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Effects of cadmium, lead, mercury and zinc on δ-aminolevulinic acid dehydratase activity from radish leaves

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Abstract

The purpose of the present study was to investigate the *in vitro* and the *in vivo* effects of cadmium, zinc, mercury and lead on δ-aminolevulinic acid dehydratase (ALA-D) activity from radish leaves. The *in vivo* effect of these metals on growth, DNA and protein content was also evaluated. The results demonstrated that among the elements studied Cd^{2+} presented the highest toxicity for radish. 50 % inhibition of ALA-D activity (IC_{50}) *in vitro* was at 0.39, 0.39, 2.29, and 1.38 mM Cd^{2+}, Zn^{2+}, Hg^{2+} and Pb^{2+}, respectively. After *in vivo* exposure Cd^{2+}, Zn^{2+}, Hg^{2+} and Pb^{2+} inhibited ALA-D by about 40, 26, 34 and 15 %, respectively. Growth was inhibited by about 40, 10, 25, and 5 % by Cd^{2+}, Zn^{2+}, Hg^{2+}, and Pb^{2+}, respectively. DNA content was reduced about 35, 30, 20, and 10 % for Cd^{2+}, Zn^{2+}, Hg^{2+}, and Pb^{2+}, respectively. The metal concentration in radish leaves exposed to Cd^{2+}, Zn^{2+}, Hg^{2+}, and Pb^{2+} was 18, 13, 6, and 7 μmol g^{-1}, respectively. The marked ability of radish to accumulate Cd^{2+} and Zn^{2+} raises the possibility of using this vegetable as a biomonitor of environmental contamination by these metals.

Additional key words: cell growth, DNA content, heavy metals, proteins, toxicity.

Introduction

δ-Aminolevulinic acid dehydratase (ALA-D) is a sulphydryl-containing enzyme that catalyses the asymmetric condensation of 2 molecules of δ-amino-levulinic acid (ALA) to porphobilinogen (Gibson *et al.* 1955). The enzyme-catalyzed condensation occurs via the formation of two successive Schiff-base intermediates (Jordan and Gibbs 1985, Jaffe 1995). This reaction is fundamental for the biosynthesis of tetrapyrroles. Due to its sulfhydryl nature (Shemin 1976, Tsukamoto *et al.* 1979, Bevan *et al.* 1980, Barnard *et al.* 1977), ALA-D activity is highly sensitive to the presence of heavy metals such as mercury (Rocha *et al.* 1993, 1995, Emanuelli *et al.* 1996), lead (Goering and Fowler 1984, 1985, Rodrigues *et al.* 1989, 1996, Rocha *et al.* 1995), copper (Nelson *et al.* 1981) and tin (Chiba and Kikuchi 1984), which present high affinity for -SH groups.

The enzyme from mammalian tissues and prokaryotes requires zinc for maximum activity (Gibbs *et al.* 1985, Chauhan and O'Brian 1995, Mitchell *et al.* 1995). In contrast, the ALA-D from plant seems to require Mg^{2+} to display maximum activity. In the plant enzyme, no cysteine residues are found in the homologous regions implicated in Zn^{2+} binding in mammalian δ-ALA D (Jaffe 1995).

In the present study, we examined the effect of various toxic metals that occur in the environment (Pb^{2+}, Cd^{2+} and Hg^{2+}) and also of Zn^{2+} on ALA-D from radish leaves. The accumulation of these metals after *in vivo* exposure was also investigated in an attempt to establish a correlation between ALA-D inhibition and metal accumulation. Growth, DNA, and protein content of exposed plants were also evaluated to obtain some information on the general effects of these metals on radish.

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Abbreviations: ALA - δ-amino-levulinic acid; ALA-D - δ-aminolevulinic acid dehydratase; PBG - porphobilinogen.

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Materials and methods

Chemicals: Delta-aminolevulinic acid and zinc chloride were purchased from *Sigma* (St. Louis, USA). Nitric acid, trichloroacetic acid and sodium carbonate were purchased from *Reagen* (Rio de Janeiro, Brazil). Sodium chloride was obtained from *Vetec* (Rio de Janeiro, Brazil). All other chemicals were purchased from *Merck* (Darmstadt, Germany).

Plant germination and metal contamination: For the *in vitro* study, radish seeds were germinated over filter paper wetted with distilled water for 5 d at 25 °C under natural irradiation. Water (1 cm^3) was added daily to plastic flasks sealed with Petri dishes. Leaves were homogenized in 140 mM Tris-HCl and 2.7 mM $MgCl_2$ (1 g of leaves in 10 cm^3 of buffer) and then centrifuged at 1000 *g* for 10 min.

For the *in vivo* exposure to metals, 1 cm^3 of water (control) or 1 cm^3 of a 1 mM solution of $CdCl_2$, $HgCl_2$, $ZnCl_2$ and $Pb(CH_3COO)_2$ were added daily for 5 d to previously water wetted filter papers. Leaf homogenates were prepared as described above (1 g of leaves in 10 cm^3 of buffer).

Growth and DNA quantification: Measuring the length of the stem assessed the effect of metals on radish growth. Leaf DNA was quantified by the method of Burton (1956) on the next day after the 5th day of exposure to the metals.

Enzyme assay: ALA-D activity was assayed according to the method of Sassa (1982) by measuring the rate of product (porphobilinogen - PBG) formation, using 140 mM Tris-HCl buffer, pH 9.0, and 3.6 mM ALA as the reaction medium (Barbosa *et al.* 1998). The reaction product was determined spectrophotometrically (CELM E 225-D, São Paulo, Brazil) at 555 nm using modified Ehrlich's reagent, with a molar absorption coefficient of 6.1×10^4 mol^{-1} cm^{-1} for the Ehrlich-porphobilinogen salt. All experiments were carried out after a 10-min pre-incubation. The reaction was started 10 min after the addition of the tissue preparation by adding the substrate. Incubations were carried out for 45 min at 35 °C. The amount of protein in samples using S_1 tissue preparations was 0.4 - 0.5 mg. S_1 is the biological material obtained as the supernatant after centrifugation in a refrigerated centrifuge at 1 000 *g* for 10 min. Protein content was determined by the method of Bradford (1976) using bovine serum albumin as standard.

IC_{50} determination: The concentration of Pb^{2+}, Zn^{2+}, Cd^{2+}, and Hg^{2+} needed to cause 50 % of enzyme activity inhibition was calculated according to the Dixon plot (Dixon and Webb 1964) using inhibitor concentration ranging from 0.05 to 4.0 mM for Pb and Hg; 0.05 to 1.0 mM for Zn and 0.05 to 0.8 mM for Cd.

Determination of cadmium, lead, mercury and zinc content: The samples were submitted to acid digestion at 80 °C in a conventional waterbath using tubes fitted with stoppers with capillaries, for gas evolution and pressure relief (Morsch and Martins 1999). A proportion of 1 g sample to 5 cm^3 concentrated HNO_3 was used and extraction was carried out for 8 consecutive hours with periodic shaking. The samples were then filtered, checked and stored in polyethylene flasks.

Cadmium, lead, zinc and mercury were determined with the aid of *GFAAS* (graphite furnace atomic absorption spectrometry) using an atomic absorption spectrometer (*Perkin Elmer 3030*, Norwalk, USA) and a graphite furnace (*HGA 400*) with an automatic sampler (*AS 10*, Norwalk, USA). The recommendations of the concept of a stabilized temperature furnace were followed. A solution of palladium nitrate (1000 μg cm^{-3}) and magnesium nitrate (1000 μg cm^{-3}) in 0.46 % nitric acid (v/v) was used as a chemical modifier. The graphite tube used was of the pyrolytic type, with an L'vov platform. A standard continuous background corrector (a deuterium arc bulb) was used. The absorbance signal was measured in the peak area and the purging gas used was pre-purified argon, with stop flow at the time of atomization.

Statistical analysis: Data were analyzed by one way *ANOVA* followed by Duncan's multiple range test when appropriate. Differences between groups were considered to be significant when $P < 0.05$.

Results

ALA-D specific activity from radish leaves increased from day 1 and reached a maximum on day 5. From that day on, the activity decreased gradually until day 10, when a sharp decrease occurred, so that on day 14 the activity was less than 20 % of the maximal activity obtained on day 5 (Fig. 1).

The potency of Hg^{2+}, Cd^{2+}, Pb^{2+}, and Zn^{2+} as *in vitro* inhibitors of ALA-D from radish leaves germinated for 5 to 6 days was characterized as the concentration at which metals caused 50 % inhibition of enzyme activity. The IC_{50} values for ALA-D inhibition by Zn^{2+} and Cd^{2+} were lower than those for Pb^{2+} and Hg^{2+}. They was 0.39 ± 0.02,

0.39 ± 0.02, 2.29 ± 0.89, and 1.38 ± 0.43 mM for Cd^{2+}, Zn^{2+}, Hg^{2+} and Pb^{2+}, respectively.

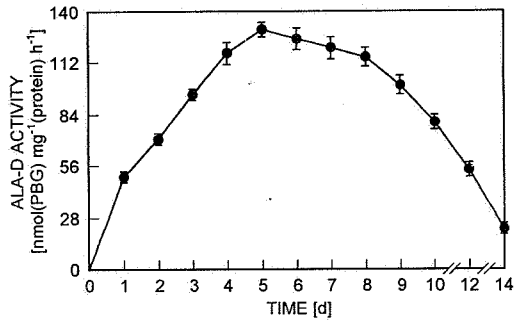

Fig. 1. ALA-D activity during ontogeny of seedling. Mean ± SE, $n = 5$.

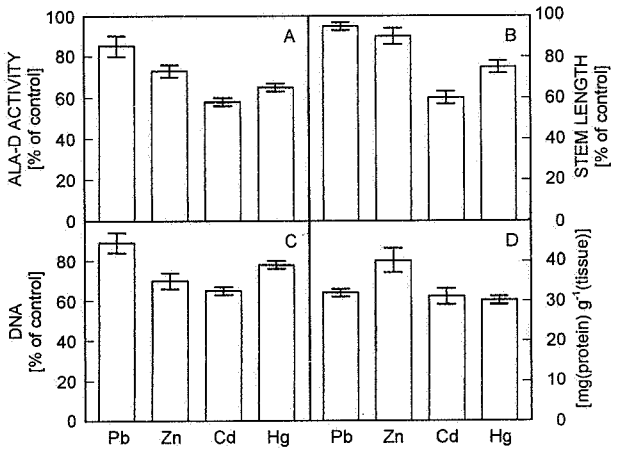

Fig. 2. Effect of Pb^{2+}, Zn^{2+}, Cd^{2+}, and Hg^{2+} 5-d exposure on ALA-D activity from radish leaves [control activity = 177.8 ± 10.4 nmol(PBG) mg^{-1}(protein) h^{-1}] (*A*), radish growth [control value = 3.42 cm] (*B*), DNA concentration [control value = 243 μg(DNA) g^{-1}(f.m.); treatment with zinc, cadmium and mercury caused a significant reduction in DNA content ($P < 0.01$, $n = 7$). Duncan's multiple range test.] (*C*), and protein content [control value = 30.81 mg(protein) g^{-1}(f.m.); zinc treatment caused a significant increase in protein content ($P < 0.05$, $n = 7$)] (*D*).

A different magnitude of inhibition of ALA-D from radish leaves was observed after seedling exposure to Hg^{2+}, Cd^{2+}, Pb^{2+} and Zn^{2+} for 5 d (Fig. 2*A*) Cd^{2+} had a maximal inhibitory effect of about 40 %, while Hg^{2+}, Zn^{2+} and Pb^{2+} caused inhibition of about 35, 25 and 15 %, respectively ($P < 0.01$).

Similar to that observed for ALA-D activity, the magnitude of inhibition of seedling growth varied depending on the metal. The reduction in growth caused by Pb^{2+} was not statistically significant, while Zn^{2+}, Hg^{2+}, and Cd^{2+} caused a significant decrease in growth of about 10, 25 and 40 % ($P < 0.01$) (Fig. 2*B*). Accordingly, the DNA concentration was also reduced by Cd^{2+} (35 %), Zn^{2+} (30 %), and Hg^{2+} (20 %) but not by Pb^{2+} (Fig. 2*C*).

In contrast to the previous results the protein concentration was not changed by exposure to Pb^{2+}, Cd^{2+}, or Hg^{2+} (Fig. 2*D*). However, Zn^{2+} caused a significant increase in protein concentration of about 30 %.

The metal content of untreated leaves was near or below the limit of detection of the method. The concentration of metals after 5-d exposure was in the following order: $Cd^{2+} \geq Zn^{2+} >> Pb^{2+} = Hg^{2+}$. With respect to the total quantity of metal added during the 5-d exposure, radish leaves accumulated about 60, 50, 25, and 20 % of Cd^{2+}, Zn^{2+}, Pb^{2+}, and Hg^{2+}, respectively (Fig. 3).

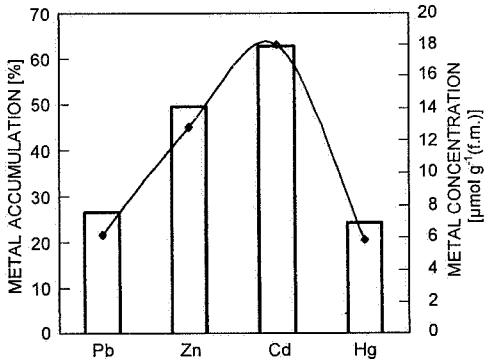

Fig. 3. Effect of Pb^{2+}, Zn^{2+}, Cd^{2+}, and Hg^2 metal content in leaf tissues and percentage of metal accumulation. All metals presented significant difference from the control group ($P < 0.01$).

Discussion

Heavy metals, which are important environmental pollutants, can inhibit the development of plants and disturb a variety of structural and biochemical processes (Chugh and Sawhney 1999, Mishra and Choudhuri 1999, Stoyanova 1999, El-Shintinawy and El-Ansary 2000, Luna *et al.* 2000, Talanova *et al.* 2000), including photosynthesis. Accordingly, in the present study we observed an inhibition of growth, DNA and ALA-D activity of radish exposed to relatively high concentration of Pb^{2+}, Zn^{2+}, Cd^{2+}, and Hg^2.

The present results demonstrate that the metals studied inhibited plant ALA-D with relatively low potency when compared to the animal enzyme (Rodrigues *et al.* 1989, Rocha *et al.* 1995). This result may indicate that the plant enzyme is less sensitive to these toxic agents, possibly due to a lower content and reactivity of the cysteinyl residues of the plant enzyme with heavy metals (Jaffe 1995). Factors other then affinity for sulfhydryl groups are involved in the inhibitory effects of the metals of group II B because Hg^{2+} is the ion with higher affinity for

-SH in this group and is the least potent inhibitor of ALA-D. Zn^{2+} and Cd^{2+}, which also have different affinities for the -SH group, had similar inhibitory potency. Consequently, other factors such as atomic radius may play an important role in determining the interaction between the ions of the II B group and the plant enzyme.

The inhibitory effect of Pb^{2+}, Hg^{2+}, Zn^{2+} and Cd^{2+} on ALA-D activity varied and Cd^{2+}, followed by Hg^{2+}, was the most potent inhibitor of the enzyme activity. In general the effect on growth and DNA was similar to that observed for ALA-D activity. The greater effect of Cd^{2+} on all these parameters can be tentatively attributed to its greater deposition in radish leaves. In fact, radish leaves accumulated 60 % of the total dose of Cd^{2+}. With respect to the other metals, the leaves accumulated a considerable quantity of Zn^{2+} (50 %) while the accumulation of Hg^{2+} and Pb^{2+} reached about 25 of the total dose. The higher effect of Cd^{+2} compared to Pb^{2+} agrees with previous published data showing that plant growth is more inhibited in Cd^{2+}-contaminated soils than in Pb^{2+}-contaminated soils (Khan and Frankland 1983, Zaman and Zereen 1998). The results of Cd^{2+} accumulation suggest higher mobility of this element when compared to Pb^{2+}, in agreement with data reported by Khan and Frankland (1983). An interesting finding of the present study was that Zn^{2+} caused a significant increase in protein concentration in radish leaves, a phenomenon not observed for the other metals.

In conclusion, the results of the present investigation suggest that ALA-D is not a good bioindicator for heavy metal intoxication. Nevertheless, the marked ability of radish to accumulate Cd^{2+} and Zn^{2+} raises the possibility of using this vegetable as a biomonitor of environmental contamination by these metals.

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