Peroxidase from Strawberry Fruit (*Fragaria ananassa* Duch.): Partial Purification and Determination of Some Properties

Pedro Marcos Civello, Gustavo Adolfo Martínez, Alicia Raquel Chaves,* and María Cristina Añón

Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), 47 y 116, (1900) La Plata, Argentina

In this work, peroxidase from strawberry fruit was detected, partially purified, and characterized. The total enzyme extract (both soluble and associated to membranes) was partially purified by means of $(NH_4)_2SO_4$ precipitation, molecular exclusion chromatography, and cationic exchange chromatography. The purification grade achieved was near 35. Effects of temperature and pH, stability against pH, and thermal stability were analyzed on both crude and partially purified extracts. The maximum enzyme activity was observed at 30 °C and pH 6.0. The enzyme showed low thermal stability and maintained activities equal to or greater than 50% of its maximum value in the 4–11 pH range. Two peroxidase isoenzymes were detected in strawberry fruit; they were of the basic type (isoelectric points 9.5–10.0) and had molecular masses of 58.1 and 65.5 kDa. Strawberry fruit peroxidase activity decreased remarkably as the fruit ripened and was found primarily in a membrane-bound form. Maximum specific activities were found at the "small green" and "large green" ripening stages.

Keywords: *Strawberry fruit; peroxidase; enzyme purification; fruit ripening*

INTRODUCTION

Peroxidase (POD, EC 1.11.1.7, donor:hydrogen-peroxide oxidoreductase) is widely distributed in the plant kingdom. The study of this enzyme in food has attracted interest because of its capacity to modify food in both desirable and undesirable ways. Deterrents of flavor, color, texture, or nutrient value (Fils et al., 1985) can be contrasted with development of desirable texture through the formation of protein cross-linking (Matheis and Whitaker, 1984). Its presence has been described in different plant parts, including climateric and nonclimateric fruits (Haard and Tobin, 1971; Gorin and Heidema, 1976; Marangoni et al., 1989; Chamarro and Molina, 1989; Biles and Martyn, 1993).

Peroxidase activity has been related to the existence of cationic and/or anionic isoenzymes (van Huystee, 1987). Moreover, a given fruit can include both isoenzyme types (Haard and Tobin, 1971; Fils et al., 1985). Generally, the enzyme is found in glycosylated form and associated to membranes, though soluble isoenzymes were also encountered in banana fruit (Haard and Tobin, 1971) and tomato fruit (Thomas et al., 1981).

Concerning the physiological role of peroxidase, it was shown that the enzyme participates in the late stages of the lignin-forming process (Mäder and Füssi, 1982; Wakamatsu and Takahama, 1993) and in the protection of tissues damaged by, or infected with, pathogenic microorganisms (Wakamatsu and Takahama, 1993; Biles and Martyn, 1993). The capacity *in vitro* of the enzyme to convert 1-aminocyclopropane-1-carboxylic acid (ACC) into ethylene has already been established (Rothan and Nicolas, 1989; Gaspar et al., 1985). Moreover, it can also degrade chlorophyll *in vitro* in the presence of phenols (Huff, 1982; Kato and Shimizu, 1985) and participate in the oxidation of indoleacetic acid (IAA) (Thomas et al., 1981; Brooks, 1986). Gaspar et al. (1985) proposed a model in which the capacity to oxide ACC and IAA was assigned to basic peroxidases, while acid peroxidases would participate in the lignification process. The control of enzymatic activity of both isoenzyme types is different. Acid peroxidases would be synthesized *de novo* as a response to different stimuli, such as hydric stress and mechanical damage. On the contrary, the increase of basic peroxidases would not be originated by *de novo* protein synthesis but by the activation of enzymes already present in the tissue.

The objective of this work was the detection, partial purification, and characterization of peroxidases from strawberry fruit.

MATERIALS AND METHODS

Plant Material. Strawberries (*Fragaria ananassa* Duch. cv. Selva) used were provided by local producers. Fruits were harvested and classified into different ripening stages according to the external coloration degree and size as small green, large green, white, 25% red, 50% red, 75% red, 100% red, and over-ripe. Fruits were washed before use, and both the calyx and peduncle were discarded.

Preparation of Acetone Powder. Ten grams of fresh fruit in the large green ripe stage was homogenized for 2 min with 40 mL of acetone precooled at −20 °C by using an Omnimixer. The homogenate was then vacuum-filtered in a Büchner funnel through filtering cloth, and the residue was washed with acetone until a colorless filtrate was obtained. The solid was allowed to dry overnight at room temperature, and the powder obtained was conserved at −60 °C until used.

Enzyme Extraction. Three types of enzymatic extracts were prepared.

(a) Soluble Extract in Phosphate Buffer. Ten grams of fresh fruit was homogenized for 2 min in an Omnimixer with 40 mL of buffer whose composition was as follows: 0.02 M Na_2HPO_4; 0.03 M NaH_2PO_4; 10 g/L polyvinylpolypyrrolidone (PVPP); pH 7.0. The suspension was allowed to rest for 3 min and then centrifuged at 8000*g* for 15 min, and the supernatant was separated.

* Author to whom correspondence should be addressed [fax (021) 25-4853; e-mail Marcos@CIDCA.Edu.Ar].

0021-8561/95/1443-2596$09.00/0

(b) Insoluble Extract in Phosphate Buffer. The solid residue obtained in the former step was washed by resuspending with 40 mL of distilled water and centrifuged at 8000*g* for 15 min, and the supernatant was discarded. This washing was repeated twice. The solid residue was poured into 40 mL of extraction medium whose composition was as follows: 0.04 M Na_2HPO_4; 0.06 M NaH_2PO_4; 0.1% v/v Triton X-100; 1 M NaCl; 10 g/L PVPP; pH 7.0. The system was left under stirring for 1 h and then centrifuged at 8000*g* for 15 min, and the supernatant was separated.

(c) Total Extract. Peroxidase enzymatic extracts were prepared from both fresh strawberries and acetone powder. The composition of the extraction buffer was as follows: 0.04 M Na_2HPO_4; 0.06 M NaH_2PO_4; 0.1% v/v Triton X-100; 1 M NaCl; 10 g/L PVPP; pH 7.0. The ratio of buffer volume (milliliters) to tissue mass (grams) was 4:1 when fresh fruit was used and 50:1 when acetone powder was used. Fresh fruits were homogenized with buffer in an Omnimixer and left under stirring for 1 h, after which time the mixture was centrifuged at 8000*g* for 15 min and the supernatant separated.

All steps in every case (a–c) were carried out at 4 °C.

Enzyme Assay. The following reaction mixture was employed: 0.02 M Na_2HPO_4–0.08 M NaH_2PO_4 buffer; 20 mM guaiacol; 4 mM H_2O_2; enzymatic extract, 300 μL; pH 6.0; total volume, 3.0 mL. The mixture was incubated at 30 °C and the enzymatic activity evaluated by measuring the increase of OD at 470 nm. For the determination of OD in fractions coming from chromatographic columns, the proportion of the enzymatic extract was increased 3 times.

The enzyme assay at pH different from 6.0 was carried out by adjusting the pH of the mixture with suitable amounts of 1.0 M NaOH, 1.0 M HCl, and 0.5 M NaCl, to keep the ionic strength constant.

The enzymatic activity unit (U) was defined as the amount of enzyme required to increase 0.001 OD unit/min at test conditions.

Enzyme Partial Purification. *(a) Precipitation with $(NH_4)_2SO_4$.* A total extract was produced by following the above-mentioned procedure (enzyme extraction c) and using 1 g of acetone powder. The supernatant thus obtained was treated with $(NH_4)_2SO_4$ up to 85% saturation and left under stirring for 5 h at 4 °C. It was then centrifuged at 9200*g* at 4 °C during 1 h and vacuum-filtered through filter paper. The retained solid was redissolved in 4–5 mL of 0.02 M Na_2HPO_4–0.08 M NaH_2PO_4 buffer, pH 6.0.

(b) Molecular Exclusion Chromatography. A 2.4 cm diameter column packed with Sephacryl S-300 (Pharmacia) up to 42.0 cm bed height was employed. It was equilibrated with 0.02 M Na_2HPO_4–0.08 M NaH_2PO_4 buffer, pH 6.0, and 2.0 mL of the extract prepared as for case a was applied. A 15 mL/h flow rate of the same buffer was used for elution, and 1.45 mL fractions were collected, on which the absorbance at 280 nm and the peroxidase activity were determined.

(c) Ionic Exchange Chromatography. A Pharmacia XK 16/20 column packed up to 15 cm bed height with CM-Sepharose Fast Flow (Pharmacia) was used. It was equilibrated with 0.03 M Na_2HPO_4–0.02 M NaH_2PO_4 buffer, pH 7.5. The fractions' pool with peroxidase activity obtained by molecular exclusion chromatography was dialyzed for 12 h at 4 °C in the presence of 0.03 M Na_2HPO_4–0.02 M NaH_2PO_4 buffer, pH 7.5. After this step, 5 mL of dialyzed aliquot was applied into the column and washed with 30 mL of the same buffer (μ = 0.116) at 14 mL/h flow rate. The retained enzyme was eluted at the same flow rate with 50 mL of 0.2–1.3 M linear gradient of NaCl in the same buffer ($\mu_{initial}$ = 0.316; μ_{final} = 1.416). Fractions of 1.9 mL were collected, on which the absorbance at 280 nm and the peroxidase activity were measured.

Enzyme Thermal Stability. Total enzymatic extracts obtained from acetone powder or, alternatively, extracts partially purified by molecular exclusion chromatography were used. Samples of 1 mL of extract were incubated for 5, 10, and 20 min at 30, 35, 40, 45, 50, 55, 60, and 70 °C. The residual enzymatic activity was then evaluated as described previously.

Enzyme Stability at Different pH Values. As in the thermal stability experiments, the total extracts from acetone powder or extracts partially purified by molecular exclusion chromatography were used as enzyme sources. Aliquots of 2.8 mL of extract were taken, and their pH values were adjusted to the desired value by adding either 1 M HCl or 1 M NaOH. In every case, suitable amounts of 0.5 M NaCl were added to keep the ionic strength constant. Samples thus prepared were incubated for 30 min at 20 °C at pH between 2 and 12. Once this last treatment was over, the pH was adjusted to 7.0 and the residual enzymatic activity was evaluated.

Molecular Mass Determination. A 1.6 cm diameter Pharmacia column packed with Sephacryl S-200 (Pharmacia) up to 33.0 cm bed height was used. The bed was equilibrated with 0.02 M Na_2HPO_4–0.08 M NaH_2PO_4 buffer, pH 6.0. The partially purified sample was concentrated by ultrafiltration, using Millipore UFC4TGC devices. Samples of 0.65 mL were applied, elution was done with the same buffer at 14 mL/h flow rate, and 1.9 mL fractions were collected. The system was calibrated with the following molecular mass markers: catalase (210 kDa), aldolase (158 kDa), bovine albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease (13 kDa).

Isoelectrofocusing. A horizontal isoelectrofocusing cell (Model 111 Mini IEF Cell, Bio-Rad) was employed, and polyacrylamide gels were prepared. Gel composition was as follows: 4.8% w/v acrylamide; 0.15% w/v bis(acrylamide); 5% w/v glycerol; 0.25 mL of ampholytes (Sigma, 3–10 range); 0.06% w/v $(NH_4)_2S_2O_8$; 0.03% v/v *N,N,N′,N′*-tetramethylethylenediamine (TEMED). [The mixture was degassed for 10 min before addition of $(NH_4)_2S_2O_8$ and TEMED.]

Samples coming from molecular exclusion chromatography were dialyzed against water for 12 h at 4 °C and freeze-dried. Then, samples were redissolved in 2 mM Na_2HPO_4–8 mM NaH_2PO_4 buffer, pH 6.0, and applied, in 5–10 μL volume aliquots, on the gel surface. Alternatively, small (3 × 5 mm sides and 1–2 mm thick) pieces of tissue were directly applied.

The run was performed by keeping the voltage constant initially at 100 V for 15 min, then at 200 V for a subsequent 15 min, and finally at 450 V for 1 h. Gels were stained with Coomassie Blue dye or with peroxidase-detecting assay. In the latter case, the gel was immersed in a solution prepared just before use, which had the following composition: 0.5% w/v benzidine; 4.5% v/v glacial acetic acid; 11.6 mM H_2O_2. The gel was photographed immediately.

SDS–PAGE. Polyacrylamide gels were prepared with SDS, according to the discontinuous buffer system of Laemmli (1970). The compositions of gels and buffers used were as follows: (*stacking gel*) 4% w/v acrylamide, 0.125 M Tris base, 0.1% w/v sodium dodecyl sulfate (SDS), 0.1% v/v TEMED, 0.025% w/v $(NH_4)_2S_2O_8$, pH 6.8; (*gradient gel*) 10–18% w/v acrylamide, 0.375 M Tris base, 0.1% w/v SDS, 0.1% v/v TEMED, 0.025% w/v $(NH_4)_2S_2O_8$, pH 8.8; (*running buffer*) 0.025 M Tris base, 0.192 M glycine; 0.1% w/v SDS, pH 8.3; (*sample buffer*) 0.0625 mM Tris base, 2% w/v SDS, 5% v/v 2-mercaptoethanol, 25 mM EDTA, 15% w/v sucrose, 0.002% w/v bromophenol blue, pH 6.8.

Electrophoresis was carried out in a refrigerated vertical slab gel cell (SE600, Hoeffer Instruments). Runs were performed at constant current intensity (15 mA/plate in the stacking and 25 mA/plate in the running), and molecular mass markers in the 14.4–94 kDa range (Pharmacia) were included. Gels were stained with silver salts according to the technique described by Blum et al. (1987).

Protein Dosage. Protein concentrations of the extracts were measured according to the modified Lowry method described by Potty (1969), measuring OD at 750 nm and using bovine albumin as standard.

Experimental Design. Each experiment was made at least three times, and similar trends were observed. To compare different tests of the same experiment, relative activities were defined as the ratios of the activities to the maximum activity obtained in each test. Values were expressed as percentages.

RESULTS AND DISCUSSION

Selection of Conditions for Enzyme Assay. For selection of measuring conditions we used a total enzymatic extract (which includes both soluble enzymes and those associated to membranes). Reaction mixtures with various guaiacol concentrations at constant (4 mM) H_2O_2 concentration were prepared, and the initial reaction rates for each condition were determined. It was observed that the initial reaction rate remained constant for guaiacol concentrations equal to or higher than 20 mM, which suggests enzyme saturation (data not shown). Under these working conditions, the optical density increased linearly up to 12 min after the start of reaction when crude enzymatic extracts were used and up to 30 min when partially purified enzymatic extracts were employed. Thus, we selected concentrations of 4 mM for H_2O_2 and 20 mM for guaiacol and reaction times according to those shown previously.

Partial Purification of the Enzyme. We started from acetone powder obtained from fruits in large green ripening stage, since they presented high specific activity of peroxidase. A total enzyme extract (both soluble and associated to membranes) was obtained and then precipitated with $(NH_4)_2SO_4$, the purification being continued by means of molecular exclusion chromatography. A typical chromatogram of the experiments carried out is shown in Figure 1a, which indicates that proteins are mainly distributed into two peaks. The peroxidase activity was detected in a group of fractions of low protein content located between such peaks.

The fractions of interest were pooled, and cationic exchange chromatography was performed. The selection of resin and working conditions was made on the basis of results obtained with isoelectrofocusing. Results obtained in a typical experiment are displayed in Figure 1b. This graph shows that most proteins were not retained and eluted into two peaks when the column was washed with buffer without NaCl ($\mu = 0.116$). On the contrary, peroxidase was strongly retained in the column, so it became necessary to use a high NaCl concentration ($\mu = 1.1$) for its elution. Moreover, it can also be observed that the peroxidase peak coincides with a small protein peak.

The purification scheme used here is summarized in Table 1, which shows that the purification grade achieved was near 35. The enzymatically active fractions arising from different chromatographic tests were analyzed by SDS–PAGE (Figure 2). The crude enzymatic extract (lane 1) reveals the presence of many polypeptides. The enzymatically active fraction coming from molecular exclusion chromatography (lane 2) shows two main polypeptides, which are accompanied by another two in less proportions. A subsequent resolution using cationic exchange chromatography led to purification of the two main polypeptides (molecular masses 58.1 and 65.5 kDa) obtained previously (lane 3).

Enzyme Characterization. All variables (temperature, pH, stability against pH, and thermal stability) were analyzed on both crude extracts obtained from acetone powder and partially purified extracts (pool of fractions coming from molecular exclusion chromatography). Conclusions drawn with both extracts were similar.

(a) Temperature. Reaction mixtures were prepared and then incubated at different temperatures. Relative enzymatic activities obtained in these tests are shown in Figure 3a. Under the working conditions used, the activity reached a maximum at 30 °C and then sharply decreased for higher temperatures, being very low (less than 20%) at 50 °C. The enzyme exhibited considerable activity at low temperatures, being about 40% at 0 °C. The behavior was similar to that observed in cauliflower peroxidase, though with a different optimal temperature (Lee et al., 1984).

(b) pH. Reaction mixtures at different pH values were prepared and then incubated at 30 °C. Results obtained for the relative enzymatic activity are displayed in Figure 3b. The enzyme showed high enzymatic activity

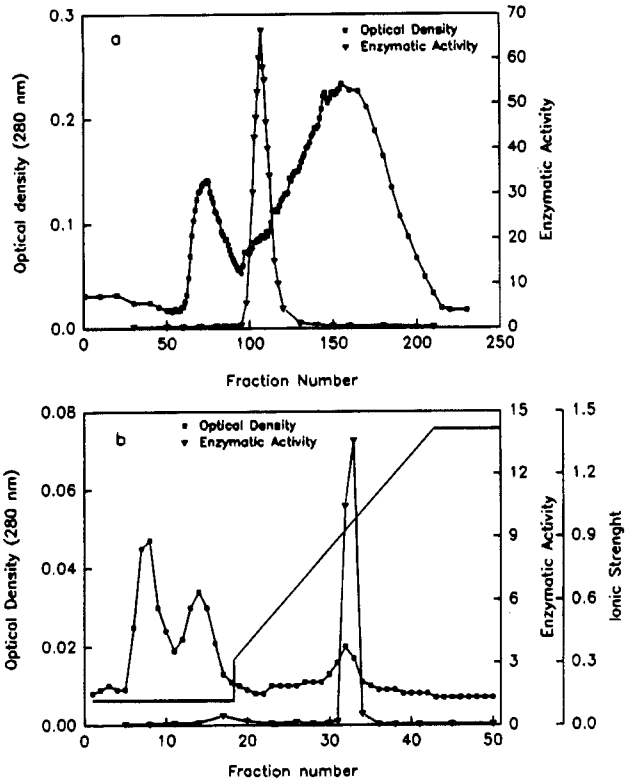

Figure 1. (a) Molecular exclusion chromatography in Sephacryl S-300. The sample was obtained from acetone powder of green strawberries, precipitated with $(NH_4)_2SO_4$ at 85% saturation and redissolved in 0.02 M Na_2HPO_4–0.08 M NaH_2PO_4 buffer, pH 6.0. The column was equilibrated and eluted with the same buffer at a flow rate of 15 mL/h. Fractions of 1.45 mL were collected, the OD was measured at 280 nm, and the peroxidase activity was determined. (b) Ionic exchange chromatography in CM-Sepharose. The sample was obtained from a pool of enzymatically active fractions arising from S-300 molecular exclusion chromatography. The column was equilibrated and washed with 0.03 M Na_2HPO_4–0.02 M NaH_2PO_4 buffer, pH 7.5, with a flow rate of 14 mL/h. Proteins retained were eluted with a NaCl linear gradient 0.2–1.3 M in the same buffer. Fractions of 1.9 mL were collected, the OD was determined at 280 nm, and dosage of the peroxidase activity was performed.

Table 1. Purification of Peroxidase from Strawberry Fruit

	crude extract	$(NH_4)_2SO_4$[a]	S-300[b]	CM-Sepharose[c]
protein (mg/mL)	0.094	0.199	0.0023	0.00034
total protein (mg)	4.00	0.995	0.173	0.0082
activity (U/mL)	0.314	1.576	0.067	0.040
total activity (U)	13.34	7.88	5.03	0.95
specific activity (U/mg of protein)	3.34	7.92	29.13	116.47
yield (%)	100	59.1	37.7	7.1
degree of purification	1	2.37	8.72	34.87

[a] Precipitation with $(NH_4)_2SO_4$ up to 85% saturation. [b] Molecular exclusion chromatography in Sephacryl S-300. [c] Ionic exchange chromatography in CM-Sepharose.

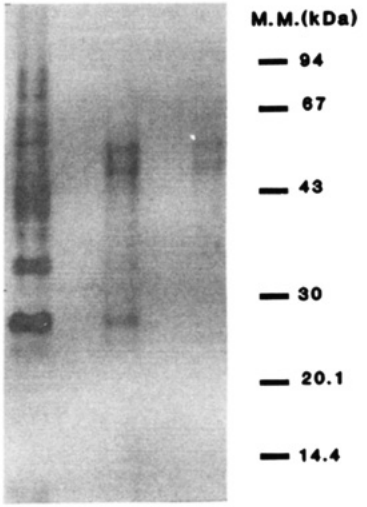

Figure 2. Polyacrylamide gel electrophoresis with SDS in a 10−18% gradient of samples obtained in the several purification stages: (lane a) crude extract; (lane b) enzymatically active fraction from S-300 molecular exclusion chromatography; (lane c) enzymatically active fraction from ionic exchange chromatography.

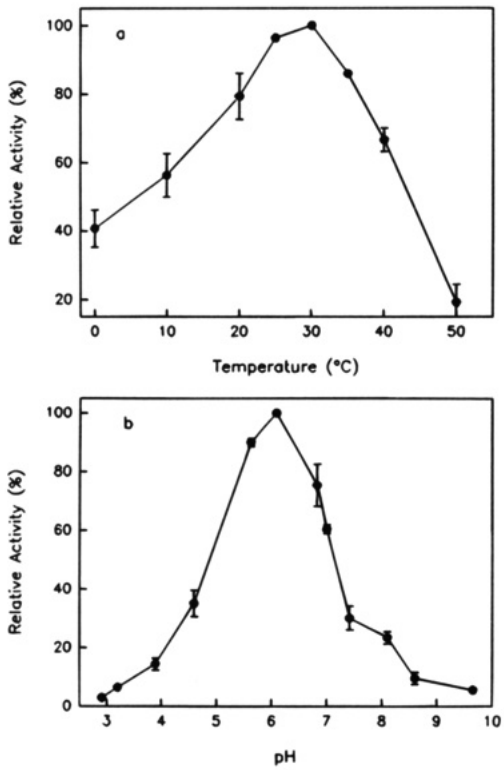

Figure 3. (a) Effect of temperature on enzymatic activity of peroxidase. Enzymatic extract was obtained from acetone powder of green fruits or from S-300 molecular exclusion chromatography. Values plotted on the graph represent relative activity, i.e. the ratio of the activity to the maximum activity (the latter at 30 °C), expressed as percentage. Vertical bars denote ± standard deviation when they exceed the size of the symbol. (b) Effect of pH on the enzymatic activity of peroxidase. Enzymatic extract was obtained from acetone powder of green fruits or from S-300 molecular exclusion chromatography. Values plotted on the graph represent relative activity, i.e. the ratio of the activity to the maximum activity (the latter at pH 6.0), expressed as percentage. Vertical bars denote ± standard deviation when they exceed the size of the symbol.

in a narrow pH range, the maximum being at pH 6. The optimum pH for peroxidase depends on the hydrogen donor. Therefore, for a given enzyme, the optimum pH changed according to whether guaiacol or pyrogallol was used (Halpin et al., 1989).

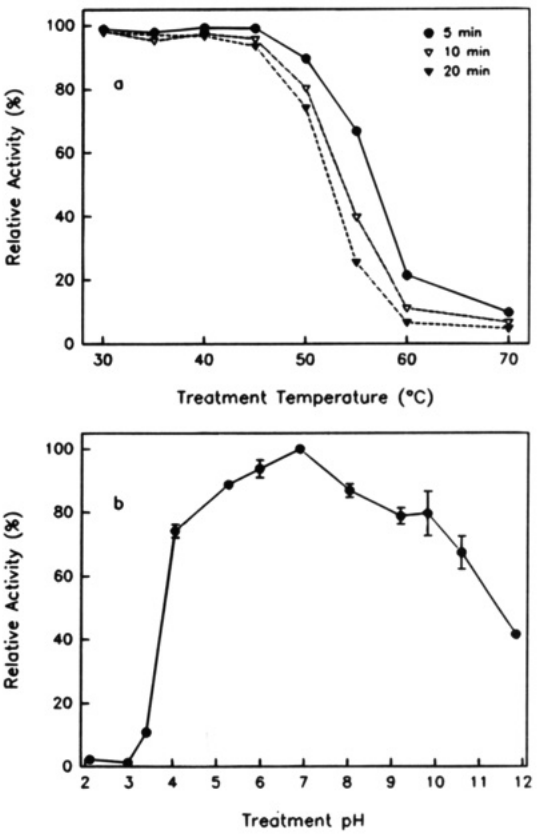

Figure 4. (a) Enzyme stability vs thermal treatment. The enzymatic extract was obtained from acetone powder of green fruits or from S-300 molecular exclusion chromatography. An extract aliquot was incubated at different temperatures and times, and the remaining enzymatic activity was measured at 30 °C. Values plotted on the graph represent relative activity, i.e. the ratio of the activity to the maximum activity (the latter at 30 °C), expressed as percentage. (b) Enzyme stability vs incubation pH. The enzymatic extract was obtained from acetone powder of green fruits or from S-300 molecular exclusion chromatography. An extract aliquot was incubated at different pH values during 30 min at 20 °C, and the remaining enzymatic activity was measured at the optimal pH (6.0) and 30 °C. Values plotted on the graph represent relative activity, i.e. the ratio of the activity to the maximum activity (the latter at pH 7.0), expressed as percentage. Vertical bars denote ± standard deviation when they exceed the size of the symbol.

(c) Thermal Stability. Results obtained after the treatments are shown in Figure 4a. The plot shows that the greater the temperature and treatment time, the smaller the residual enzymatic activity, which reflects lower stability. The enzyme kept almost its original activity after treatments up to 45 °C for 20 min. At higher temperatures, treatment time becomes more important. For instance, at 55 °C, a 5 min treatment led to a residual enzymatic activity of 68%, while with a 20 min treatment, the final activity was 23%. Treatments done at temperatures higher than 60 °C caused an almost complete loss of activity, even at short times (5 min).

Peroxidase is considered to be a stable enzyme when undergoing a thermal treatment. So, the reduction of its activity has been used as a measure of adequate blanching (Halpin et al., 1989; Polyák-Fehér et al., 1992). However, the resistance to treatments depends on the source of the enzyme. Moreover, in a given

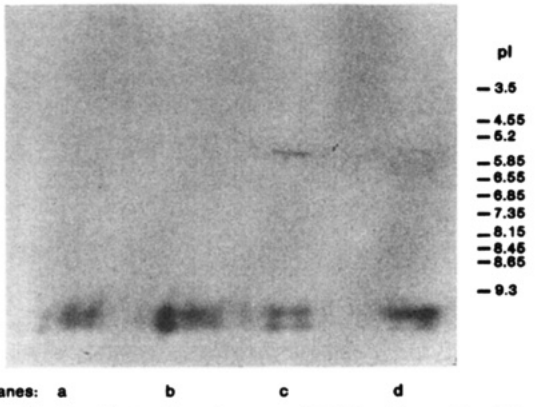

Figure 5. Isoelectrofocusing on 4.95% polyacrylamide gel, 3–10 pH range. Pieces of fruit (3 × 5 mm side, 1–2 mm thick) at different ripening stages were applied: (lane a) large green; (lane b) white; (lane c) 50% red; (lane d) over-ripe. The enzyme was detected with benzidine–acetic acid–H_2O_2.

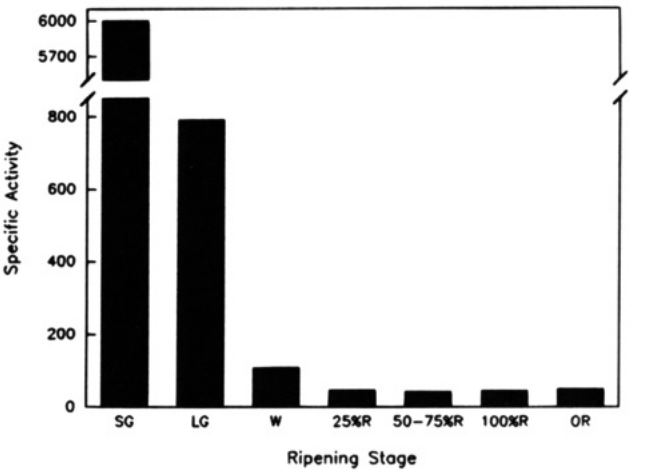

Figure 6. Evolution of specific enzymatic activity of peroxidase during ripening of strawberry fruits. The extracts were obtained from fresh fruit in the following stages: small green, large green, white, 25% red, 50–75% red, 100% red, and over-ripe.

source, it varies from one isoenzyme to the other. Our results show that the thermal stability was greater than that described for cauliflower (Lee et al., 1984) and than those reported for two basic isoenzymes present in green peas. In the last case it was observed that the basic isoenzymes were much more thermally resistant than other neutral isoenzymes, also present in the system (Halpin et al., 1989).

(d) Stability vs pH. After incubation at different pH values the resultant relative activities were plotted in Figure 4b. The graphs show that the enzyme preserves activities equal to or greater than 50% of its maximum activity in the pH 4–11 range. The enzyme is more stable at pH between 6 and 7.5, keeping relative activities higher than 90% of the maximum value. Moreover, the enzyme stability is totally lost at pH lower than 3.0. This effect, already reported for other systems, has been ascribed to the loss of the heme group at low pH (Burnette, 1977).

(e) Molecular Mass Determination. The partially purified samples were concentrated by ultrafiltration to make enzyme detection easier. The molecular exclusion chromatography analysis allowed us to estimate the molecular mass of the enzymatically active fraction as 56 ± 3 kDa. This value is similar to those obtained by means of SDS–PAGE (Figure 2) for purified polypeptides after ionic exchange chromatography (58.1 and 65.5 kDa). The result is higher than those of tomato peroxidase (46 kDa) (Jen et al., 1980; Heidrich et al., 1983; Fils et al., 1985) and horseradish peroxidase (32 kDa) (van Huystee, 1987).

(f) Isoelectric Point Determination. Results obtained from pieces of fresh fruits were similar to those of concentrated column eluates. In both cases, we obtained two bands assigned to isoenzymes of p*I* 9.5 and 10.0. Such isoenzymes were found in all ripening stages, i.e., from large green to over-ripe (Figure 5). In some experiments, a weak band of lower p*I* was also observed, suggesting the existence of an anionic isoenzyme (results not shown).

Peroxidases found in higher plants include isoenzymes with basic, neutral, or acid isoelectric points. A given fruit can show several isoenzymes with very different isoelectric points (Rothan and Nicolas, 1989; Halpin et al., 1989). The analysis made here on strawberries along the course of ripening did not detect differences in the isoenzyme profile among the different stages, starting from the large green stage. This is similar to what occurs in banana fruit, in which the same six isoenzymes are present in the several ripening stages (Haard and Tobin, 1971). On the contrary, in tomato the profile of peroxidase isoenzymes changes during ripening (Thomas et al., 1981).

(g) Analysis of Soluble and Associated-to-Membranes Enzymes. We attempted to determine whether the strawberry peroxidase was soluble or attached to membranes. To this end, we prepared extracts from fresh fruit in several ripening stages by following the procedure already described under Materials and Methods. The amount of protein extracted with phosphate buffer (soluble fraction) was greater than that obtained in the presence of Triton X-100 and NaCl (fraction associated to membranes).

Values of the specific enzymatic activity of both soluble fractions and fractions associated to membranes for fruits in the large green ripening stage were 5.7 and 10 824 U/mg of protein, respectively. In the remaining ripening stages, the specific enzymatic activity of the fraction associated to membranes was always 100–1000 times greater than that of the soluble fraction. From our study, we conclude that strawberry fruit peroxidase is mainly found associated to membranes. In other systems, enzymes in both soluble form and associated to membrane were found in comparable proportions. Some authors have described this enzyme as associated to internal membranes of chloroplast (Huff, 1982), to cellular walls, and to internal membrane of tonoplast (Thomas et al., 1981). Furthermore, an important activity of soluble peroxidase was encountered in orange flavedo (Chamarro and Molina, 1989), rice leaf (Kar and Mishra, 1976), pineapple fruit (Van Lelyveld et al., 1991), tomato (Thomas et al., 1981), apple (Gorin and Heidema, 1976), and banana (Haard and Tobin, 1971).

Variation of the Enzymatic Activity during Ripening. Total enzymatic extracts from fruits in different ripening stages were prepared and the specific enzymatic activities were measured. Results are plotted in Figure 6, where the existence of very high specific enzymatic activity can be observed for fruits in small green and large green ripening stages. The figure also shows a low, yet clearly detectable, specific enzymatic activity in the remaining ripening stages.

Conclusions. In this study for strawberry fruit, two peroxidase isoenzymes were detected. These isoenzymes are basic ones, and their isoelectric points differ

by 0.5 unit, while their molecular masses were 58.1 and 65.5 kDa. The peroxidase activity is mainly found associated to membranes. In addition, a marked decrease of the specific enzymatic activity was observed along the course of ripening.

LITERATURE CITED

Biles, C. L.; Martyn, R. D. Peroxidase, polyphenoloxidase, and shikimate dehydrogenase isozymes in relation to tissue type, maturity and pathogen induction of watermelon seedlings. *Plant Physiol. Biochem.* **1993**, *31* (4), 499–506.

Blum, H.; Beier, H.; Gross, H. J. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **1987**, *8*, 93–99.

Brooks, J. L. Oxidase reactions of tomato anionic peroxidase. *Plant Physiol.* **1986**, *80*, 130–133.

Burnette, F. S. Peroxidase and its relationship to food flavor and quality: a review. *J. Food Sci.* **1977**, *42*, 1–6.

Chamarro, J.; Molina, I. Oxidation of indoleacetic acid by an apparently homogeneous peroxidase from the flavedo of washington navel oranges (*Citrus cinensis*). *J. Food Biochem.* **1989**, *13*, 361–375.

Fils, B.; Sauvage, F. X.; Nicolas, J. Tomato peroxidases, purification and some properties. *Sci. Aliments* **1985**, *5* (2), 217–232.

Gaspar, T.; Penel, C.; Castillo, F. J.; Greppin, H. A two-step control of basic and acidic peroxidases and its significance for growth and development. *Physiol. Plant.* **1985**, *64*, 418–423.

Gorin, N.; Heidema, F. T. Peroxidase activity in golden delicious apples as a possible parameter of ripening and senescence. *J. Agric. Food Chem.* **1976**, *24* (1), 200–201.

Haard, N. F.; Tobin, C. L. Patterns of soluble peroxidase in ripening banana fruit. *J. Food Sci.* **1971**, *36*, 854–857.

Halpin, B.; Pressey, R.; Jen, J.; Mondy, N. Purification and characterization of peroxidase isoenzymes from green peas (*Pisum sativum*). *J. Food Sci.* **1989**, *54* (3), 644–649.

Heidrich, E.; Lorenz, G.; Schreier, P. Ultrathin layer isoelectric focusing of partially purified peroxidase from tomato fruit. *Food Chem.* **1983**, *10*, 285–296.

Huff, A. Peroxidase-catalyzed oxidation of chlorophyll by hydrogen peroxide. *Phytochemistry* **1982**, *21* (2), 261–265.

Jen, J. J.; Seo, A.; Flurkey, W. H. Tomato peroxidase: purification via hydrophobic chromatography. *J. Food Sci.* **1980**, *45*, 60–63.

Kar, M.; Mishra, D. Catalase, peroxidase and polyphenoloxidase activities during rice leaf senescence. *Plant Physiol.* **1976**, *57*, 315–319.

Kato, M.; Shimizu, S. Chlorophyll metabolism in higher plants VI. Involvement of peroxidase in chlorophyll degradation. *Plant Cell Physiol.* **1985**, *26* (7), 1291–1301.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **1970**, *227*, 680–685.

Lee, C. Y.; Pennesi, A. P.; Dickson, M. H. Characterization of the cauliflower peroxidase isoenzyme. *J. Agric. Food Chem.* **1984**, *32*, 18–21.

Mäder, M.; Füssi, R. Role of peroxidase in lignification of tobacco cells II. Regulation by phenolic compounds. *Plant Physiol.* **1982**, *70*, 1132–1134.

Marangoni, A. G.; Brown, E. D.; Stanley, D. W.; Yada, R. Y. Tomato peroxidase: rapid isolation and partial characterization. *J. Food Sci.* **1989**, *54* (5), 1269–1271.

Matheis, G.; Whitaker, J. R. Modification of proteins by polyphenol oxidase and peroxidase and their products. *J. Food Biochem.* **1984**, *8*, 137–162.

Polyák-Fehér, K.; Rácz-Horváth, B.; Kecskés, T. Effect of blanching and ripeness on green pea peroxidase activity. *Acta Aliment.* **1992**, *21*, 261–274.

Potty, V. H. Determination of protein in the presence of phenols and pectins. *Anal. Biochem.* **1969**, *29*, 535–539.

Rothan, C.; Nicolas, J. Changes in acidic and basic peroxidase activities during tomato fruit ripening. *Hortic. Sci.* **1989**, *24* (2), 340–342.

Thomas, R. L.; Jen, J. J.; Morr, C. V. Changes in soluble and bound peroxidase-IAA oxidase during tomato fruit development. *J. Food Sci.* **1981**, *47*, 158–161.

van Huystee, R. B. Some molecular aspects of plant peroxidase biosynthetic studies. *Annu. Rev. Plant Physiol.* **1987**, *38*, 205–219.

Van Lelyveld, L. J.; Visser, G. J. Swarts, D. H. The effect of various storage temperatures on peroxidase activity and protein PAGE gel electrophoresis in Queen pineapple fruit. *J. Hortic. Sci.* **1991**, *66* (5), 629–634.

Wakamatsu, K.; Takahama, U. Changes in peroxidase activity and in peroxidase isozymes in carrot callus. *Physiol. Plant.* **1993**, *88*, 167–171.

Received for review December 27, 1994. Accepted June 12, 1995.[⊗] This work was supported by grants from CONICET.

JF940729P

[⊗] Abstract published in *Advance ACS Abstracts,* August 1, 1995.