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Purification, characterization and catalytic activity of anionic zucchini peroxidase

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The isolation and purification, by preparative electrofocusing, of the major anionic (ZPOA) and cationic (ZPOC) isoenzymes, collected from young zucchini squash, are reported. The M_r and sugar content are similar to those found previously for the major isoenzymes from the ripe fruits and in the range commonly observed for plant peroxidases. The amount of the two cationic enzymes was very low compared with that of anionic ZPOA. The anionic enzyme has been characterized by electronic, circular dichroism, proton NMR and electron paramagnetic resonance spectroscopy. The spectra are qualitatively similar to those of the corresponding anionic horseradish peroxidase (HRPA) derivatives, with minor differences attributable to the particular protein environment around the heme. The kinetics of the enzymatic oxidation of a series of phenols by H_2O_2 have been studied. ZPOA shows a parallel behavior to HRPA, but it is systematically more active than HRPA, indicating that the zucchini enzymes have a marked tendency to carry out oxidation of this type of compounds.

Keywords: heme proteins, peroxidase activity, peroxidase isoenzymes, spectroscopic characterization, zucchini peroxidase

Introduction

Peroxidases (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7) have been isolated from various plant tissues and extensively studied over the years (Dunford & Stillman 1976, Frew & Jones 1984, Greppin *et al.* 1986). Although the role of these enzymes in plant metabolism is varied and not completely established, they appear to be involved in the process of lignification in trees (Harkin & Obst 1973, Peter 1989) and in chlorophyll catabolism (Huff 1982). The cell wall is sometimes considered as the site of primary action of plant peroxidases (Gaspar *et al.* 1986), but the presence of isoenzyme families and the possible contamination of plant cell wall fragments by cytoplasmic or organellar components complicates the understanding of the physiological role of these enzymes. In particular, it is not clear how the variability in the peroxidase isoenzyme composition depends on natural factors and experimental techniques, and to what extent they represent the products of several genes or the post-translationally modified products of a few genes (McNeil *et al.* 1984).

Detailed catalytic and physicochemical studies on horseradish peroxidase (HRP) isoenzymes have shown that different catalytic activities can be associated with structural differences of the isoenzymes near the heme prosthetic group (Kay *et al.* 1967, Strickland *et al.* 1968, Aibara *et al.* 1981, 1982, Kato *et al.* 1984, Gonzalez-Vergara *et al.* 1985).

It would be desirable to have this type of information for other plant peroxidases. We previously reported the purification and spectral characterization of the major cationic peroxidases isoenzyme form zucchini (*Cucurbita pepo*) ripe fruits (Casella *et al.* 1986). Like other basic isoenzymes

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from plant sources (Theorell 1947, Mazza *et al.* 1968, Nakajima *et al.* 1969, Ricard *et al.* 1972, Yamazaki 1974), this exhibited paraperoxidase character, i.e. an absorption spectrum similar to those of low-spin cytochromes *b*, and gave rise to the formation of Compound III as the major steady-state form of the enzyme during activity. The latter result is consistent with the idea that paraperoxidase isoenzymes may exhibit mixed peroxidase–oxidase activity *in vivo* (Yamazaki 1974, Frew & Jones 1984). Such activity is apparently associated with aging of the fruit, since it was noted that enzymatic fractions rich in paraperoxidases isoenzymes increase with ripening (Casella *et al.* 1986). In this paper we continue our characterization of zucchini peroxidase (ZPO) by reporting the catalytic activity and spectral properties of the major anionic isoenzyme of the fruit collected after fecundation and before the full development process leading to maturation. These properties are compared with those of the commercially available anionic HRPA isoenzyme in order to assess the potential usefulness of the enzyme from the easily accessible zucchini source in biotechnological applications.

Materials and methods

Materials

The anionic HRP isoenzyme (HRPA) was purchased from Sigma (St Louis, MO, USA; type VIII, RZ = 3.0). The lyophilized enzyme was dissolved in 0.1 M phosphate buffer, pH 7.0, and dialyzed against 0.1 M EDTA and then phosphate buffer. Protein concentrations were estimated using $\varepsilon_{403} = 103\ \text{mM}^{-1}\,\text{cm}^{-1}$ (Kato *et al.* 1984). Phenol, *p*-cresol, *p*-hydroxybenzoic acid, salicylic acid, hydroquinone, catechol, resorcinol and 4-methylcatechol were obtained from Aldrich and crystallized twice. *o*-Dianisidine, 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (MBTH) and dimethylaminobenzoic acid (DMAB) are Sigma products of analytical grade. The hydrogen peroxide solutions were freshly made from Merck (Darmstadt, Germany) 30% H_2O_2 solution preserved at 4 °C. All the buffers and other solutions were made using double distilled water.

Purification of ZPO isoenzymes

The crude enzyme material obtained from the peel of 2 kg of young squash fruits (5–20 cm long), following the procedure described before for enzyme extraction from ripe zucchini (Casella *et al.* 1986), was suspended in water (1:1, w/v) and dissolved by addition of solid sodium borate up to pH 8.2. Then chilled (−30 °C) acetone (1.0:0.9, v/v) was added to reprecipitate the crude proteins and the mixture was filtered. All subsequent purification operations were performed at 4 °C. The precipitate was treated with 10 mM Tris–HCl buffer, pH 7.6, and the solution was concentrated by ultrafiltration (membrane cut 10 kDa). The enzymatic fraction was loaded on a G100 Sephadex (coarse) column (2 × 100 cm) and eluted with 0.1 M phosphate buffer, pH 6.8. The eluate was divided in three large portions according to the relative molecular weight of the components. The first fraction contains products with molecular weight above 50 kDa and includes the enzyme ascorbate oxidase. The second fraction contains the peroxidase enzymes and the third fraction contains low molecular weight material and phenolic compounds.

The second and main fraction containing the peroxidase enzyme (~0.3 mg ml^{-1} protein; RZ = 0.4), after dialysis against 10 mM phosphate buffer, pH 7.0, was concentrated on a collodion bag and centrifuged at 20000 r.p.m. for 20 min. The solution was then loaded on a CM-Sephadex C50 column (4 × 20 cm), equilibrated with 10 mM phosphate buffer, pH 7.0. The cationic fraction (ZPOC) was retained by the column while the main anionic fraction (ZPOA) was found in the eluate. ZPOC was eluted by a linear gradient obtained by increasing the sodium phosphate concentration from 10 to 100 mM. The solution containing ZPOA was loaded on a DEAE–Sephadex A50 column and eluted with the same 10–100 mM sodium phosphate gradient. The purification procedure of the anionic zucchini isoenzyme with respect to activity, measured by using the DMAB–MBTH system (Ngo & Lenhoff 1980) as donor substrate, and RZ in the various steps of the preparation is reported in Table 1.

Preparative electrophoresis

Preparative electrophoresis was carried out in solution using a Rotophor system (Biorad) equipped with two electrodes, immersed in 0.1 M NaOH and 0.1 M H_3PO_4, respectively, at 2 °C using a constant power of 8–10 W for a total time of 4 h. The partially purified ZPOA and ZPOC fractions to be subjected to electrophoresis were previously concentrated to a volume containing 1 mg ml^{-1} enzyme and centrifuged as before. Then to 60 ml of the solution was added 2% ampholyte (pH 3–10) and the solution was introduced in the Rotophor cell. At the end of the electrophoretical cycle two main red fractions were found in the acid and basic regions of the electrophoresis cell, respectively. These solutions were centrifuged and dialyzed against 25 mM phosphate buffer, 100 mM EDTA and again 100 mM phosphate buffer, pH 7.0. The anionic protein solution exhibited a RZ value of 2.0 and a concentration of 1.4 mg ml^{-1} of ZPOA.

Assays

The molecular weights of the ZPO isoenzymes were determined by SDS–PAGE in 12% polyacrylamide gels in the presence of low range molecular weight standards (Biorad) according to the method of Weber & Osborn (1969); molecular weights in the range 30000–50000 can be determined with an accuracy of at least ±5%.

The isoelectric points (p*I*) of the ZPO isoenzymes were determined with the Pharmacia isoelectric focusing (IEF)

system, according to the method of Hoyle (1977), using LKB ampholyte PAG plates (pH 3.5–9.5), Biorad IEF standards (p*I* 4.6–9.6), Pharmacia low p*I* calibration kit (p*I* 2.5–6.5), and the isoenzymes HRPA and HRPC (Sigma) as control proteins, with 60 W constant power in 90 min time.

The carbohydrate content of ZPOA was determined by the method of Dubois *et al.* (1956), with slight modification, using a 1:1 mixture of D-galactose and D-mannose as standard, and turned out to be about 16%.

The protein content of ZPOA was estimated to be around 78% using the method of Lowry *et al.* (Rieder 1974), with crystalline serum albumin (Fluka) as standard.

Peroxidase activity of ZPOA and HRPA was investigated in the pH interval 5.0–8.0 using two different methods. The first involved the use of hydroquinone or *o*-dianisidine and hydrogen peroxide as substrates (Kuo & Fridovich 1988), the second used the coupled MBTH–DMAB and hydrogen peroxide system (Ngo & Lenhoff 1980). Specific activities were measured at pH 7.0 and 25 °C. Then, 3 ml of reaction mixture in 0.1 M phosphate buffer, pH 7.0, contained the donor substrate (4×10^{-5} M), H_2O_2 (2×10^{-4} M) and HRPA (1×10^{-9} M) or ZPOA (5.3×10^{-10} M). The reaction was initiated by the addition of the enzyme and followed at the fixed wavelength ($\Delta\varepsilon$, $M^{-1}\,cm^{-1}$) $\lambda = 288$ nm (−2680) for hydroquinone, $\lambda = 460$ nm (+11300) for *o*-dianisidine and $\lambda = 590$ nm (+47600) for DMAB–MBTH. The specific activities were calculated in units per milligram of enzyme, which are micromoles of substrate oxidized in one minute by 1 mg ml^{-1} of ZPOA or HRPA.

Kinetic and binding studies

The kinetic studies on ZPOA and HRPA using hydroquinone, catechol, 4-methylcatechol and *p*-hydroxybenzoic acid as donor substrates were performed spectrally at 25 °C and pH 7.0. Then, 3 ml of reaction mixture in 0.1 M phosphate buffer, pH 7.0, contained the donor substrate (2×10^{-4} to 2×10^{-5} M), H_2O_2 (2×10^{-4} M) and ZPOA or HRPA (4×10^{-8} M), (1.44×10^{-7} M in the case of HRPA/*p*-hydroxybenzoic acid). Higher hydrogen peroxide concentration did not increase the reaction rate. The reaction was initiated by the addition of the enzyme and followed by the decrease of the absorption of the donor substrate. The monitored wavelengths and the molar extinction coefficients of the substrates (calculated in the experimental conditions employed) were as follows [λ_{max} in nm (ε in $M^{-1}\,cm^{-1}$)]: hydroquinone 288 (2680), catechol 275 (2540), 4-methylcatechol 281 (2530) and *p*-hydroxybenzoic acid 246 (11500). The primary kinetic data were fitted to the Michaelis–Menten equation, since the hydrogen peroxide concentration was saturating, and the kinetic parameters K_M and V_{max} were determined.

Substrate binding studies to the native enzymes were performed by optical difference spectra (enzyme–substrate versus enzyme), by titrating 2 ml samples of enzyme solution (~10^{-6} M) in 0.1 M phosphate buffer, pH 7.0, with small volumes of concentrated solutions of the substrates in the same buffer. The binding constants (K_B) were calculated by double reciprocal plots of absorbance against substrate concentration as described previously (Casella *et al.* 1991). The stoichiometry of formation of enzyme–substrate complexes was established by Hill plots (Casella *et al.* 1991). Since the spectral variations observed in these experiments are very small, reliable binding data could not be obtained for catechol and 4-methylcatechol due to some interference by absorbing quinone compounds formed by air oxidation of these derivatives during the experiments.

Spectral measurements

Optical absorption spectra were recorded on a Kontron UVIKON 930 double beam or a HP 8452A diode array spectrophotometer. Circular dichroism (CD) spectra were obtained on a Jasco J-500 C dichrograph; the CD spectra are reported in terms of the differential molar extinction coefficient ($\Delta\varepsilon$, $M^{-1}\,cm^{-1}$). The molar absorption coefficient of ZPOA was determined on the basis of the pyridine hemochrome spectrum; the pyridine hemochrome derivative was prepared according to a known procedure (Fuhrhop & Smith 1975). For the spectral measurements of reduced enzyme solutions, quartz cells fitted with Schlenk connections were used. Reduction was carried out by adding a slight excess of degassed sodium dithionite solution in water to the enzyme solution under an inert atmosphere. The carbon monoxide adducts of reduced peroxidases were obtained by exposing the degassed enzyme solution to carbon monoxide at 1 atm. The cyanide adduct of ferric ZPOA was prepared by adding a few crystals of potassium cyanide to the enzyme solution and the corresponding ferrous cyanide adduct was obtained by adding a few crystals of sodium dithionite under nitrogen to the deaerated ferric cyanide enzyme solution.

The Compound I derivative of ZPOA and HRPA was prepared as follows. The enzyme solution in 0.1 M phosphate buffer, pH 7.0, was pretreated with hydrogen peroxide (1.1:1.0 mol ratio of H_2O_2/protein) and allowed to stand at 4 °C for 12 h. A second aliquot of hydrogen peroxide corresponding to a 1.1:1.0 molar ratio was added to the enzyme solution at 10 °C and the spectrum recorded within a few seconds. The Compound II derivative of peroxidases was prepared by adding 2 mol equivalents of substrate (hydroquinone) and 2 mol equivalents of hydrogen peroxide to the enzyme solution in 0.1 M phosphate buffer, pH 7.0, at 10 °C.

Electron paramagnetic resonance (EPR) spectra were recorded on a Varian X band spectrometer equipped with an Oxford Instruments ESR900A continuous flow cooling system (3.8–300 K) and a Stelar AQM Auriga/AT data system. DPPH was used as standard in the reference cavity.

Proton NMR spectra were recorded at 200 MHz on a Bruker AC 200 spectrometer employing 41 kHz sweep width and accumulating 10000 transients over 8 K data points. Samples for paramagnetic NMR spectra were prepared by ultrafiltrating the aqueous solution of the

enzymes and exchanging water with deuterated 0.1 M phosphate buffer, pD 7.0 (uncorrected for the smaller isotope effect). The residual monodeuterated water (HDO) signal was presaturated for 1 s before acquisition. Temperature was kept constant at 298 K by means of a variable temperature unit. Chemical shifts are referenced to the DSS signal. The downfield region of the NMR spectra was Fourier-transformed by applying a line broadening function of 50 Hz, while that upfield was processed with 5 Hz line broadening.

Results and discussion

Purification of ZPO *isoenzymes*

The purification procedure followed here to obtain the isoenzymes differs to some extent from that described previously for enzyme extraction from ripe fruits (Casella *et al.* 1986). The tissues of young squash release pectinaceous substances that tend to pack strongly the ion exchange columns; for this reason fast eluting columns, with a large filtrating surface, are required. The two columns of CM–Sephadex and DEAE–Sephadex, used in succession, selected the two classes of acid and basic peroxidases. No significant amount of paraperoxidase isoenzyme was found in the present preparation. The anionic (ZPOA) and cationic (ZPOC) isoenzymes were completely separated and purified in the preparative electrofocusing cell. This improves the isoenzyme separation with respect to the more traditional preparative SDS technique. The two enzymatic components, ZPOA and ZPOC, after extensive dialysis to eliminate ampholytes, and centrifugation, show RZ values of 2.0 and 1.6, respectively. The ZPOA isoenzyme was homogeneous by the criteria of SDS–PAGE and isoelectric focusing, whereas ZPOC displayed two very close bands (Figure 1). The molecular weights calculated with an accuracy of ±3% (as described in Materials and methods) and isoelectric points corresponding to these peroxidase isoenzymes are reported in

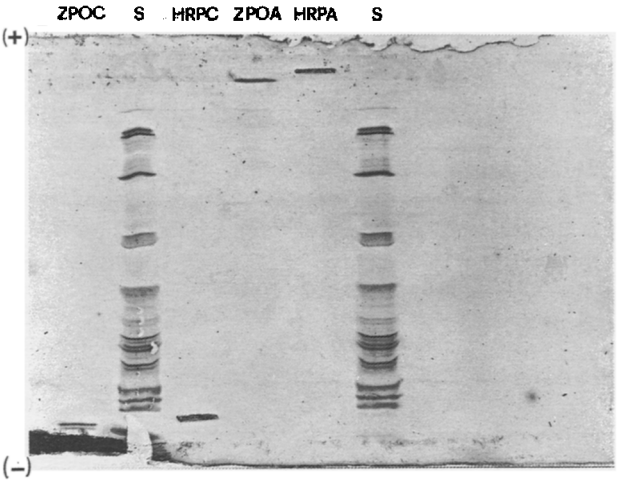

Figure 1. Isoelectric focusing of ZPOA and ZPOC after preparative electrophoresis in the presence of HRPA, HRPC (Sigma) and S (IEF Standards, p*I* 4.6–9.6, Biorad). Cathode (+) at top of gel; anode (−) at bottom of gel. The running conditions are described in Materials and methods.

Table 2. The M_r values and sugar content of the present ZPO isoenzymes are similar to those found previously for the major isoenzymes of ripe fruits (Casella *et al.* 1986) and fall in the range commonly observed for plant peroxidases (Chibbar & Van Huystee 1984, Frew & Jones 1984, Welinder 1985, Marangoni *et al.* 1989). With regard to the two cationic isoenzymes, $ZPOC_1$ and $ZPOC_2$, their relative amount was very low with respect to the

Table 2. Physicochemical properties of the purified isoenzymes ZPOA and ZPOC

Isoenzyme	RZ	M_r	p*I*
ZPOA	2.0	38800	3.8
$ZPOC_1$	1.6	41200	9.2
$ZPOC_2$	1.6	40000	9.0

Table 1. Purification of ZPOA

	Volume (ml)	Activity (units ml^{-1})	Proteins (mg ml^{-1})	Total protein (mg)	Total activity[a] (10^3 units)	Specific activity[a] (units mg^{-1})	RZ
After acetone precipitation, dialysis	200	321	40.2	8040	64	8	0.1
Sephadex G-100	105	545	20.2	2121	57.2	27	0.4
DEAE–Sephadex A50	60	357	1.7	102	21.4	210	1.0
Preparative IEF	30	620	1.4	42	18.6	443	2.0

[a]DMAB–MBTH as donor substrates.

anionic ZPO isoenzyme in the present preparation, and only their UV-visible spectra have been obtained with Soret maximum at 403 nm, typical of high-spin peroxidases.

Catalytic properties of ZPO and HRP anionic isoenzymes

In order to gain an assessment of the overall properties of ZPO we performed comparative catalytic, substrate binding and spectral studies between ZPOA and HRPA. The p*I* of our sample of HRPA was very close to that of ZPOA. As shown in Figure 2, ZPOA and HRPA exhibit a parallel, broad activity versus pH profile, using hydroquinone as donor substrate; the pH of maximum activity is found near pH 7. The specific activity depends markedly on the substrate, and in particular ZPOA and HRPA exhibit an opposite behaviour towards *o*-dianisidine and hydroquinone (Table 3). In order to establish whether the higher activity of ZPOA towards hydroquinone reflects a more general tendency of this enzyme to oxidize phenolic compounds with respect to HRPA, we investigated in more detail the kinetics of peroxidase-catalyzed oxidation of a series of phenols by hydrogen peroxide. The kinetic parameters for hydroquinone, 4-methylcatechol, catechol and *p*-hydroxybenzoic acid are collected in Table 4. The data are expressed in terms of k_{cat}, the turnover number, i.e. V_{max}/enzyme and k_{cat}/K_M, that can be considered as the second order kinetic constant when the concentration of phenolic substrate is much lower than K_M. ZPOA also shows activity towards other phenolic substrates like phenol, resorcinol and *p*-cresol. However, in this case we could not measure the kinetic parameters because the substrate absorption is superimposed to that of the reaction products. By contrast, salicylic acid seems to be completely unaffected by ZPOA.

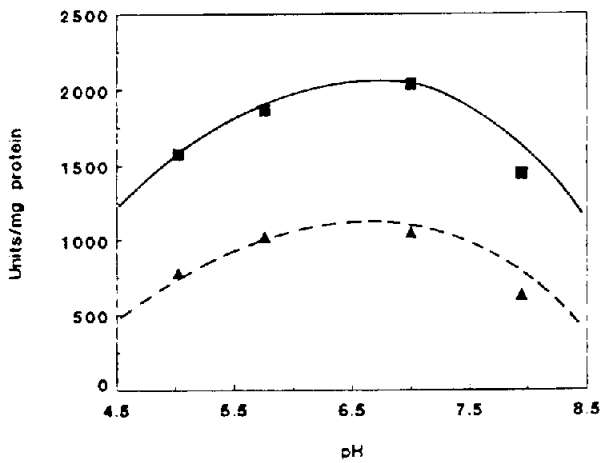

Figure 2. Specific activity of ZPOA (■, ——) and HRPA (▲, – – –) with hydroquinone in the pH range 4.5–8.5.

Table 3. Specific activity of ZPOA and HRPA in 0.1 M, phosphate buffer, pH 7.0 and 25 °C

Substrate	Enzyme	Specific activity (units mg^{-1} protein)
DMAB–MBTH	HRPA	1700
	ZPOA	440
o-Dianisidine	HRPA	4430
	ZPOA	1920
Hydroquinone	HRPA	1050
	ZPOA	2030

It is well established that oxidation of substrates (SH_2) by hydrogen peroxide catalyzed by peroxidases proceeds through two intermediates, called Compound I and Compound II, which are two and one oxidation equivalents above the native ferric heme, respectively, with the following mechanism (Dunford & Stillman 1976):

$$\text{Enzyme} + H_2O_2 \rightarrow \text{Compound I} \quad (1)$$
$$\text{Compound I} + SH_2 \rightarrow \text{Compound II} + SH^{\cdot} \quad (2)$$
$$\text{Compound II} + SH_2 \rightarrow \text{Enzyme} + SH^{\cdot} \quad (3)$$
$$2\,SH^{\cdot} \rightarrow \text{Products} \quad (4)$$

The mechanism implies binding of both the oxidizable substrate and hydrogen peroxide to the enzyme. Under hydrogen peroxide saturating conditions and assuming the reaction of Compound II as the slow step it is possible to treat the kinetic data under the classical Michaelis–Menten scheme. In the case of HRP the intermediates Compound I and Compound II have been characterized with a variety of spectroscopic techniques (Dunford & Stillman 1976, Browett *et al.* 1988, Dawson 1988), and we will give spectral characterization for the corresponding intermediates of ZPOA in the next section. Generally, the primary product of substrate oxidation is a free radical species ($SH^{\cdot}$). This is formed in a fast reaction by Compound I in step (2) and in a slower reaction by Compound II in step (3) (Job & Dunford 1976, Berry & Boyd 1984, Dunford & Adeniran 1986, Sakurada *et al.* 1990), both reactions being favored by electron donating substituents on the substrate.

The data in Table 4 show a parallel behaviour of ZPOA and HRPA in the oxidation of the series of phenolic compounds; however, ZPOA is systematically more active than HRPA, indicating that the

Table 4. Catalytic properties of ZPOA and HRPA in the oxidation of phenolic compounds by hydrogen peroxide in 0.1 M phosphate buffer, pH 7.0 and 25 °C

Substrate	Enzyme	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (mM^{-1}/min^{-1})
Hydroquinone	HRPA	0.15	1200	8000
	ZPOA	0.14	13200	94300
4-Methylcatechol	HRPA	0.29	1700	5800
	ZPOA	0.23	10800	47000
Catechol	HRPA	0.35	280	800
	ZPOA	0.33	3000	9100
p-Hydroxybenzoic acid	HRPA	0.36	20	60
	ZPOA	0.29	580	2000

zucchini enzyme has a marked propensity to carry out oxidation of this type of compound. The higher efficiency of ZPOA is determined by larger k_{cat} values, for each individual substrate, with respect to HRPA. The larger k_{cat} values probably reflect a higher reactivity of Compound II intermediate towards the reducing substrates. The assessment of this point requires fast kinetic measurements that are beyond the scope of the present investigation. It is interesting to note that although the differences in K_M are probably not significant, some differences in the way the phenolic compounds bind to the enzymes are evidenced by binding experiments to resting ZPOA and HRPA. Titration of the enzymes with the donor molecules produces small spectral perturbations in the Soret absorption bands, indicating that these molecules do not bind directly to the heme iron but rather in the heme proximity. Spectral perturbations of this type were in fact observed for the binding of several aromatic donor molecules to HRP and lactoperoxidase (Paul & Ohlsson 1978, Sakurada *et al.* 1986, Hosoya *et al.* 1989, Modi *et al.* 1989, Casella *et al.* 1991). As shown in Table 5 for the most rapidly reacting (hydroquinone) and the most slowly reacting (*p*-hydroxybenzoic acid) substrates, these molecules bind more strongly to ZPOA than HRPA. Since the binding process involves interactions between protein residues and functional groups of the substrates (Sakurada *et al.* 1986, Casella *et al.* 1991), it appears that the active site of ZPOA is structurally better suited than that of HRPA for complexing phenolic substrates.

Spectral properties of ZPO and HRP anionic isoenzymes

The electronic absorption spectra of native ZPOA, the reduced enzyme, and its CO and CN^- complexes are similar to those of HRPA and its corresponding derivatives (Table 6). Since the alkaline pyridine hemochrome assay of ZPOA identifies the heme group of this enzyme as protoheme IX, the spectral similarities between ZPOA and HRPA indicate that the proximal ligand for iron in the zucchini enzyme is probably a histidine imidazole. By recording the spectra of ZPOA in the presence of hydrogen

Table 5. Difference spectra characteristics and apparent binding constants (K_B) of ZPOA–donor complexes and HRPA–donor complexes in 0.1 M phosphate buffer, pH 7.0 and 25 °C

Enzyme	Donor	Spectrum of the complex (nm)		$\Delta\varepsilon$[a] ($mM^{-1}\,cm^{-1}$)	K_B (mM^{-1})	n[b]
		λ_{min}	λ_{max}			
ZPOA	hydroquinone	422	412	4.5	0.122	1.00
	p-hydroxybenzoic acid	422	402	5.3	0.075	0.99
HRPA	hydroquinone	408	382	3.3	0.048	0.95
	p-hydroxybenzoic acid	416	380	5.8	0.026	0.98

[a]Calculated from the extrapolated ΔA_∞ values; $\Delta\varepsilon = |\Delta\varepsilon_{peak} - \Delta\varepsilon_{trough}|$ for two-signed difference spectra.
[b]Hill coefficient.

Table 6. Electronic absorption spectra of ZPOA and HRPA in 0.1 M phosphate buffer, pH 7.0

Derivative	Enzyme	Absorption λ_{max}, nm (ε, mM^{-1} cm^{-1})			
Native	ZPOA	403 (106)	493 (11.5)	540[a]	645 (3.2)
	HRPA	403 (103)	495 (10.5)	540[a]	640 (3.1)
Fe(III)–CN^-	ZPOA	420 (110)	554 (12.0)	585[a]	
	HRPA	422 (107)	540 (11.9)	580[a]	
Fe(II)	ZPOA	435 (96)	556 (12.5)	590[a]	
	HRPA	436 (89)	556 (11.6)	585[a]	
Fe(II)–CO	ZPOA	422 (159)	542 (13.6)	572 (13.9)	
	HRPA	422 (152)	540 (12.4)	572 (13.3)	
Fe(II)–CN^-	ZPOA	434 (155)	534 (14.8)	566 (20.6)	
	HRPA	432 (151)	536 (14.8)	566 (20.2)	
Compound I	ZPOA	400			
	HRPA	400 (52)			
Compound II	ZPOA	420 (105)	532 (10.0)	556 (10.0)	
	HRPA	419 (95)	528 (8.8)	557 (9.0)	

[a]Shoulder.

peroxide it was not possible to observe stable Compound I spectra as it was for HRPA, because of the too fast conversion of this derivative to Compound II. Upon adding hydrogen peroxide in stoichiometric amounts to ZPOA and recording the spectrum in a few seconds at 10 °C, the intensity of the Soret band was reduced to about 70% that of the native enzyme, with two apparent maxima at 400 and 420 nm that can be associated to Compound I and Compound II derivatives, respectively. The spectrum of ZPOA Compound II could be obtained by adding a 2-fold excess of hydrogen peroxide to the enzyme in the presence of the reducing substrate hydroquinone.

Figure 3 shows the CD spectra of native ZPOA and its reduced and carbon monoxide derivatives. These spectra bear qualitatively close relationships not only with those of HRP isoenzymes (Strickland *et al.* 1968, Willick *et al.* 1969) but also with those of the acidic (P_1) isoenzyme of turnip peroxidase (Job & Dunford 1977). The main differences between the CD spectra of these proteins are observed in the otherwise almost featureless curves of the reduced derivatives and in the asymmetry of the Soret CD couplet of the carbonyl derivative of reduced ZPOA, exhibiting a weaker positive limb at lower energy and a stronger negative limb at higher energy. The latter feature was previously observed in the CD spectrum of the analogous derivative of ZPO paraperoxidase (Casella *et al.* 1986) and appears, therefore, a common characteristic of the

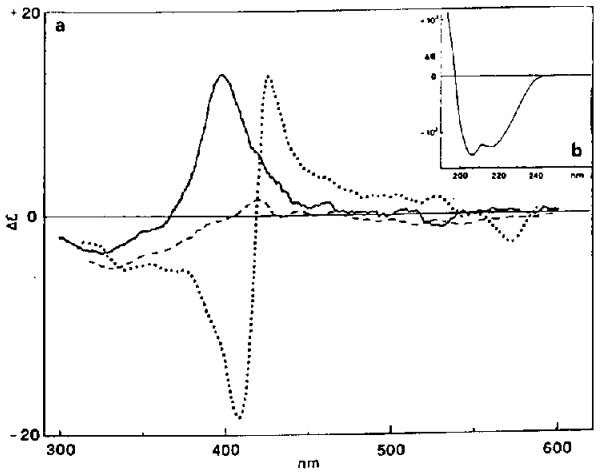

Figure 3. (**a**) CD spectra of ZPOA in 0.1 M phosphate buffer, pH 7.0 (2.5×10^{-6} M): (——) native (Fe^{3+}), (– – –) reduced (Fe^{2+}) and (· · · · ·) carbonyl derivative of reduced (Fe^{2+}–CO) enzyme. (**b**) Far-UV region of ZPOA in 0.1 M phosphate buffer, pH 7.0 (8×10^{-7} M).

zucchini enzymes. The remaining portion of the CD spectrum of carbon-monoxy ferro-ZPOA, including the negative peak near 570 nm, is similar to those of the turnip (Job & Dunford 1977) and HRPA derivatives (data not shown). The far-UV CD spectrum of ZPOA (Figure 3b) is consistent with the emerging overall structural similarity of the three plant peroxidases and shows close resemblance with those of ZPO paraperoxidase (Casella *et al.* 1986),

HRP isoenzymes (Strickland *et al.* 1968) and turnip peroxidase (Job & Dunford 1977). An estimate of the α-helix content of ZPOA from the ellipticity at 222 nm (Chen *et al.* 1972), using a mean residue weight of 112 determined as before (Casella *et al.* 1986) on the basis of the amino acid composition of HRP (Welinder 1979), gives about 30% helical content, a value in the range predicted or estimated for plant and yeast peroxidases (Welinder 1985).

The proton NMR spectra of ZPOA and HRPA are presented in Figure 4. The spectrum of HRPA exhibits far downfield and near upfield features that are very similar to those reported before for HRP isoenzyme A_3 at 360 MHz (Gonzalez-Vergara *et al.* 1985). On the basis of the relative intensities we assign the peaks at 83, 72, 66 and 52 p.p.m. of the ZPOA spectrum to the porphyrin ring methyl groups. These signals occur at significantly different positions with respect to the corresponding HRPA signals, and in particular the two central methyl peaks are much better separated than in any of the HRP isoenzymes spectra (Gonzalez-Vergara *et al.* 1985). Assuming that the assignment of the porphyrin methyl signal made by La Mar on HRPC (La Mar *et al.* 1980), and used by Goff for the HRP anionic isoenzymes (Gonzalez-Vergara *et al.* 1985), is generally valid for plant peroxidases, we can attribute the above signals of ZPOA to methyl groups at positions 5, 1, 8 and 3 of the porphyrin ring, as well as the low intensity peak at 63 p.p.m. to the CH group of the 2-vinyl residue. Two other signals between 48 and 45 p.p.m. probably belong to the 4-vinyl group, while the signals in the 40–25 p.p.m. region are probably due to the propionic acid side chains and proximal histidine CH_2 group (Gonzalez-Vergara *et al.* 1985). Several peaks appear in the near upfield region of the ZPOA spectrum. As for the corresponding spectra of HRP isoenzymes (Gonzalez-Vergara *et al.* 1985), this spectral region contains signals for the CH_2 vinyl groups and several signals attributable to alkyl groups of amino acid residues in proximity to the heme prosthetic group, that are obviously impossible to assign.

The EPR spectrum of ZPOA isoenzyme at low temperature (4 K), reported in Figure 5, shows absorption signals in the $g \sim 6$ and $g \sim 2$ regions. The absorption in the low field region clearly indicates the presence of a high spin heme Fe(III) species and exhibits an evident well resolved rhombic splitting ($g_x = 6.30$, $g_y = 5.57$). The weaker $g = 2$ signal arise from high spin heme iron, while the equally weak signals $g = 2.04$ and $g = 4.3$ arise from a small amount of non-heme Fe(III) contaminant species (Blumberg *et al.* 1968). This spectral pattern is similar to that of HRP isoenzymes (Blumberg *et al.* 1968, Dunford & Stillman 1976) and cytochrome *c* peroxidase (Dunford & Stillman 1976), but it is different from those of manganese peroxidase (Mino *et al.* 1988) and lignin peroxidase (Andersson *et al.* 1985), which exhibit axial EPR geometry. The rhombicity $R\%$ ($R\% = (g_x - g_y)/16 \times 100$; Blumberg *et al.* 1968, Ryu & Dordick 1992) of the ZPOA heme iron, as present in the $g \sim 6$ region of the spectrum, has been calculated to be 4.5%, of the same order of that found from the spectrum of HRPC ($R\% = 4.4$) obtained in our laboratory in the same experimental conditions (data not shown), and of that from the reported (Blumberg *et al.* 1968) EPR spectrum under similar experimental conditions. The rhombic character of the EPR spectra of peroxidases may arise from the fact that the histidine and water (where present) axial ligands are more distorted from the heme

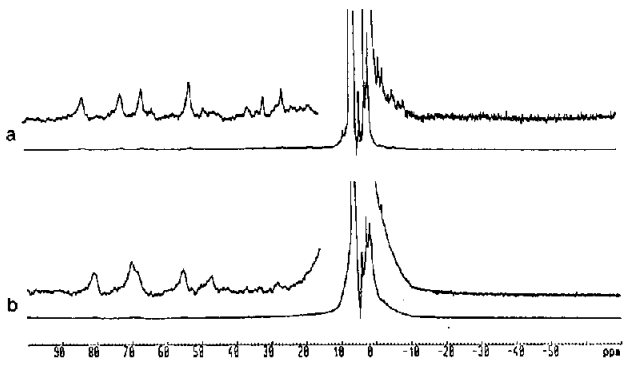

Figure 4. Proton NMR spectra of ZPOA (10^{-4} M) (**a**) and HRPA (10^{-4} M) (**b**) isoenzymes in deuterated 0.1 M phosphate buffer, pD 7.0.

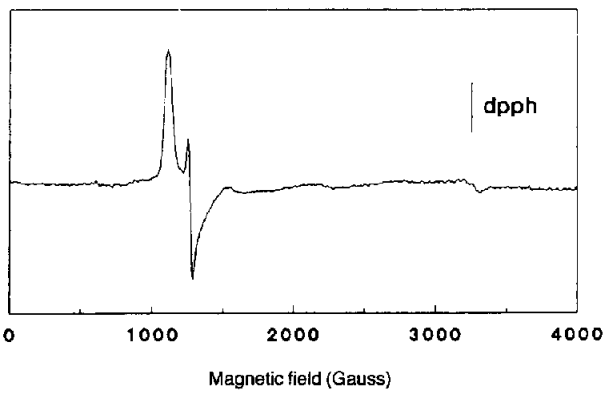

Figure 5. EPR spectrum of ZPOA (10^{-5} M) in 0.1 M phosphate buffer, pH 7.0, at 4 K; power 10 mW, receiver gain 10^3, modulation 10 G.

normal than, for instance, in the met heme globins (Brill *et al.* 1986).

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