J. Enzyme Inhibition, 2000, Vol. 15, pp. 171–183
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CADMIUM INHIBITION OF A STRUCTURAL WHEAT PEROXIDASE

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(Received 18 March 1999; In final form 16 June 1999)

The major peroxidase from 15-day-old wheat plants was purified to homogeneity by FPLC ion exchange and molecular exclusion chromatography. It consists of a single polypeptide of M_r 37,500 according to gel filtration and SDS-PAGE and has a pI of 7.0. Kinetics of pyrogallol peroxidation showed that the enzyme follows the accepted mechanism for peroxidase, with kinetic constants $k_1 = 4.4 \times 10^6\,M^{-1}\,s^{-1}$ and $k_3 = 8.6 \times 10^5\,M^{-1}s^{-1}$. The effect of different metal ions was assayed on peroxidase activity. None of the ions used had any effect on enzyme activity, except for Cd(II), which was an inhibitor. This was an unexpected and novel finding for a peroxidase. The kinetics of pyrogallol peroxidation at different concentrations of Cd(II) have been studied and a mechanism for Cd(II) inhibition proposed. The results obtained could explain, in part, cadmium-induced oxidative stress.

Keywords: Cadmium inhibition; Kinetics; Peroxidase; Wheat

INTRODUCTION

Peroxidase (EC 1.11.1.7) is an enzyme that catalyzes the H_2O_2-dependent oxidation of a wide variety of substrates, mainly phenolics.[1] These enzymes have been found in all higher plants studied. Many isozymes are expressed in each plant, their pattern of expression is tissue-specific and changes upon

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development or in response to external stimuli.[2,3] Several functions are ascribed to peroxidases: auxin metabolism, lignification, wound healing and defence against pathogens.[4–7] Although much is known about the catalytic mechanism and molecular structure of peroxidases, the existence of so many molecular forms is intriguing. Since peroxidase virtually lack substrate specificity, it is very difficult to refer a particular function to a particular isozyme. Basic isozymes have been associated with auxin catabolism[4] and acidic isozymes with lignification,[5] but the true function and mechanisms of control at the isozyme level remains unknown.

We believe that a molecular characterization of different isoperoxidases will shed light on the above mentioned problem. Recent studies carried out in our laboratory have shown that peroxidase isozymes in wheat germ, which are expressed independently of development and external stimuli, are mainly basic.[8] We have purified and characterized the main isozyme, which turned out to be quite different from classical peroxidases. Among other distinctive features, it bears a regulatory Ca(II) binding site, different from the structural Ca(II) site present in peroxidases, which could be of physiological relevance in controlling activity.[9] We have also described the isozymes that appear in wheat upon development, which are mainly acidic.[10]

Due to the increasing evidence for the involvement of metal ions in oxidative stress associated with molecular damage in higher plants, it is of considerable interest to evaluate the effect of metal ions on isolated enzymes which have been associated to the oxidative response, such as peroxidases. Although some heavy metals are essential as micronutrients, uptake of toxic quantities can be harmful to most plants. Metal toxicity in plants may result from complex interactions of the major toxic metal ions with other soil components or environmental factors. Wheat and other important crops are often cultivated in agricultural environments showing low levels of metals. However, low metal concentrations can result in significant accumulation in plant tissues and different species show different responses to metal toxicity.

The oxidative damage caused by several metals such as Cu and Fe can be explained directly as involving changes in redox state (Fenton reaction). The molecular mechanisms of Cd toxicity are not known with any certainty. So far the influence of Cd(II) on reactive oxygen species levels in plant tissues has been described in only a few reports.[11–16]

In view of these considerations, in this paper we describe the purification and properties of the major structural peroxidase from wheat plants, and its behavior in the presence of different metal ions. The enzyme was inhibited by Cd(II), a novel feature for a peroxidase, and therefore the kinetics of this inhibition was studied.

MATERIALS AND METHODS

Plants

Wheat (*Triticum aestivum* L.) seeds supplied by Cargill S.A. were grown in a controlled climate chamber at $24 \pm 2°C$, 50% relative humidity, a photoperiod of 16 h and a light intensity of 175 $\mu mol\, m^{-2}\, s^{-1}$, in plastic boxes containing vermiculite and watered daily with Hoagland solution prepared according to Hoagland and Arnon.[17]

Enzyme Purification

15-day-old whole wheat plants were homogenized using a Sorvall Omni-Mixer, in 50 mM Tris-HCl buffer (pH 7.4) using 5 ml buffer/g of fresh weight. Homogenates were centrifuged for 15 min at 26,800× *g*. The supernatant was chromatographed on a DEAE-cellulose column equilibrated with 50 mM Tris-HCl buffer (pH 7.4). The fraction eluting with equilibrium buffer was concentrated with polyethyleneglycol and chromatographed on a Sephadex G-200 column. Fractions with peroxidase activity were further purified using an FPLC apparatus. A mono P HR 5/5 column equilibrated with 50 mM Tris-HCl buffer (pH 7.4), was used. The peroxidase was eluted with a linear gradient of 0–200 mM NaCl in the same buffer.

Molecular Properties

Molecular weight was determined using an FPLC apparatus with a Superose 12 column calibrated with Blue Dextran, bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and cytochrome c (12.4 kDa). It was also determined by SDS-PAGE as shown below.

Electronic absorption spectrum of the purified enzyme was obtained with a Hitachi U-2000 spectrophotometer.

Enzyme Assays

Pyrogallol oxidation was determined in a reaction mixture containing 20 mM potassium acetate buffer (pH 5.5), 5 mM pyrogallol, 0.6 mM H_2O_2 and 1.2 nM enzyme in a final volume of 1 ml. Activity was measured by

following the increase in absorbance at 430 nm due to the formation of purpurogalline ($\varepsilon = 2.47\,mM^{-1}\,cm^{-1}$).

When the kinetics of pyrogallol oxidation were performed, different concentrations of pyrogallol and H_2O_2 were used as indicated in Results. H_2O_2 concentration was determined in a spectrophotometer by measuring the absorbance[18] at 240 nm, using $\varepsilon = 43.6\,M^{-1}\,cm^{-1}$. When metal ions were added to the incubation medium, they were used at concentrations between 0 and 20 mM. Experimental data were fitted to the equations using the program Enzfitter from Elsevier Biosoft.

PAGE

SDS-PAGE in discontinuous buffer was performed as described by Laemmli[19] in a LKB 2001 vertical unit. Molecular weight markers used for the M_r determination were lysozyme (14.4 kDa), lactalbumin (18 kDa), trypsinogen (24 kDa), pepsin (34.7 kDa), ovalbumin (45 kDa) and bovine serum albumin (66 kDa).

IEF were performed in a Multiphor II unit (Pharmacia) thermostatized at 10°C. One hundred and fifty µl samples were applied to 2 mm thick gels containing ampholytes in the pH range 3–10 (Pharmalyte 3–10) and run for 3 h at 25 W.

RESULTS

Purification and Molecular Properties

In a previous paper,[10] we showed that more than 15 peroxidase isozymes were revealed in 15-day-old wheat plant extracts by specifically stained native PAGE.[10] One of the major isoperoxidases, which is present in roots, stems and leaves of wheat plants, was purified to homogeneity as described in Materials and Methods. Figure 1 shows a Coomassie blue stained SDS-PAGE of the purified enzyme. According to MW markers, the peroxidase molecular weight was 38,000 Da. A value of 37,500 Da for the molecular weight of the enzyme was obtained by gel filtration, using a Superose 12 column in an FPLC equipment (Figure 2), thus indicating the monomeric nature of the enzyme.

The enzyme has a pI close to 7, according to an IEF-PAGE (not shown). Figure 3 shows the electronic absorption spectrum of the peroxidase.

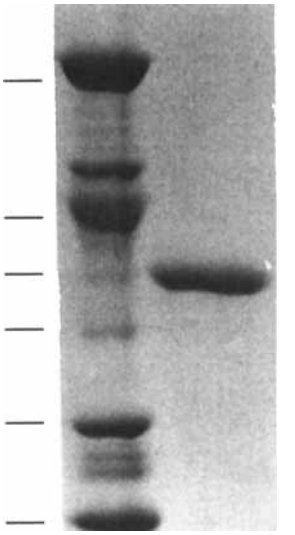


FIGURE 1 SDS-PAGE of the purified peroxidase. Molecular weight markers used are described in Materials and Methods.

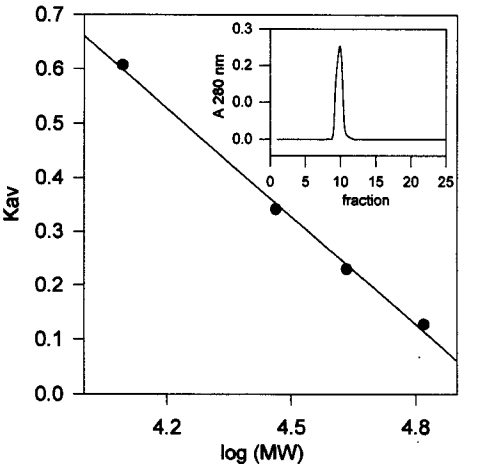


FIGURE 2 Plot of K_{av} as a function of the log of MW. Inset: absorbance at 280 nm of the fractions eluted from Superose 12 column. The column was calibrated as shown in Materials and Methods.

As can be seen, it is a typical spectrum of a high spin ferric hemoprotein. The Soret absorption band shows a maximum at 407 nm, while visible maxima are near 495 and 636 nm. The spectrum is similar to that of wheat germ peroxidase.[20]

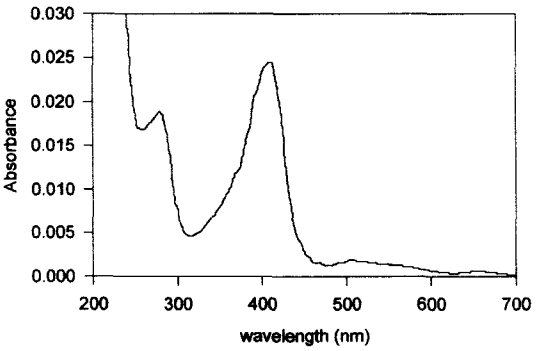


FIGURE 3 Electronic absorption spectrum of wheat peroxidase.

Kinetics of Pyrogallol Oxidation

Equations (1)–(4) represent the accepted mechanism for horseradish peroxidase:[21]

$$E + H_2O_2 \xrightarrow{k_1} \text{compound I} + H_2O \tag{1}$$

$$\text{compound I} + S: \xrightarrow{k_2} \text{compound II} + S\cdot \tag{2}$$

$$\text{compound II} + S: \xrightarrow{k_3} E + S\cdot \tag{3}$$

S is the second substrate, here the pyrogallol

$$2S\cdot \longrightarrow P \tag{4}$$

Under steady state conditions, assuming $k_2 \gg k_3$ (as is the case with peroxidases), the following Equation (5) can be derived from Equations (1)–(4):

$$d[P]/dt = \frac{k_3[S][E]_t}{1 + (k_3[S]/k_1[H_2O_2])}. \tag{5}$$

In this way, kinetic parameters $V_{max\,app}$ and $K_{m\,app}$ can be defined as:

$$V_{max\,app} = k_3[S][E]_t, \tag{6}$$

$$K_{m\,app} = k_3[S]/k_1. \tag{7}$$

To characterize the peroxidase mechanism, steady state kinetics of pyrogallol oxidation was performed. Figure 4 shows the experimental curves

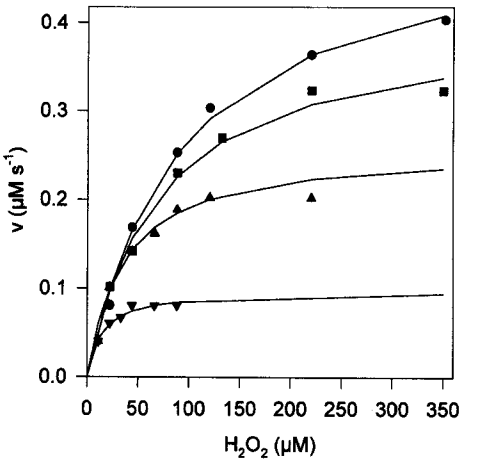


FIGURE 4 Plots of v as a function of H_2O_2 concentration at different pyrogallol concentrations. Pyrogallol concentrations used were 100 (▼), 200 (▲), 350 (■) and 500 (●) μM.

obtained for velocity as a function of H_2O_2 concentration for different concentrations of pyrogallol. All the curves could be fitted to rectangular hyperbolae according to:

$$v = \frac{V_{\max app}}{1 + (K_{m app}/[H_2O_2])}. \quad (8)$$

Values of the kinetic parameters $V_{\max app}$ and $K_{m app}$ were obtained by nonlinear regression of experimental data.

From the plots of $V_{\max app}$ as a function of pyrogallol concentration and of $K_{m app}$ (Figure 5A and B), values of $K_3 = 8.6 \times 10^5\,M^{-1}\,s^{-1}$ and of $k_1 = 4.4 \times 10^6\,M^{-1}\,s^{-1}$ respectively were obtained. Thus, the three irreversible steps mechanism accepted for HRP and other peroxidases can also be verified for the wheat peroxidase.

Effect of Metal Ions on the Wheat Peroxidase Activity

The effect of metals usually found as soil contaminants such as Co(II), Cd(II), Cu(II), Ni(II), and Zn(II) was assayed with wheat peroxidase. Since we have recently described that some cationic wheat germ peroxidases are directly activated by Ca(II), independently of the stabilizing effect of structural Ca(II) present in plant peroxidases,[9] the effect of Ca(II) and Mg(II) was also studied. None of the ions used had any effect on enzyme activity,

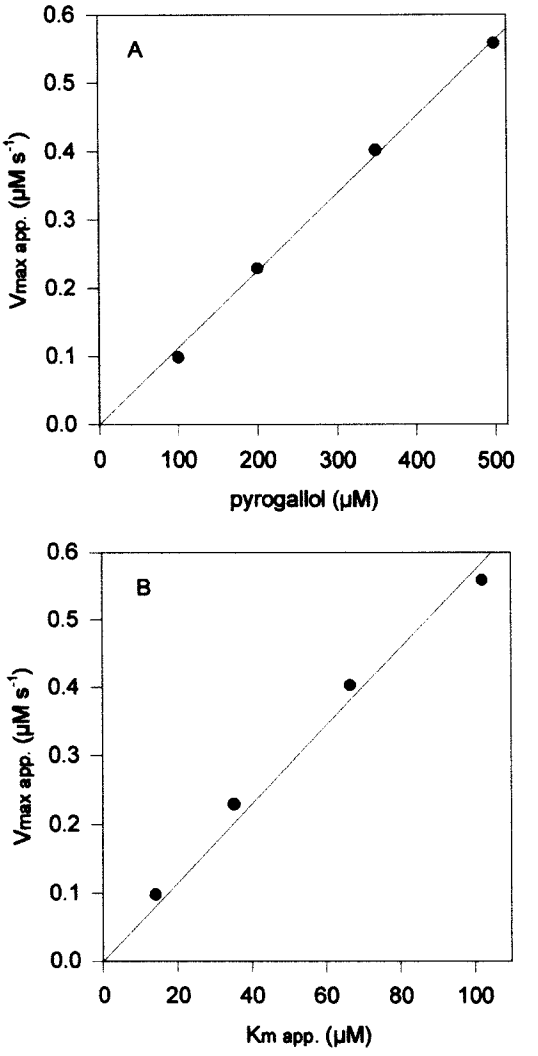


FIGURE 5 Plots $V_{max\,app}$ as a function of pyrogallol concentration (A) and $K_{m\,app}$ (B).

except for Cd(II) which, at a concentration of 4 mM, produced a 50% inhibition on peroxidase activity. Thus, the activity of the enzyme is reduced but not abolished even at very high Cd(II) concentration. Similar results were obtained when either ascorbic or ferulic acid were used as substrates. This inhibition was reversible, and the enzymatic activity was totally recovered after dialysis or Sephadex G-25 filtration. A plot of v as a function of Cd(II) concentration looks like a rectangular hyperbola (Figure 6) and the data

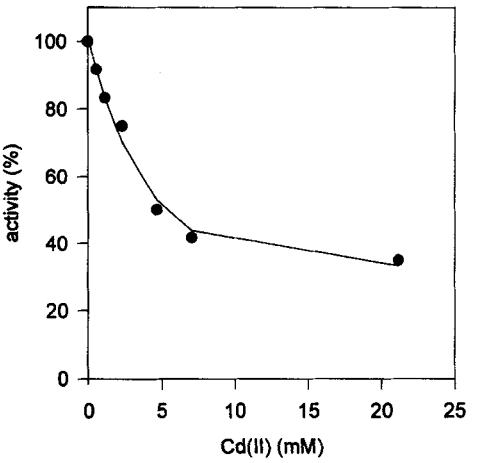


FIGURE 6 Peroxidase activity as a function of Cd(II) concentration.

can be fitted to the empirical equation:

$$v = v_0 - \frac{v_0 - v_\infty}{1 + (K/[Cd^{2+}])} \tag{9}$$

where v_0 and v_∞ are the values of v respectively in the absence and at saturation of Cd(II). K is independent of $[Cd^{2+}]$. Its value is numerically equal to $[Cd^{2+}]$ that produces 50% of the maximum inhibition. The results suggest that Cd(II) binds to one site or to non-interacting identical sites.

Mechanism of Peroxidase Inhibition by Cd(II)

Kinetics of pyrogallol peroxidation were studied at different Cd(II) concentrations and a fixed concentration of H_2O_2 (Figure 7). Experimental data were fitted by non-linear regression for each Cd(II) concentration to the equation:

$$v = \frac{V_{\max app}}{1 + (K_{m\,app}/[\text{pyrogallol}])}. \tag{10}$$

Cd(II) produced a decrease in $V_{\max app}$ but had no effect on $K_{m\,app}$ (Table I), indicating that Cd(II) induces a decrease in k_1.

Assuming that the enzyme can bind Cd(II) in its three redox states, a reaction model in the presence of Cd(II) may be proposed (Scheme 1).

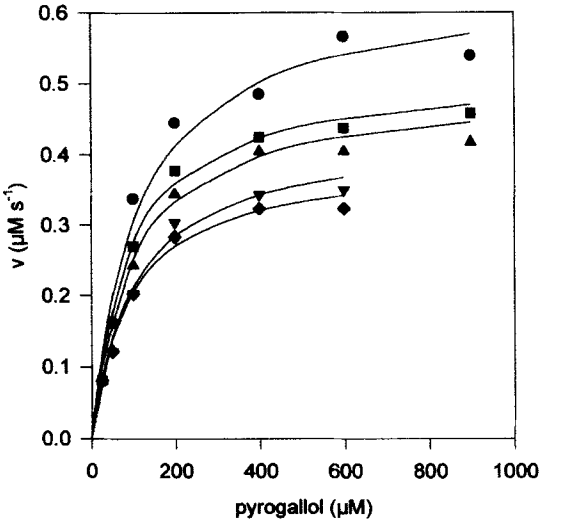
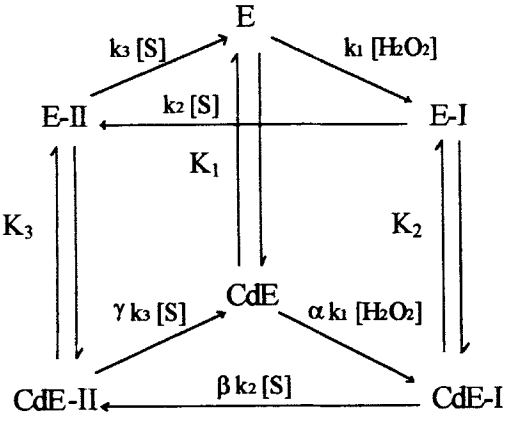


FIGURE 7 Plots of v as a function of pyrogallol concentration for 0 (●), 1.2 (■), 2.4 (▲), 4.8 (▼) and 11.8 (◆) mM Cd(II). The constant value of the H_2O_2 concentration was 0.6 mM.

TABLE I Effect of Cd(II) on the steady state kinetic parameters of pyrogallol peroxidation catalyzed by wheat peroxidase

Cd(II) (mM)	$K_{m\,app}$ (μM)	$V_{max\,app}$ (μM s^{-1})
0	111 ± 10	0.095 ± 0.006
1.2	88 ± 6	0.077 ± 0.007
2.4	97 ± 10	0.063 ± 0.005
4.8	104 ± 9	0.054 ± 0.006
11.8	92 ± 8	0.043 ± 0.004

H_2O_2 concentration was 0.6 mM. Values ± SE were estimated by the best fit to Equation (10).



SCHEME 1

E, E–I and E–II represent native enzyme, compound I and compound II, respectively; Cd–E, Cd–E–I and Cd–E–II represent the same intermediates with bound Cd(II); K_1, K_2 and K_3 are the corresponding equilibrium dissociation constants; k_1, k_2 and k_3 are kinetic constants and α, β and γ are parameters that modify kinetic constants.

Since the enzyme remains partially active at very high Cd(II) concentration, the inhibition is not dead-end. Thus, there are at least two enzyme–inhibitor complexes. Because V_{max} is influenced by the inhibitor concentration the complex cadmium–native enzyme certainly exists.

From this model, the following velocity equation may be derived:

$$\frac{[E]_t}{v} = \frac{1}{k_1'[H_2O_2]} + \left(\frac{1}{k_2'} + \frac{1}{k_3'}\right)\frac{1}{[S]} \tag{11}$$

with:

$$k_1' = k_1 \frac{\alpha[Cd^{2+}] + K_1}{[Cd^{2+}] + K_1}, \tag{12}$$

$$k_2' = k_2 \frac{\beta[Cd^{2+}] + K_2}{[Cd^{2+}] + K_2}, \tag{13}$$

$$k_3' = k_3 \frac{\gamma[Cd^{2+}] + K_3}{[Cd^{2+}] + K_3}. \tag{14}$$

Assuming that $k_2 \gg k_3$ and that β is not much smaller than one, the quantity $1/k_2'$ vanishes in the above formula (11).

It is possible to compare the theoretical predictions with the experimental results given in Table I:

$$V_{max\,app} = [E]_t[H_2O_2]k_1',$$
$$K_{m\,app} = [H_2O_2]k_1'/k_3'.$$

Since $K_{m\,app}$ is constant (Table I), K_1 equals K_3 and α equals γ. Because of these circumstantial equalities the theoretical curve in Figure 6 is rigorously a rectangular hyperbola. The plot $\{(1/[Cd^{2+}]);(v_0/(v_0 - v))\}$ gives $K_1 = 2\,mM$ and $\alpha = 0.4$. Theoretical values of $V_{max\,app}$ may then be calculated: 0.093, 0.072, 0.063, 0.054 and $0.045\,\mu M\,s^{-1}$ for 0, 1.2, 2.4, 4.8 and 11.8 mM of Cd(II) concentrations, respectively. These values are in agreement with the experimental results (Table I).

DISCUSSION

Although peroxidases have been extensively studied, their true physiological functions and control mechanisms at the isozyme level remain unclear. We have studied wheat germ peroxidases, where mainly cationic isozymes are expressed.[8] According to the two step mechanism of peroxidase expression,[22] anionic isozymes appear upon development. We have characterized isozyme expression in wheat upon development and found at least 18 bands of different electrophoretic mobility.[10] In the present work, the main isozyme of 15-day-old wheat plants has been purified and characterized. It is a typical high spin ferric hemoprotein of 37.5 kDa.

The effect of several metal ions, essentially those found as soil pollutants, was studied on the wheat peroxidase activity. Surprisingly, the enzyme was inhibited by Cd(II), so far a not described feature for a peroxidase.

Cadmium is an abundant element that is generating concern due to its accumulation in the environment as a result of industrial practices, and it is a major environmental pollutant present in areas with heavy road traffic as well as near smelters and sewage sludge areas. Although not essential for plant growth, this metal is readily taken up by roots and translocated to aerial organs in many species. The presence of Cd(II) in the environment has increased in some areas to levels which threaten the health of aquatic and terrestrial organisms. The biochemical mechanisms of its toxicity are not well understood. Cadmium does not appear to generate free radicals,[23] but does elevate lipid peroxidation in plants and tissues soon after exposure.[11–14] Previous studies in our laboratory[14] have shown that free radical scavengers and antioxidants are useful in protecting against cadmium toxicity. Cd(II) has been reported to bring about oxidative stress, although unable to trigger Fenton's reaction.[12,14,24]

Although the *in vivo* relevance of the Cd(II) inhibition remains to be studied, it could explain in part cadmium-derived oxidative stress through peroxidase inhibition, thus increasing levels of H_2O_2.

A model for the enzyme inhibition by Cd(II) was proposed. This model assumes that Cd(II) binds, reversibly, to the three intermediates of peroxidase reaction, and that the intermediates are active with Cd(II) bound.

The finding that peroxidases have sites for metal binding is a contribution to the study of the mechanisms of control of these enzymes at the isozyme level.

Acknowledgments

This work was supported by grants from Universidad de Buenos Aires (Argentina) and Consejo Nacional de Investigaciones Científicas y Técnicas

(CONICET) (Argentina). M.L.T. is a career investigator from CONICET and D.A.C. is a fellow from CONICET.

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