 ml of 4.6 mM *p*-phenylenediamine hydrochloride solution and 1 ml of 8.8 mM H<sub>2</sub>O<sub>2</sub> solution were added to 9.7 ml of 0.06 M phosphate buffer (pH 6.8). A 2.9-ml aliquot of this mixture accompanied by 0.1 ml of enzyme solution was added to a glass cuvette (1-cm path). The changes in the OD were measured at 485 nm with a Perkin-Elmer 124 spectrophotometer with recorder. Changes of OD higher than 0.4/min were avoided by diluting the enzyme. Assays were carried out at 24 C. Units of peroxidase activity were calculated according to the empirical formula:

$$\text{Units/ml} = \frac{\Delta \text{OD/min} \times \text{dilution factor} \times 1,000}{\text{ml of enzyme used in the assay}}$$

**IAA Oxidase Assay.** Reagents were mixed in the reaction chamber of a Gilson 20 oxygraph to give a solution of the following composition: 2.5 mM citrate buffer (pH 4.0), 0.075 mM DCP, 0.025 mM MnCl<sub>2</sub>, 0.75 mM IAA, and 120,000 units of peroxidase. The total volume of the reaction mixture was 1.6 ml. The reaction was started by the addition of the enzyme, and O<sub>2</sub> uptake was monitored. The temperature of the reaction was kept at 24 C by circulating water.

**Protein Determination.** Protein was determined as described by Bradford (1).

**Polyacrylamide Gel Disc Electrophoresis.** Disc electrophoresis of peroxidases was carried out by a simplified variation of the method described by Davis (4). Gels were stained for protein as described in the same method; peroxidase isozymes were visualized by immersing the developed gels in a solution of acetate buffer (pH 6.0) containing 1% benzidine and 0.3% H<sub>2</sub>O<sub>2</sub> for 30 sec. Gels were stored in water.

**SDS-Polyacrylamide Gel Electrophoresis.** SDS electrophoresis of peroxidases was done as described by Fairbanks *et al.* (7). Mol wt were estimated by using appropriate standards.

**HRP.** Horseradish peroxidase was purchased from Worthington Co. (HPOD 553441). The R<sub>z</sub> (A<sub>403</sub>/A<sub>280</sub>) value of its solutions was 1.4, and it contained 2.5 × 10<sup>8</sup> units of peroxidase activity/μmol.

**Concentration of Peroxidase in Solutions.** The HRP concentration was determined from the A at 403 nm, using a millimolar A coefficient of 107 (21) for the ferric form of the enzyme at that wavelength. The concentration of the tomato peroxidase was estimated in the same way.

## RESULTS

**Purification.** A summary of the extraction and purification procedures for the tomato pericarp soluble and ionically bound peroxidases is given in Figure 1. The purification scheme of a typical anionic peroxidase preparation from ripe tomato pericarp is shown in Table I. Similar purification schemes were obtained for preparations from green tomato pericarp at various stages of development. The peroxidase activity of the soluble fraction from the green fruits was always considerably higher due to the anionic peroxidase which then declined with ripening of the fruit.

**QAE-Sephadex Chromatography of Soluble Peroxidases.** The elution pattern of the ammonium sulfate-fractionated soluble peroxidase fraction from ripe tomato pericarp is shown in Figure

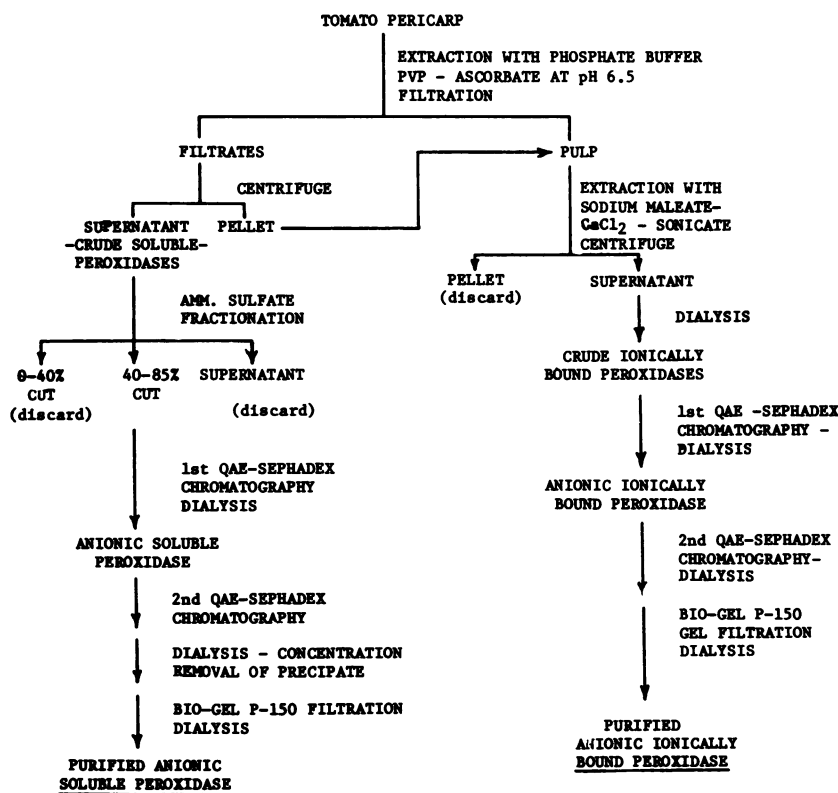


FIG. 1. Outline for extraction and purification of anionic tomato peroxidases.

2. The bulk of the peroxidase activity, due to anionic peroxidase, was eluted when the NaCl gradient reached 0.31 M, while traces of peroxidase activity appeared scattered in the first 100 ml of the elution volume. Rechromatography of the major peroxidase peak under similar conditions yielded no significant loss of peroxidase activity and gave no evidence of interconversion of peak material, while a considerable reduction of the protein content of the peak was observed. Preparations extracted from green tomato pericarp gave similar elution patterns.

**QAE-Sephadex Chromatography of Ionically Bound Peroxidases.** Figure 3 shows the elution pattern of the crude ionically bound peroxidase fraction from ripe tomato pericarp to be essentially the same as that of soluble peroxidase. Although peroxidase

activity eluted in the first 100 ml was 18% of the activity eluted in the main peak, it was still negligible when compared with the activity of the soluble anionic peroxidase. Rechromatography of the major peroxidase peak under similar conditions did not give any significant improvement to the specific activity of the peroxidase. Crude ionically bound peroxidase fraction from green tomato pericarp gave similar elution patterns.

**Bio-Gel P-150 Filtration.** Soluble or ionically bound anionic peroxidase subjected to Bio-Gel P-150 filtration showed one major peak of protein which contained all of the peroxidase activity. When preparations purified according to the alternative method were subjected to the same treatment, an additional protein peak preceded the peroxidase peak. In either case the  $R_z$  value of the eluted peroxidase was 1.6.

During Bio-Gel filtration of anionic peroxidase, the enzyme always appeared at elution volume 1.5. BSA (mol wt 70,000) subjected to Bio-Gel filtration under the same conditions also had an elution volume of 1.5 which suggests that the tomato anionic peroxidase has a mol wt similar to that of BSA.

**Polyacrylamide Gel Disc Electrophoresis.** Purified soluble or ionically bound anionic peroxidase preparations from ripe and green tomato pericarp were tested for purity and isozyme composition by disc electrophoresis. All of the gels contained one major band when stained for either protein or peroxidase activity (Fig. 4). Scanning of the gels stained for protein in a Gelman gel scanner indicated that the major band contained 76 to 87% of the total protein. Minor protein impurities appeared in all preparations. Higher purities were observed for the soluble anionic peroxidase preparations.

Gels stained for peroxidase activity revealed the existence of one major peroxidase band which accounted for at least 85% of the color. This band was always accompanied by four (satellite) bands.

**SDS-Polyacrylamide Gel Electrophoresis.** When preparations of anionic peroxidase similar to those used for disc electrophoresis were subjected to SDS electrophoresis in the presence of DTT, two major bands appeared in the gels stained for protein. The

Table I. Purification of Soluble and Ionically Bound Peroxidases from Ripe Tomato Pericarp

Fraction	Total Activity Units x $10^{-5}$	Total Protein mg	Specific Activity Units x $10^{-5}$ /mg	Purification Factor	Recovery %
<u>Soluble</u>					
40% $(\text{NH}_4)_2\text{SO}_4$ Supernatant	105	462.1	0.23	----	----
40-85% $(\text{NH}_4)_2\text{SO}_4$ Cut	99	128.0	0.77	3.35	94.3
1st QAE-Sephadex	78	53.2	1.47	6.37	74.3
2nd QAE-Sephadex	77	6.2	12.42	54.00	73.3
After Dialysis and Concentration	77	4.0	19.25	83.69	73.3
Bio-Gel P-150	56	2.2	25.45	110.65	53.3
<u>Ionically Bound</u>					
Crude	8	102.3	0.08	----	----
1st QAE-Sephadex	7	2.1	3.33	41.66	87.5
2nd QAE-Sephadex	6	1.0	6.00	75.00	75.0
After Dialysis	6	0.8	7.50	93.00	75.0

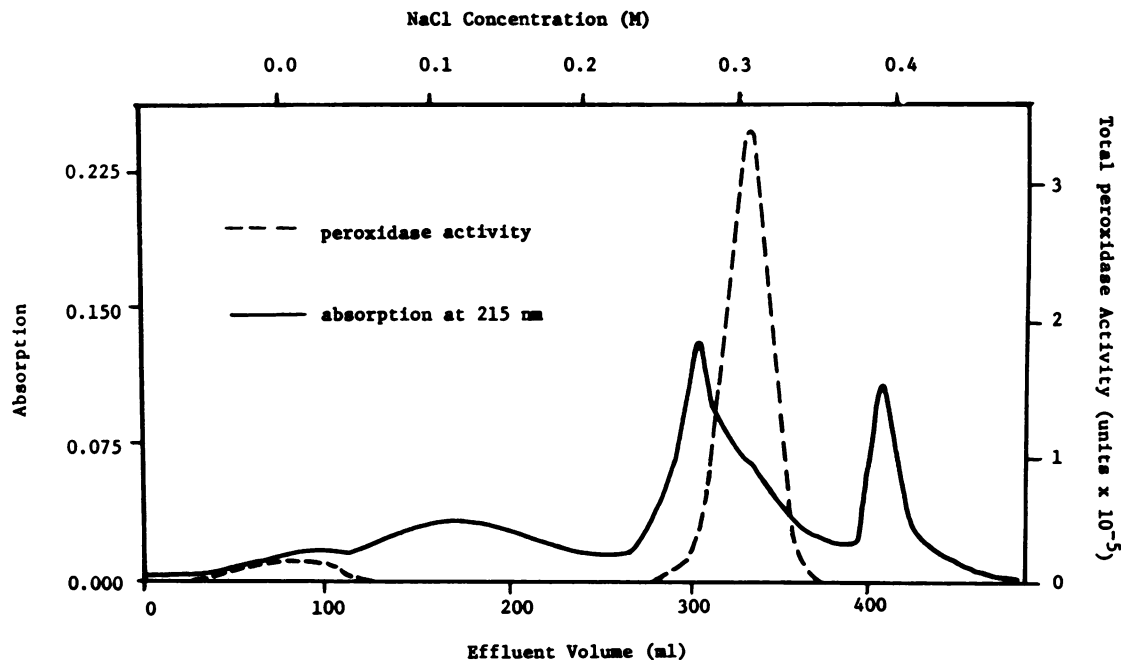


FIG. 2. Ion exchange chromatography of the 40 to 85% cut of the crude soluble peroxidase from ripe tomato pericarp on QAE-Sephadex. The 80-ml sample having  $9.9 \times 10^6$  units of peroxidase activity and containing 128 mg of protein in 5 mM phosphate buffer at pH 6.5 was applied on the column ( $40 \times 2.5$  cm) and eluted with 5 mM phosphate buffer (pH 6.5) with a NaCl linear gradient. Fractions (3 ml) were collected and assayed for their  $A$  at 275 nm and total peroxidase activity.

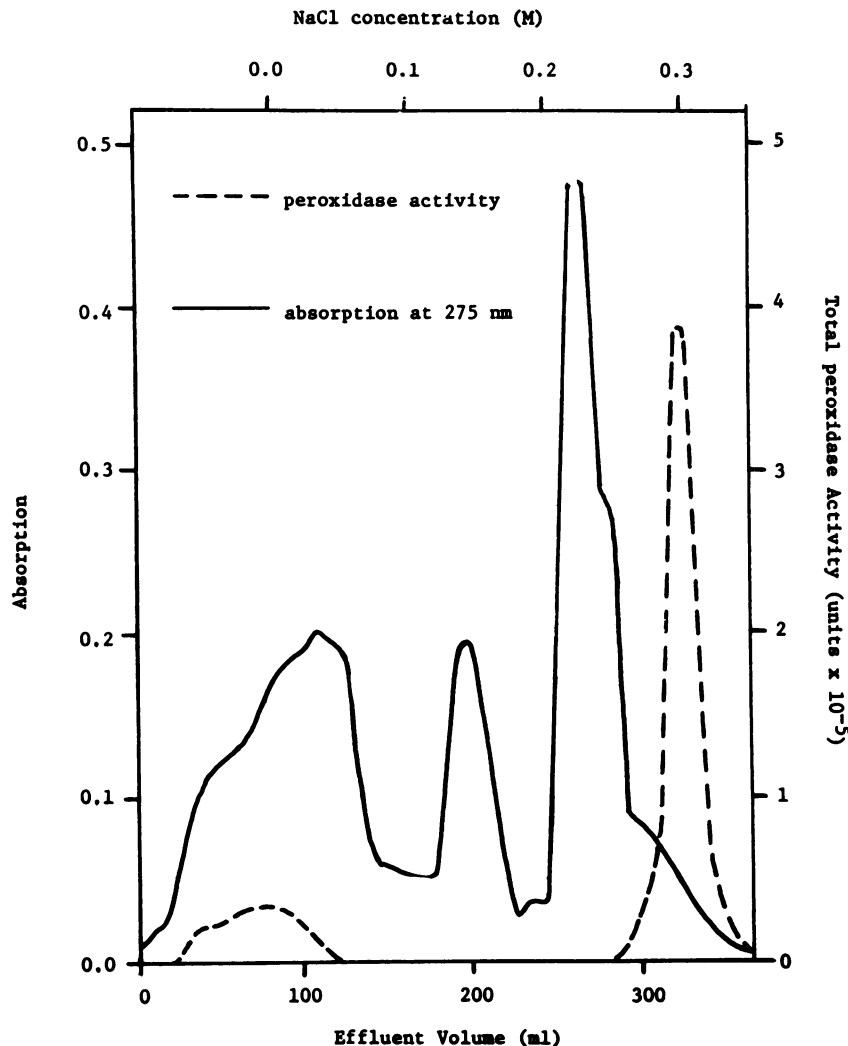


FIG. 3. Ion exchange chromatography of the crude ionically bound peroxidase from ripe tomato pericarp on QAE-Sephadex. The 80-ml sample having  $0.8 \times 10^6$  units of peroxidase activity and containing 100 mg of protein in 5 mM phosphate buffer at pH 6.5 was applied on the column ( $40 \times 2.5$  cm) and eluted with 5 mM phosphate buffer (pH 6.5) with a NaCl linear gradient. Fractions (3 ml) were collected and tested for their  $A$  at 275 nm and total peroxidase activity.

relative mobility values ( $R_f$ ) of 0.58 and 0.80 for the two bands correspond to approximate mol wt of 46,000 and 19,000, respectively. Commercial HRP, on the other hand, gave one band that corresponded to a mol wt of 40,000 (2, 16).

**Activity of Tomato Anionic Peroxidase.** Purified ionically bound and soluble anionic peroxidase preparations from ripe or green pericarp were found to have specific activity of  $2.0 \pm 0.1 \times 10^8$  units/ $\mu$ mol, while the commercial HRP had a specific activity of  $2.5 \pm 0.1 \times 10^8$  units/ $\mu$ mol, with enzyme concentrations being calculated from the heme group absorbance.

A specific activity of  $1.77 \times 10^8$  units/ $\mu$ mol calculated from the data in Table I and an estimated mol wt of 70,000 are consistent with a preparation of 84 to 89% purity.

**Effect of DTT on Tomato Anionic Peroxidase.** Horseradish peroxidase has the capacity to oxidize DTT especially at high pH values (20). The oxidation of this substrate as catalyzed by HRP causes the conversion of the enzyme to compound III, which is a complex of the ferropoxidase with molecular  $O_2$  and which accumulates in the reaction mixture.

Addition of DTT to solutions of the anionic peroxidase from tomato pericarp resulted in the conversion of the enzyme to its compound III form while oxidation of DTT took place. In this

respect the two peroxidases behaved similarly. After the exhaustion of the  $O_2$  in the reaction mixture the tomato peroxidase compound III did not return to the ferric form as did HRP compound III; instead, it was converted to a form that absorbed at 442 nm (Fig. 5). The existence of such a form is unknown in the case of HRP or any other peroxidase, and therefore was given the name  $P_{442}$ .

**Bio-Gel P-150 Filtration of  $P_{442}$ .** Immediately after treating tomato anionic peroxidase with DTT, the resulting  $P_{442}$  was subjected to Bio-Gel P-150 filtration (Fig. 6A). Figure 6B shows the elution pattern of untreated peroxidase from the same source and identical purity. The elution volume of the peroxidase was increased from 1.5 to 1.8 upon treatment with DTT, while the protein content of the peroxidase peak was considerably reduced. Treatment of peroxidase with DTT caused a reduction of the mol wt of the protein. SDS-polyacrylamide electrophoresis of the fractions having peroxidase activity revealed that during filtration of the  $P_{442}$ , the small polypeptide chain (mol wt 19,000) was removed and that the reduced peroxidase peak was composed of the large polypeptide chain (mol wt 46,000) and the heme group.

Treatment of the tomato anionic peroxidase with DTT reduced its activity to  $\frac{1}{10}$  of that of the native enzyme. The activity was not



FIG. 4. Polyacrylamide gels showing relative migration of anionic peroxidases purified from tomato pericarp. a: Gels stained for protein; b: gels stained for peroxidase activity; 1: soluble peroxidases from ripe pericarp; 2: soluble peroxidases from green pericarp; 3: ionically bound peroxidase from ripe pericarp; 4: ionically bound peroxidase from green pericarp.

regained after the removal of the excess DTT by gel filtration which suggests that dissociation of the two polypeptide chains caused the loss of the peroxidase activity.

**Compounds I, II, and III of Tomato Anionic Peroxidase.** Compounds I and III of the tomato anionic peroxidase formed by the addition of  $H_2O_2$  to the ferric form of the enzyme, as well as compound II produced by one electron reduction of compound I by a suitable electron donor (ascorbate), had spectra similar to the spectra reported for the respective compounds of HRP (3, 10). Although in the case of both peroxidases the reduction of compound I to compound II was too fast to be measured by our equipment, rate differences were measured when the two compound II's were reduced to their respective ferric forms. The addition of excessive amounts of ascorbate to tomato peroxidase compound I and HRP compound I revealed that the decomposition rate of the tomato peroxidase compound II to the free ferric form was much slower than that of HRP (11).

**Reduction of Tomato Anionic Peroxidases by Dithionite.** Dithionite is a strong reducing agent capable of converting the ferric heme iron of a peroxidase into the ferro form (25). Such a conversion is followed by changes in the spectrum of peroxidase. Both HRP and tomato anionic peroxidase give similar spectra upon reduction by dithionite (Fig. 7).

**IAA Oxidase Activity of Tomato Anionic Peroxidase.** The anionic peroxidases extracted from the pericarp of the tomato fruit at all stages of ripeness were tested for their capacity to oxidize IAA in the presence of DCP and  $Mn^{2+}$ . The rate of this oxidation reaction, expressed in terms of  $O_2$  consumption instead of destruction of IAA, and the total  $O_2$  consumed before the reaction came to an end, are shown in Table II. In comparison to the rate of  $O_2$  uptake observed when HRP was utilized to catalyze the reaction,

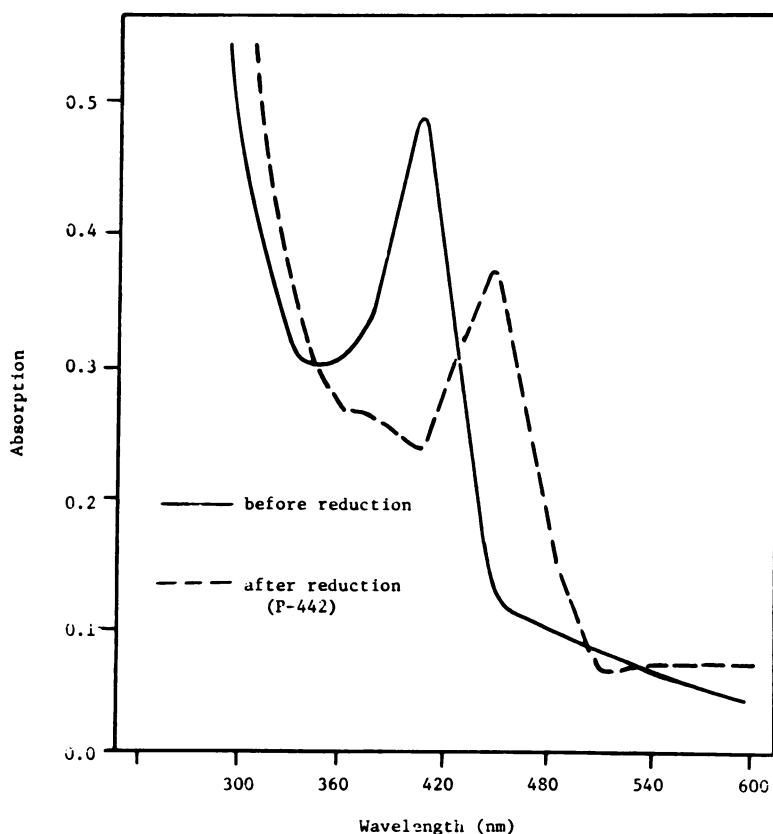


FIG. 5. A spectra of tomato anionic peroxidase from green tomato pericarp subjected to two QAE-Sephadex fractionations according to the alternative method, before and after reduction with DTT. Three ml of enzyme solution having  $3 \times 10^6$  units of peroxidase activity and 2.3 mg of protein in 0.1 M phosphate buffer (pH 7.6) were mixed with 50 mg of solid DTT in a 1 cm-light path cuvette. The spectrum of  $P_{442}$  was recorded 5 min later.

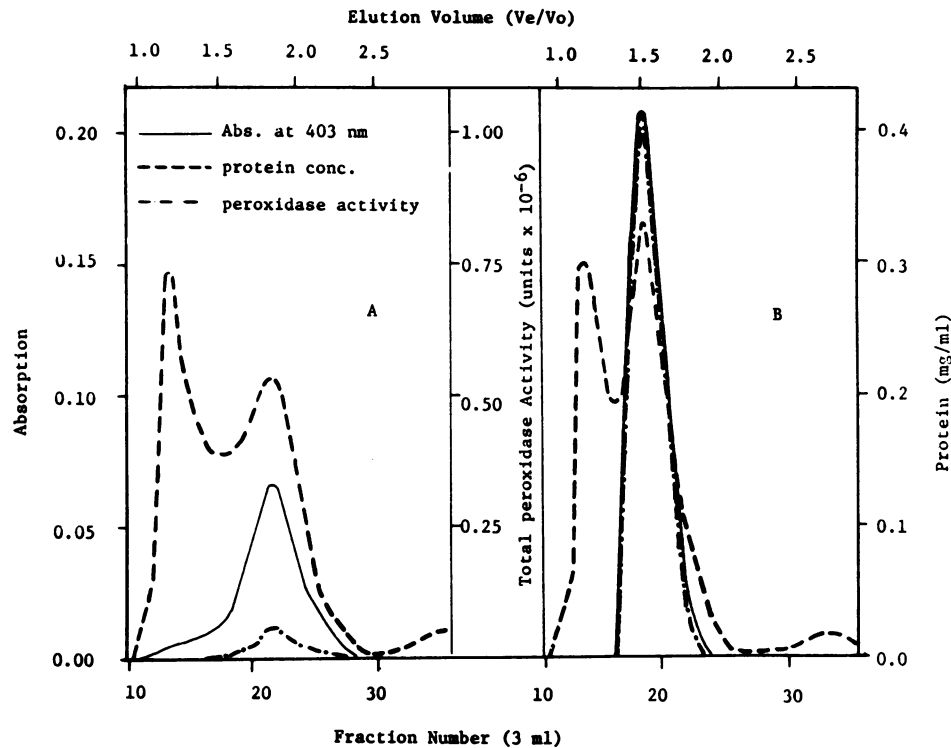


FIG. 6. Bio-Gel P-150 filtration of tomato anionic peroxidase from green tomato pericarp subjected to two QAE-Sephadex fractionations according to the alternative method, before (A) and after (B) reduction with DTT. The 3-ml samples initially having  $3 \times 10^6$  units of peroxidase activity and containing 2.3 mg of protein in 0.1 M phosphate buffer at pH 7.6 were applied on the columns ( $70 \times 1.5$  cm) and eluted with 0.1 M phosphate buffer at pH 7.6. Fractions (3 ml) were collected and were tested for their A at 403 nm, protein content, and total peroxidase activity. Sample B was reduced by the addition of 50 mg of DTT 5 min prior to its application on the column.

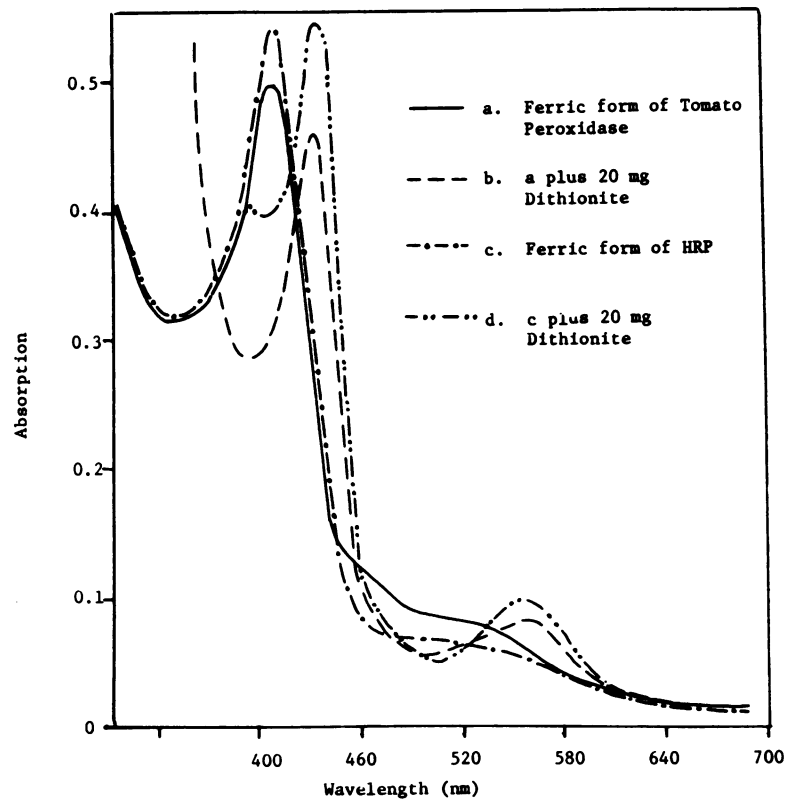


FIG. 7. Spectra of ferro and ferric forms of tomato anionic peroxidase and HRP under anaerobic conditions. a: Solution of 1.8 mg of HRP in 3 ml of 0.1 M phosphate buffer at pH 7.4; b: a plus 20 mg of dithionite; c: solution of 2 mg of purified tomato anionic peroxidase in 3 ml of 0.1 M phosphate buffer at pH 7.4; d: c plus 20 mg dithionite.

Table II. IAA Oxidase Activity of the Tomato Anionic Peroxidase and HRP. Reaction mixtures contained 0.075 mM DCP, 0.025 mM MnCl<sub>2</sub>, 0.75 mM IAA, 2.5 mM citrate buffer (pH 4.0), and 1.2 × 10<sup>5</sup> units of peroxidase.

Peroxidase	Reaction Rate (umoles O <sub>2</sub> /min)	Total Oxygen Consumed (umoles)
Anionic Soluble (Ripe pericarp)	0.003	0.018
Anionic Soluble (Climacteric pericarp)	0.004	0.021
Anionic Soluble (Green pericarp)	0.003	0.019
HRP	0.153	0.390

the tomato enzyme seems relatively incapable of catalyzing an O<sub>2</sub>-consuming oxidation of IAA under the reaction conditions tested.

### DISCUSSION

The results indicate that more than 80% of the peroxidase activity in tomato fruit extracts was due to a single peroxidase isozyme, which presumably corresponds to the tomato peroxidase B of Evans (5). Enzyme preparations from mature green fruits and from ripe fruits appeared to be identical in their properties, although the yield of enzyme per gram of tissue decreased with fruit ripening.

Studies with the purified tomato anionic peroxidase indicated that the enzyme is composed of two polypeptide chains with mol wt of 46,000 and 19,000, respectively, linked by disulfide bonds. This structure is most likely to have arisen by proteolytic cleavage of a protein consisting of one polypeptide chain with disulfide bonds, and might indicate that we are dealing with an enzyme which had been degraded during isolation. This point is under investigation. Alternately, this structure may represent the physiologically active form of the enzyme, produced by proteolytic cleavage of a precursor protein. The production of active forms of enzymes by proteolytic cleavage is well documented in animals and has been occasionally reported in higher plants (9, 23).

Tomato anionic peroxidase was similar to HRP in its ability to oxidize *p*-phenylenediamine and in its ability to form compound I, compound II, compound III, and reduced enzyme. The inability of the tomato enzyme to oxidize IAA rapidly under the test conditions used here was probably related to the low rate at which its compound II form was reduced by ascorbate as well as by various phenols (Kokkinakis, unpublished results). This is at least consistent with the theory of Yamazaki and Yamazaki (26) which emphasizes the importance of a highly reactive compound II in the autocatalytic oxidation of IAA by peroxidases.

The low reactivity of tomato anionic peroxidase as an IAA oxidase relative to HRP is not unique, as peroxidases vary greatly in their ability to oxidize IAA autocatalytically. Extracts of pea (15) and of sorghum (24) each contain peroxidase isozymes show-

ing high and low ratios of IAA oxidase activity to peroxidase activity. The extremely low rate of IAA oxidation observed for tomato anionic peroxidase under the test conditions used here does not eliminate the possibility that the enzyme serves as the physiological agent for the oxidation of IAA *in vivo*. The tomato enzyme is an effective IAA oxidase in the presence of H<sub>2</sub>O<sub>2</sub> (unpublished data) or thiol compounds (manuscript in preparation).

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