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Peroxidase Activity in the Xerophytic «Cardon» (*Pachycereus pringlei*), a Cactaceae of the Sonoran Desert of Mexico

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Summary

Several isoperoxidase activities (EC 1.11.1.7. donor: H_2O_2 oxidoreductase) were detected in branch extracts of the cardón cactus (*Pachycereus pringlei*) of the Sonoran Desert, México. The whole enzyme activity in the crude extract was separated into several anionic and cationic isoenzymes. These isoforms exhibited characteristics similar to those of other plant peroxidases, i.e. i) optimal pH, ii) high activity when using guaiacol as hydrogen donor, iii) lower V_{max} for DOPA and iv) isoperoxidases were inactivated at high concentrations of the substrate hydrogen peroxide. However, cardón peroxidases were denatured at 70 °C. In FPLC Mono S, the main active fractions were separated into an anionic and two cationic isoperoxidases. When using isoelectric focusing, the anionic isoperoxidase was separated into two bands with slightly different isoelectric points. The partial purification of the crude extract using ammonium sulfate fractionation showed a highly atypical behavior, yielding a purification factor of 12 at 40–90 % saturation range.

Key words: Baja California, cactus, cordón, FPLC, IEF, isoperoxidases, Pachycereus pringlei, peroxidase activity, Sonoran Desert.

Abbreviations: CPO = cardón peroxidase; c = 90 %-fraction; a = anionic; c1 = cationic 1; c2 = cationic 2 fractions already obtained by FPLC Mono S; DOPA = dihydroxyphenil alanine; FPLC = fast protein liquid chromatography; IEF = isoelectric focusing; isoPO = isoperoxidase; K_m = Michaelis-Menten constant; PAGE = polyacrylamide gel electrophoresis; OPD = ortho-phenilen diamine; PO = peroxidase; V_{max} = highest velocity of activity.

Introduction

Peroxidases (PO) (EC 1.11.1.7. donor: H_2O_2 oxidoreductase) are important enzymes that allow aerobic organisms to survive in the presence of an oxidative atmosphere. The primary role of POs is the degradation by reduction of the highly toxic hydrogen peroxide. This is done using a variety of hydrogen donors co-substrates present in cellular fluids. Moreover, POs play relevant roles in the general metabolism of plants (Malik and Kaur, 1980; Fry, 1979; Stahmann and Demorest, 1972; Rubin and Ivanova, 1963; Kay and Basile, 1987 and Ferrer et al., 1990). Peroxidases have been used as indicators of plant response to external stimuli such as light, cold, heat, drought, hypoxia, salt, and pollution (Gaspar et al., 1982, García-Carreño and Ochoa, 1991). This could be because plants, as immobilized and autotroph organisms, are extremely dependent upon environmental conditions. They must often adjust themselves quickly in response to external changes, modulating the primary molecules of gene expression, the enzymes. Peroxidase activity is one of the fastest mechanisms for metabolic regulation in plants (Gaspar et al., 1982).

The Cactaceae is one of the most predominant families of plants in the arid regions of America. The most representa-

tive columnar cacti in the Mexican Sonoran Desert is *Pachycereus pringlei* (S. Watson. Britton et Rose, 1909), usually called cardón. In spite of this cacti is common through all plant communities on the Baja California Peninsula (Wiggins, 1980), there are few basic studies about its biology. The plant is succumbing to the ravages of development and many of them are dying from what appears to be an infectious disease. The survival and reproduction of cardón in desert ecosystems will depend largely on future studies of its ecology and physiology.

The cardón is distinguished from other members of the same family by its particular response to injury. When a portion of the plant is severed, the exposed portion rapidly turns brown. This response, which is largely enzyme dependent, may involve peroxidase activity. This research focuses on the isolation, separation of isoenzymes, and kinetic characterization of isoperoxidases of cardón branch juice. As far as we know, this is the first report on peroxidase activity from a member of the Cactaceae.

Materials and Methods

Plant material

Plants were studied in the Comitán area surrounding the Centro de Investigaciones Biológicas de Baja California Sur (24° 8′ N; 110° 23 W), 18 km west La Paz City, BCS, México (García-Carreño et al., 1992). Branches were collected from both flowering and non-flowering adult plants measuring more than 5 m in height.

Enzyme extract

Usually, one kilogram of the tip of a branch was used. Thorns were removed manually and the cleaned specimen was cut into small pieces and homogenized in a household fruit juice extractor (Molinex, France). Since the plant is a succulent one, the addition of any solution was unnecessary. In order to eliminate tissue debris, the extract was filtered through two layers of cheesecloth and centrifuged at 3000 rpm for 20 min. The supernatant was stored at 10 °C for up to several months, with no appreciable reduction in the enzyme activity.

Partial purification

The crude extract was fractionated by a salting-out procedure using solid ammonium sulfate. The precipitate of 0–40 % saturation was eliminated. The precipitate pellet of 40–90 % of saturation, which possessed the main peroxidase activity, was obtained by centrifugation at 3000 rpm for 20 min. It was dissolved in 50 mM acetate buffer, pH 5 and desalted by further dialysis in the same buffer.

Protein concentration determination

Protein content in the crude extract and in purified fractions was measured following the recommendations of Bradford (1976). Robinson (1979) demonstrated that this technique avoids any interference from phenolic compounds present in plant extracts. The relative concentration of protein in FPLC fractions was further determined by spectrophotometry at 280 nm.

Enzyme assay

Enzyme activity was measured, in the standard assay using guaiacol as co-substrate. This hydrogen donor is highly specific for peroxidases, because it is not oxidized by other enzymes which are present in plant extracts such as polyphenoloxidases (van Loon, 1971). For the quantification of substrate specificity of the purified fractions, several hydrogen donors such as DOPA, OPD, and guaiacol were used with a constant substrate concentration of 2 mM in 50 mM acetate buffer, pH 5. The response of the peroxidases to the concentration of the substrate hydrogen peroxide was assayed with guaiacol (16 mM , in 50 mM acetate buffer, pH 5) as hydrogen donor.

The standard assay was performed as follows: 2 mL of a solution containing the co-substrate in 50 mM acetate buffer, pH 5 were mixed in a spectrophotometer cell with 0.01 mL of the sample. Then, 0.005 mL of 30 % H_2O_2 was settled on the top inner wall of the spectrophotometer cell. The reagents were mixed by inverting the capped cell. Immediately, the cell was placed in the spectrophotometer (Spectronic 2000 by Bauch and Lomb) and the increase in the absorbance caused by oxidation of the co-substrate recorded at 470, 444, 490 nm for guaiacol, OPD, and DOPA, respectively.

The rapid localization of the peroxidase activity in the thirty fractions obtained after separation by ion-exchange chromatography was performed by placing 0.1 mL of a mixture of H_2O_2-guaiacol in the wells of a 96-well plate. Then, mixing 0.01 mL of every fraction in a respective well. A change from colorless to yellow-to-brown color, after a few minutes, was taken as positive for the PO activity. The specific activity was defined as the change in absorbance per minute per mg of protein.

The determination of the apparent Michaelis-Menten constant (K_m) for different co-substrates was made by measuring the enzyme activity at increasing concentrations of the hydrogen donor at 25 °C. The values of the K_m and V_{max} were estimated using an iterative regression analysis PC program in Basic, written by Page (1987).

The influence of pH on the peroxidase activity was assayed in the 90 %-fraction in 50 mM acetate buffers at 3.5–6.5 pH values, using the standard method.

Thermostability of the enzymes was determined by incubating the enzyme solution for 70 minutes in a waterbath at different temperatures and taking samples every ten min. Then, assaying the residual peroxidase activity, using the standard method.

FPLC

Isoenzymes were separated using ion exchange chromatography in Mono S ($-CH_2-SO_3^-$) FPLC column (Pharmacia Fine Chemicals, Sweden). The 90 %-fraction was dialyzed against 50 mM MES/NaOH buffer pH 6.0. The sample (0.1 mL) was 3.13 g per L of protein before loading to the application loop. The chromatographic separation was performed according to the recommendation of the manufacturer. The cationic isoPOs were eluted by 1 M NaCl lineal gradient in the MES buffer. Thirty fractions of 1 mL were collected, including those not absorbed to the matrix of the Mono S column.

Isoelectric focusing

Thin-layer isoelectric focusing (IEF) was performed in PAGE (5 % acrylamide) using pH 3 to 10 ampholytes. Electrophoresis was carried out at constant power of 5 W. The separation was stopped when the voltage became constant, i.e. 1.5 h, according to the application notes from LKB products (Sweden).

Table 1: Purification chart for cardón peroxidases using selective precipitation by ammonium sulfate and FPLC mono S. Activity assay was done using guaiacol as hydrogen donor.

Fraction	activity abs/min	specific activity	purification factor
crude extract	0.32	13.9	1
40 %-precipitate	0.14	3.9	0.2
90 %-precipitate	0.87	174	12.5
90 %-supernatant	0.0	0.0	0.0
FPLC:			
anionic	0.043	48.8	23.75
cationic 1	0.83	5593	2735
cationic 2	0.048	1230	601.2

Substrate-electrophoresis

After the IEF separation, the gels were immersed in a freshly prepared solution of 16 mM guaiacol in 50 mM acetate buffer, pH 5. After 15 min, the co-substrate solution was drained off and replaced with 0.03 % H_2O_2 solution. The isoPO active zones were visible after a few minutes as brown color bands. Their intensity was proportional to the peroxidase activity. The location and intensity of the bands were recorded immediately.

Staining for protein was carried out in the same gels after being treated for peroxidase activity. The gels were immersed in a Bradford reagent and allowed to stand for 5 min in a microwave oven at mild power and overnight at room temperature. The bands of protein lacking PO activity were stained in a blue color. Their intensity was proportional to the protein concentration. All experiments were repeated at least five times to assure validity.

Results

The partial purification of protein by selective precipitation using $(NH_4)_2SO_4$ as salting-out reagent rendered a saturation range of 40–90 % fraction. In it, about 87 % of the total peroxidase activity was present, but, only 11 % of the total protein. The specific activity in the crude extract was 14 and 174 in the 90 %-fraction precipitate. This treatment rendered in a purification factor of 12. In Table 1, the standard results of the purification procedure are presented.

Cation-exchange chromatography on the Mono S FPLC column rendered the separation of three fractions bearing peroxidase activity: a fraction in the non-adsorbed protein; the so called anionic peroxidase fraction, c1 fraction eluted during the application of a NaCl linear gradient at 0.28 M; the so called cationic 1 and c2 fraction released at 0.48 M of NaCl; the so called cationic 2. Figure 1 shows the chromatogram obtained in the separation technique.

The analysis of the isoperoxidases using isoelectric focusing on polyacrylamide gels rendered three isoPO active zones when assaying the 90 %-fraction, two cationic and one anionic. The a fraction obtained in the Mono S ion exchange FPLC column was resolved by IEF in two zones of PO activity. Each of the two peroxidase NaCl-released fractions from FPLC, rendered one active zone with cathodic movement. These active zones correlated with those present in the 90 %-fraction. However, only one of the anionic fractions correlated with those present in the 90 %-fraction. Several other bands, which were stained as protein, were present in the 90 %-fraction. They did not demonstrate any peroxidase activity. Figure 2 shows the isoelectrophoregram obtained for the analyzed samples.

Studies on the pH dependence of the peroxidase activity showed that CPO had a maximal activity at pH 5. Figure 3 shows the enzyme activity at different pH from 2.5 to 6.5. At pH 3 the peroxidase activity was null; at pH 4 there was an increasing slope up to 5. Then, a decrease to 6.5. At basic pH the PO activity was negligible.

The thermostability assay showed that cardón peroxidases are completely active when incubated at 50 °C up to 70 min. The enzyme preparation preserved 75 % of its activity when treated at 60 °C for up to 70 min and was completely inactiv-

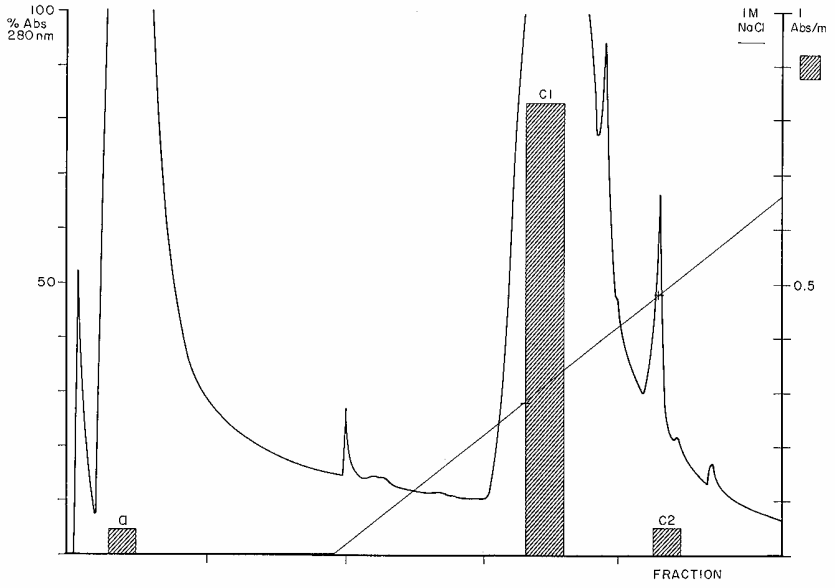


Fig. 1: Chromatogram of cardón isoperoxidases using FPLC mono S column. For conditions see materials and methods. a is anionic, c1, and c2 cationic 1, and cationic 2 fractions.

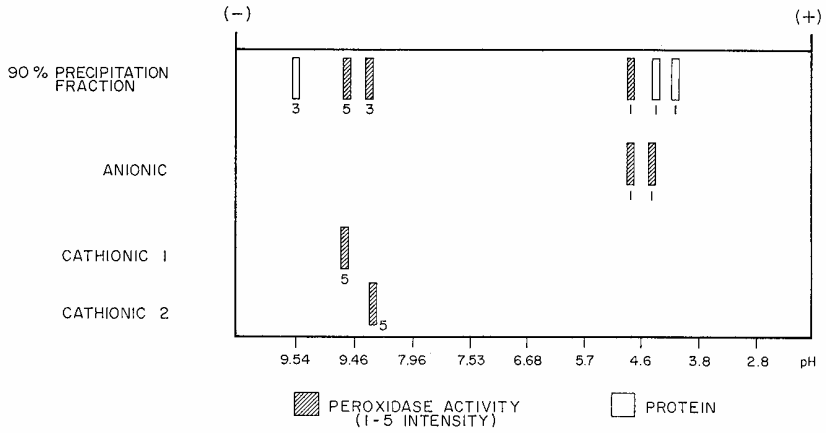


Fig. 2: Isoelectrophoregram of cardón fractions using isoelectric focusing (pH 3–10). Intensity of staining is suggested using numbers from weak (1) to very intense (5) for peroxidase containing activity bands and protein staining.

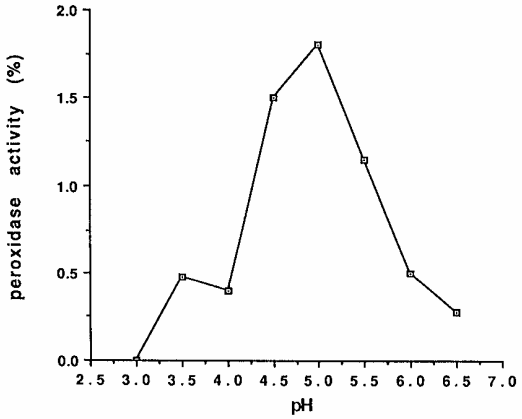


Fig. 3: pH dependence of cardón peroxidase activity on the 90 %-fraction. For experimental conditions see materials and methods.

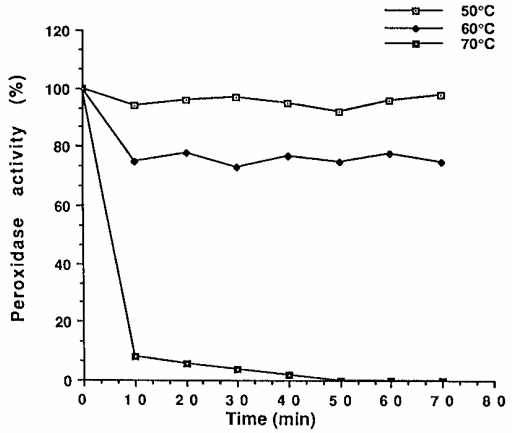
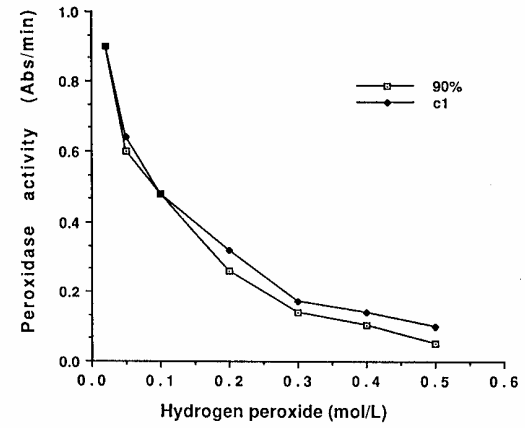


Fig. 4: Thermostability of cardón peroxidase on 90 %-fraction. For experimental conditions see materials and methods.



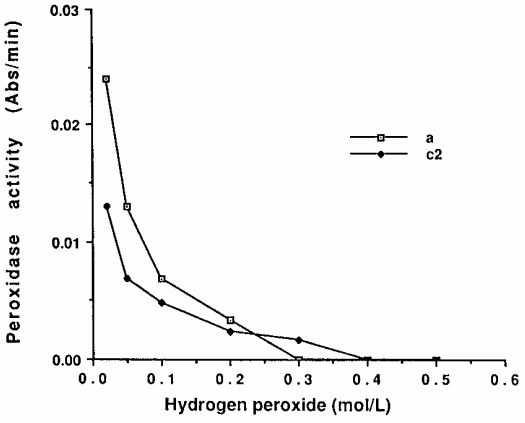
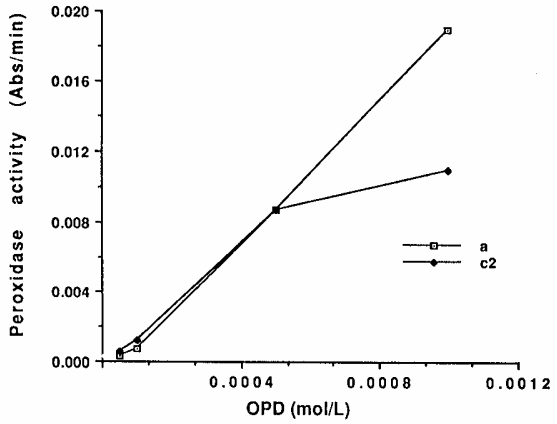
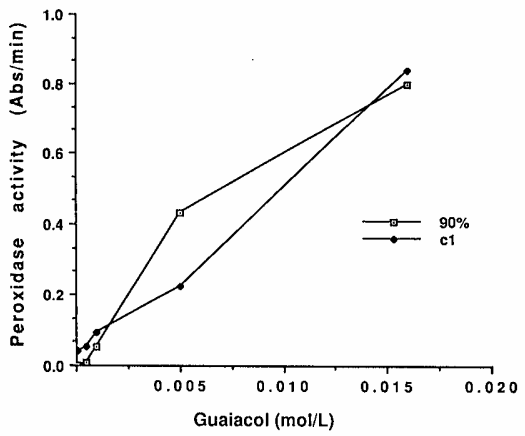


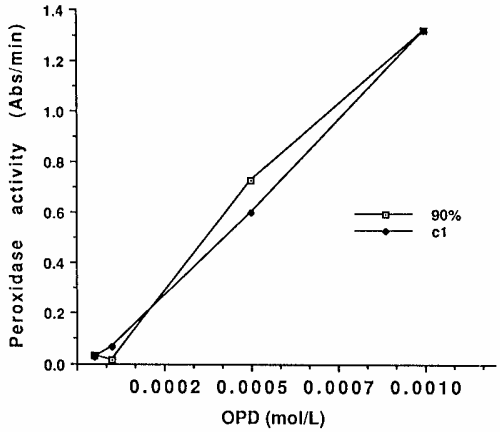
Fig. 5: Effect of hydrogen peroxide concentration on cardón isoperoxidases and 90 %-fraction. For experimental conditions see materials and methods.

ated at 70 °C, after a few minutes. Figure 4 shows the percent of remained PO activity after treatment at different temperatures.

The PO activity at increasing concentrations of the substrate is presented in Figure 5. It shows an inhibitory rate, both in the 90 %-fraction and in the fractions obtained after







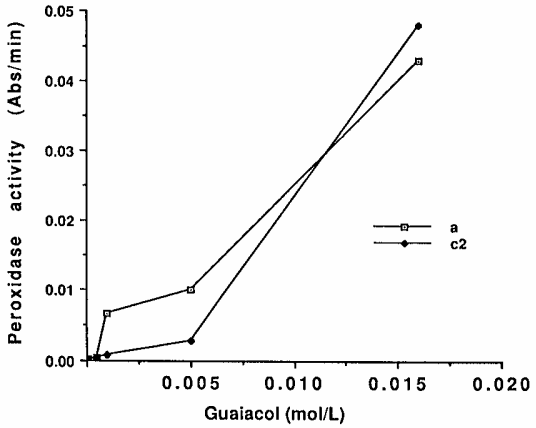


Fig. 6 a: Effect of OPD concentration on cardón isoperoxidases and 90 %-fraction. For experimental conditions see materials and methods.

Fig. 6 b: Effect of guaiacol concentration on cardón isoperoxidases and 90 %-fraction. For experimental conditions see materials and methods.

ion exchange chromatography. When treating with high concentrations of the substrate, the anionic peroxidase was the most sensitive fraction. The anionic fraction was completely inhibited at concentration of 0.3 M of the peroxide substrate, whereas the 90 %-fraction and c1 fraction remained active at concentrations as high as 0.5 M.

The PO activity at increasing concentrations of several hydrogen donors such OPD and guaiacol are presented in Figure 6, which shows the dependence of enzyme activity on the substrate. In both cases, the main isoPO activity was from the c1 fraction, and it was almost always equal to the total activity found in the 90 %-fraction. The activity on the a and c2 fractions was always ten to hundred times smaller for guaiacol and OPD.

The enzyme response of FPLC fractions using DOPA, a recognized substrate for other oxidoreductases, such as polyphenol oxidases, exhibited negligible activity (Fig. 7). The enzyme activity in this case never matched the activity recorded in the 90 % fraction. There was no activity when the FPLC enzyme fractions were assayed in the absence of hydrogen peroxide.

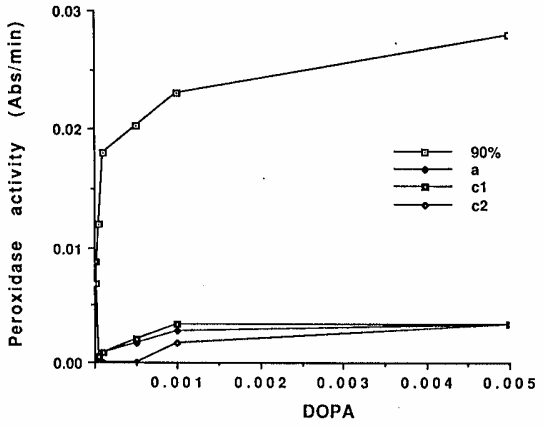


Fig. 7: Effect of DOPA concentration on cardón isoperoxidases and 90 %-fraction. For experimental conditions see materials and methods.

Table 2a: The cardón isoperoxidases apparent K_m and V_{max} for hydrogen peroxide.

Fraction	K_m ($\times 10^8$)	standard error	V_{max}	standard error
90 %-precipitation fraction	−2.0	0.005	0.231	0.074
anionic	−1.6	0.003	0.0063	0.002
cationic 1	−1.8	0.0044	0.27	0.072
cationic 2	−1.6	0.0024	0.0033	0.0086

Table 2b: The cardón isoperoxidases apparent K_m and V_{max} for DOPA.

Fraction	K_m ($\times 10^{-4}$)	standard error	V_{max}	standard error
90 %-precipitation fraction	4.5	0.000014	0.025	0.0018
anionic	4.3	0.00012	0.0037	0.00034
cationic 1	27	0.00034	0.0135	0.00092
cationic 2	16	0.0001	0.0045	0.0001

Table 2c: The cardón isoperoxidases apparent K_m and V_{max} for guaiacol.

Fraction	K_m ($\times 10^{-2}$)	standard error	V_{max}	standard error
90 %-precipitation fraction	1.3	0.0031	1.49	0.219
anionic	2.7	0.0048	0.139	0.253
cationic 1	1.8	0.0026	1.99	0.263
cationic 2	0.72	0.0019	0.0069	0.0013

Table 2 shows the K_m and V_{max} of the cardón isoPOs for the hydrogen donors used in this study, i.e. DOPA and guaiacol, as well as for the substrate. The apparent K_m for the substrate was negative since the enzymes showed reducing activity at increasing concentrations of the H_2O_2.

Discussion

The cardón stem and its juice show a rapid and spontaneous browning after cutting, which may be a response to injury. However, this reaction is not limited to the cardón, but other plant tissues. Peroxidases have been associated with cicatrization (Gaspar et al., 1982) and the bruising and browning of fruits and vegetables (Burnett, 1977). Preliminary experiments showed that this browning may be enzymatic and vulnerable to high temperatures. A sample of one Kg of cardón branch that was autoclaved for 15 min at 15 psi, did not brown, and peroxidase activity was not detected in its extracted juice.

Cardón peroxidases were precipitated from crude extract at 40 to 90 % saturation with $(NH_4)_2SO_4$ yielding 87 % of PO activity but only 11 % of protein present in the original extract. The factor of purification, i.e. 12, is abnormally high, unlike most of the enzymes already purified. This behavior is specific to the CPOs and contrary to the average factor, i.e. about 3, reported for this technique of purification. But it is in the same order of magnitude as the maximum purification factor already reported by Bonnerjea (1986).

The FPLC rendered two main peaks of protein along with other minor ones. The peroxidase activity was found in one of the non-absorbed protein peaks and in the two peaks eluted at 0.28 and 0.48 M of the sodium chloride lineal gradient.

The isoelectric focusing analysis of the crude, 90 %-fraction and FPLC fractions revealed that the peroxidase activity present in the 90 %-fraction could be separated by ion exchange chromatography in several active fractions. Each FPLC isoPO had its corresponding active zone in substrate-PAGE IEF. Moreover, IEF showed more resolution power in the separation of the anionic fraction than could be obtained by FPLC. The anionic fraction was separated by IEF into two bands bearing PO activity.

The IEF isoPO composition of the cardón extract is quite different from that found in mezquite (*Prosopis articulata*), another xerophytic plant from the Sonoran Desert, which possesses mainly anionic isoPO in the leaf extract (García-Carreño and Ochoa, 1991).

The pH of maximum activity for CPO found in this study was 5, and the range where the enzyme kept at least 20 % of its activity was from pH 4 to 6. This finding agrees with the pH in which the conformation of most POs is preserved. At alkaline pHs, the protohemin IX is gradually destroyed (Livron, 1986).

CPO activity was stable at 50 °C for 70 min. When the temperature increased to 60 °C, the enzyme lost 25 % of the initial activity, but the peroxidases were very sensitive to 70 °C. This behavior is quite different from POs of other sources. For example, horseradish peroxidase keep some activity at temperatures up to 100 °C (Livrom, 1986).

The enzyme kinetic analysis using different hydrogen donors showed that the main activity in the crude extract was associated with the c1 fraction. Almost all the enzyme activity present in this fraction matched the 90 %-fraction. In contrast, the activity associated with the a and the c2 fractions was insignificant. They were two orders of magnitude smaller than c1, regardless of the hydrogen donor used. In both, substrate-IEF and standard assay in test tube, the c1 fraction always showed the highest intensity or activity.

The assays using DOPA as hydrogen donor demonstrated that the enzyme activity measured in the FPLC fractions were peroxidases. The DOPA activity in the 90 %-fraction was ten times higher than the anionic (a) and cathodic 2 (c2) fractions and three times higher than those present in the cationic 1 (c1) fraction. Moreover, the enzyme activity against DOPA was null in all FPLC fractions when hydrogen peroxide was not included in the reaction, which could be expected for real peroxidases. This result is a demonstration that the 90 %-fraction may contain polyphenol oxidase activity, but no the FPLC fractions.

The cardón isoPOs showed a distinctive kinetic behavior against the assayed substrate and co-substrates. The inhibition rate observed for the increasing concentrations of the substrate was notable in the anionic and cathodic 2 FPLC fractions. By the contrary, cationic 1 fraction kept some ac-

tivity i.e. 0.176 Abs per min, at 0.3 M of the substrate. At the same concentration of the substrate, the cathodic 2 activity fell to 0.0017 Abs per min and anionic activity was null. These results agreed with the reduction in the catalytic power of PO reported by Paul (1963).

The data reported in this paper about the characteristics of CPOs contribute to understand some catalytic properties of these multipurpose enzymes in cardón. However, the involvement of POs in the browning and cicatrization of injured cardón needs further study. Such a protective role may be the result of metabolic functions that contribute to develop a barrier after mechanical damage. These functions possibly involve the recruitment of phenol species and the cross-linking of the cell wall monomers. The involvement of peroxidases in metabolic functions and its protective role in cardón require further investigation.

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References

BRADFORD, M.: A rapid and sensitive method for the measurement of micrograms quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry *72*, 248–254 (1976).

BONNERJEA, J.: Protein purification: the right step at the right time. Biotechnology *4*, 954–958 (1986).

BURNETT, F.: Peroxidase and its relationships to food flavor and quality. J. Food Sci. *42*, 1 (1977).

GARCÍA-CARREÑO, F. and J. L. OCHOA: The effect of saline stress on peroxidase activity in the mezquite (*Prosopis articulata*) leaf. J. Arid Environments *20*, 313–319 (1991).

GARCÍA-CARREÑO, F. L., E. TROYO-DIEGUEZ, and J. L. OCHOA: Relationships between saline ground water, soil composition, and leaf tissue of the phreatophyte mezquite in Baja California Sur, México. Journal Ground Waters *30*, 676–682 (1992).

GASPAR, T., C. PENEL, T. THORPE, and H. GEPPIN: Peroxidases 1970–1980. Universite de Geneve, Switzerland (1982).

FERRER, M., M. PEDREÑO, R. MUÑOZ, and A. ROS BARCEÑO: Oxidation of coniferyl alcohol by cell wall peroxidases at the expense of indole-3-acetic acid and O_2. FEBS Letters *276*, 127–130 (1990).

FRY, S.: Phenolic components of the primary cell wall and their possible role in the hormonal regulation of growth. Planta *146*, 343–351 (1979).

KAY, L. and D. BASILE: Specific peroxidases isoenzymes are correlated with organogenesis. Plant Physiol. *84*, 99–105 (1987).

LIVRON, D.: Effects de la temperature sur l'activite peroxydasique. Travail presente a la Faculte des Sciences pour l'obtention du Diplome de Biologiste. Universite de Geneve (1986).

MALIK, C. and S. KAUR: Changes in levels of auxins and inhibitor during seed development in okra (*Abelmoschus sculentus*). Plant Biochem. J. *7*, 5–14 (1980).

PAGE, A.: A non-linear regression program in basic for estimating K_m and V_{max}. Cambios Communications *3*, 49–51 (1987).

PAUL, K.: Peroxidases. In: The Enzymes. BOYER, P., H. LARDY, and K. MYRBACK (eds.). Academic Press *7*, 227–275 (1963).

ROBINSON, T.: The determination of proteins in plant extracts that contain polyphenols. Plant Sciences Letters *15*, 211–216 (1979).

RUBIN, B. and T. IVANOVA: On the oxidase function of plant peroxidases. Life Sci. *4*, 281–289 (1963).

STAHMANN, M. and D. DEMOREST: Changes in isoenzymes of host and pathogen following some fungal infections. Symp. Biol. Hung. *13*, 355–365 (1972).

VAN LOON, L.: Tobacco polyphenoloxidase: a specific staining method indicating non-identity with peroxidases. Phytochemistry *10*, 503–507 (1971).

WIGGINS, I. L.: Flora of Baja California. Stanford University Press. Stanford, Ca. USA. 1025 pp. (1980).