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STUDIES ON THE INHIBITION BY ZINC OF L-MALATE AND L-GLUTAMATE DEHYDROGENASES EXTRACTED FROM THE ROOTS OF ZINC TOLERANT AND NON TOLERANT CLONES OF *ANTHOXANTHUM ODORATUM* L.

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Abstract

In vitro studies on the inhibition of L-malate and L-glutamate dehydrogenases by zinc revealed that the two enzymes from both tolerant and non tolerant clones were equally sensitive to 1mM zinc. No evidence of enzymic tolerance was found. Kinetic studies showed that the inhibition was of mixed type and that the kinetic parameter of importance was the dissociation constant of the reaction $EI \rightleftharpoons E + I$ (K_I). It is suggested that in the zinc tolerant clones of *A. odoratum* enzymes are protected from high levels of metal in the cytoplasm. This protection of enzymes and other sensitive sites in the cytoplasm may be achieved by the removal of excess metal from the cytoplasm and complexing agents like organic acids and proteins may be involved.

Introduction

Metal tolerance confers on cells the ability to function in the presence of elevated and normally toxic levels of metals. In physiological terms this ability could be achieved by (a) evolution of tolerant enxymes (Wainwright & Woolhouse, 1975) which can function normally in the presence of high levels of metals and/or (b) by evolution of an ability to complex and compartmentalise the metals, thereby providing a protection to the enzymes (Mathys, 1977; Qureshi, 1982).

Previous attempts of Mathys (1975) to identify enzymic tolerance in zinc tolerant *Silene cucubalus* were unsuccessful. He studied *in vitro* inhibition by zinc of malate dehydrogenase, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase and nitrate reductase and concluded that the enzymes from the tolerant plants were as sensitive to zinc as the enzymes from the non tolerant plants. Wainwright & Woolhouse (1975) studied the kinetics of cell wall acid phosphatases from the roots of copper tolerant and non tolerant *Agrostis tenuis* in the presence of copper. They identified enzymic tolerance in this species for copper. However, in a similar study using zinc tolerant and non tolerant *A. tenuis* no such enzymic tolerance was observed (Wainwright & Woolhouse, 1978).

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In an earlier report, Cox, Thurman & Brett (1976) showed no differences in the *in vitro* zinc inhibition of root acid phophatase activity of zinc tolerant and non tolerant *A. odoratum* when the plants were grown in the absence of zinc. However, in a more recent report it has been shown (Cox & Hutchinson, 1980) that significant difference were apparent in the degree of copper inhibition of soluble acid phosphatase activity in the two populations of *Deschampsia caespitosa* only after the plants were grown in the presence of sublethal copper concentration.

The two enzymes, MDH and GDH were chosen for the present study because they are present as soluble enzymes also and are likely to be influenced by any changes in the cytoplasmic levels of metals and thus may provide some answers about the possible adaptations against the metal exposure in *A. odoratum*.

Material and Methods

(1) *Plant material*

Four different clones of *A. odoratum* were chosen for this study, three tolerant to zinc (T92, T79, T48) and one non tolerant (S20). The tolerants were originally collected from a zinc/lead mine site in Wales (U.K) and the non tolerant clone from an uncontaminated pasture. The details of collection, material maintainance and tolerance testing are given in Cox (1976).

(2) *Root growth*

Healthy tillers of these clones were obtained and their roots removed prior to their growth in the culture solution. Individual tillers were allowed to grow in the 1/2 strength Hoagland and Arnon (1938) solution for two weeks. Roots were excised 2cm from the tip and these tips used for enzyme extraction.

(3) *Extraction of malate (MDH) and glutamate (GDH) dehydrogenases*

Excised roots were weighed and ground in 0.1M Tris/HCl buffer for MDH and 0.2M Tris/Acetate buffer, pH 7.5 for GDH for three minutes at 4°C. The ratio of buffer to tissue was $10cm^3/500mg$ for MDH and $10cm^3/1000mg$ for GDH The homogenate was then centrifuged for five minutes at 1000 g using a MSE bench centrifuge. The supernatant was used as the crude enzyme extract.

(4) *Effect of different levels of zinc on the activity of MDH*

Crude enzyme extracts were subjected to different levels of zinc and the activity of the enzymes was measured using the following assay at 20°C.

The reaction mixture consisted of 2.7 cm^3 0.1M Tris/HCl buffer pH 7.5, 0.1 cm^3 0.2 mM NADH, 0.1 cm^3 0.4 mM oxaloacetate, and 0.1 cm^3 enzyme extract. The concentrations of oxaloacetate and NADH were saturating. Activity of the enzyme was determined as changes in units of absorbance^{-1} at 340 nm.

(5) *Effect of different levels of zinc on the activity of GDH*

Different zinc concentrations were prepared in Tris/Acetate buffer pH 7.5 and the activity of the enzyme was assayed at each level using the following method at 20°C.

The reaction mixture consisted of 2.6 cm^3 0.2 M Tris/Acetate buffer pH 7.5, 0.1 cm^3 0.2 mM NADH, 0.2 cm^3 1.5 M $NH_4(SO_4)_2$, 0.1 cm^3 15 mM α-ketoglutaric acid and 0.1 cm^3 enzyme extract. Assay conditions were optimised before by using different concentrations of substrate, ammonia and NADH. Activity of the enzyme was determined as changed in 0.1 units of absorbance min^{-1} at 340 nm.

(6) *Determination of Michaelis constant K_m for oxaloacetic acid and α-keroglutaric acid*

Preparation of extracts was achieved in a similar manner as described in section three. Assay conditions were identical to the previous experiment except that for this study a range of substrate concentrations was used, 0.05 mM to 0.4 mM oxaloacetate for MDH and 5.0 to 15 mM α-ketoglutaric acid for GDH. For each concentration the assay was replicated at least three times. Activity of the enzyme was determined as changes in 0.1 units of absorbance min^{-1}. Mean activity at each substrate concentration was calculated and plotted by the methods of Cornish-Bowden (1976), Dixon (1953) and Lineweaver & Burk (1934), and confidence limits at p = 0.05 calculated.

(7) *Determination of inhibitor constants K_i and K_I*

Estimates of K_i were made by using the method of Dixon (1953). In this case plots of $\frac{1}{V} x \frac{1}{S}$ provide the value of $-K_i$. Values were compared by using the same data and by plotting the slopes against the inhibitor levels. Slopes in this case were calculated from the primary double reciprocal plots. Determination of K_I was achieved by plotting $\frac{S}{V}$ x I (Cornish-Bowden, 1976). Also the K_I values were obtained by using the method of Cleland (1963) by plotting intercepts against the inhibitor levels. The intercepts were calculated from the primary double reciprocal plots. Values obtained by these two methods were compared.

Results

(1) *Effect of different levels of zinc on the activities of MDH and GDH*

The activity of MDH was assayed in the presence of 0.0, 0.5 and 1.0 mM zinc using the crude enzyme extracts of roots of three zinc tolerant (T92, T79, and T48) clones and one non tolerant (S20) clone. The results are expressed as percent inhibition of activity over controls (Table 1). The results indicate a similar effect of zinc upon the activity of MDH in all four clones. Slight differences were apparent but none statistically significant.

For the study of the inhibition of GDH activity by zinc, crude enzyme extract was obtained from the roots of one tolerant clone (T92) and one non tolerant clone(S20) only. Zinc levels were the same as those used for MDH. At 0.5 mM zinc the enzyme activity of the S20 clone was inhibited more than that of T92 enzyme, but no difference in the inhibition was detected at 1.0 mM zinc (Table 1).

The results indicate that both of these enzymes from the tolerant and non tolerant clones behave similarly *in vitro* in the presence of zinc.

(2) *Kinetic studies*

(a) *Determination of Michaelis constant K_m of MDH and GDH*

Values of K_m were determined for the two enzymes, MDH and GDH using different levels of oxaloacetic acid and α-ketoglutaric acid respectively. The K_m values

Table 1. Effect of zinc on the *in vitro* activity of MDH and GDH extracted from the roots of tolerant and non-tolerant clones of *A. odoratum* grown in the absence of zinc. Values are expressed as percent inhibition of activity over control. Confidence limits at p = 0.05 are shown with each value.

Enzyme	Clones	0.5 mM Zn	1.0 mM Zn
MDH	T92	15.21 ± 2.25	30.88 ± 4.78
	T79	16.01 ± 2.41	32.21 ± 3.56
	T48	20.70 ± 4.06	33.47 ± 3.24
	S20	17.17 ± 3.52	34.92 ± 4.03
GDH	T92	20.87 ± 1.36	24.10 ± 2.66
	S20	24.81 ± 1.50	24.50 ± 0.60

Table 2. Determination of Michaelis Constant (K_m) for MDH and GDH extracted from the roots of tolerant and non-tolerant clones of *A. odoratum* grown in the absence of zinc. Confidence limits are shown against each value, p = 0.05.

Clones	MDH K_m (mM Oxaloacetic acid)	GDH K_m (mM α-ketoglutaric acid)
T92	0.082 ± 0.009	3.95 ± 0.019
T79	0.107 ± 0.013	–
T48	0.127 ± 0.047	–
S20	0.147 ± 0.047	4.75 ± 0.274

obtained are presented in Table 2. The K_m values of oxaloacetic acid for MDH were similar for T79, T48 and S20; whereas value for T92 was less than that of S20 enzyme.

The K_m values of α-ketoglutaric acid for GDH were different for the two clones. The enzyme extracted from the tolerant clone had a significantly lower K_m for the substrate (Table 2).

(b) *Determination of inhibitor constants K_i and K_I of MDH and GDH*

The enzyme sources were similar to the ones described in the last section. Inhibition results were plotted by using the method of Dixon (1953) for K_i estimates (Figs. 1 A – F) where $\frac{1}{V}$ against I plots gave the value of $-K_i$ on or above the abscissa. Values were compared by plotting the same data as slopes against I, slopes were calculated from the double reciprocal plots (Figs. 2 A – F). For MDH these values vary between the tolerant clones, highest being 1.5 mM for T79 and lowest being 0.88 mM for T92 (Table 3).

GDH behaved similarly, the K_i value of the enzyme of the tolerant clone being less than the value for the enzyme from the non tolerant clone (Table 4).

The results for the K_I determination were plotted by the method of Cornish-Bowden (1976) and the values of K_I obtained from the plots of $\frac{S}{V}$ against I (Figs. 3 A – F). For comparison, the results were also plotted by the method of Cleland (1963) where the values were determined from the plots of intercepts against inhibitor concentration (Figs. 2 A – F). Intercepts were calculated from the double reciprocal plots. Values K_I are presented in Tables 3 & 4.

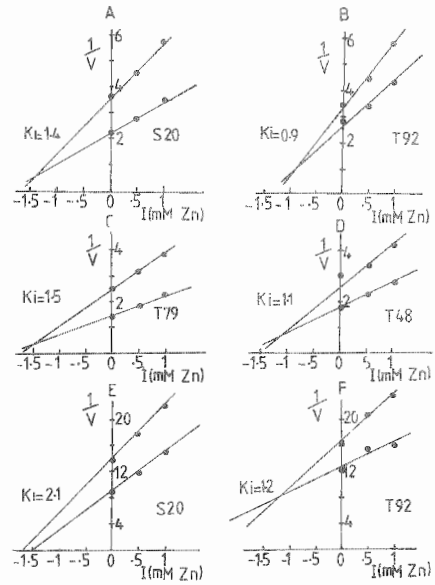

Fig. 1. Plots of $\frac{1}{V}$ against I for MDH (A – D) and GDH (E & F) activity of enzymes estracted from the roots of zinc tolerant and non tolerant clones of *A. odoratum* at two substrate concentrations in the presence of three zinc concentrations.

In all cases it is apparent that the values of the dissociation constant of the reaction $EI \rightleftharpoons E + I\ (K_i)$ are lower than the values of the dissociation constant of the reaction $ESI \rightleftharpoons ES + I\ (K_I)$. This observation clearly indicates that the kinetic parameter of importance in the inhibition of MDH and GDH by zinc is the dissociation constant K_i. The lower value of K_i indicates a higher affinity of the inhibitor for the enzyme alone and not the enzyme: substrate complex.

Table 3. Estimation of inhibitor constants K_i and K_I for MDH extracted from the roots of tolerant and non-tolerant clones of *A. odoratum* grown in the absence of zinc.

Clones	K_i (mM zinc)		K_I (mM zinc)	
	1/v vs I	Slope vs I	s/v vs I	Intercept vs I
T92	0.90	0.88	2.00	1.52
T79	1.50	1.52	2.50	2.50
T48	1.10	0.85	2.30	3.10
S20	1.42	1.42	1.88	1.95

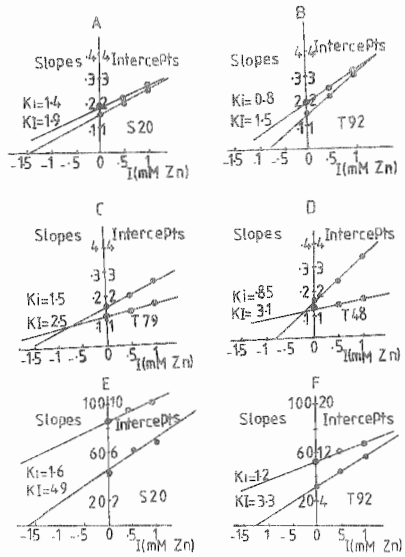

Fig. 2. Secondary plots of slopes (closed circles) and intercepts (open circles), derived from primary double reciprocal plots, against inhibitor concentration. These plots were used in the determination of K_i and K_I for the zinc inhibition of MDH (A – D) and GDH (E & F) extracted from the roots of zinc tolerant and non tolerant clones of *A. odoratum*.

Table 4. Estimation of inhibitor constants K_i and K_I for GDH extracted from the roots of tolerant and non-tolerant clones of *A. odoratum* grown in the absence of zinc.

Clones	K_i (mM zinc)		K_I (mM zinc)	
	1/v vs I	Slope vs I	s/v vs I	Intercept vs I
T92	1.20	1.25	4.72	3.32
S20	2.10	1.62	7.00	4.95

Discussion

Studies on the *in vitro* inhibition by zinc of malate and glutamate dehydrogenases from the roots of zinc tolerant and non tolerant clones of *A. odoratum* have demonstrated that the two enzymes from both the tolerant and non tolerant populations are equally sensitive to 1mM zinc. This observation is in agreement with the earlier findings of Mathys (1975), who tested a number of enzymes of *Silene cucubalus* and found no

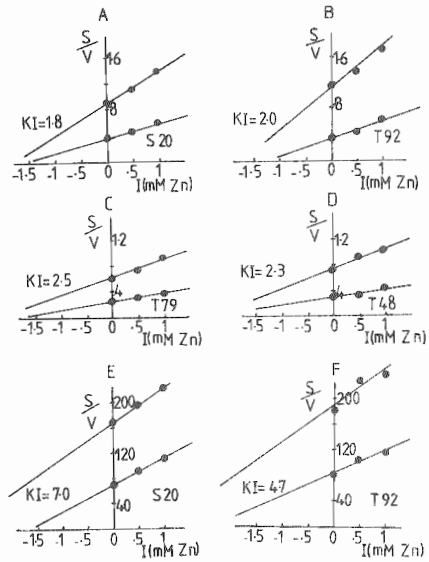

Fig. 3. Plots of $\frac{S}{V}$ against I for MDH (A – D) and GDH (E & F) activity of enzymes extracted from the roots of zinc tolerant and non tolerant clones of *A. odoratum* at two substrate concentrations in the presence of three zinc concentrations.

in vitro enzymic tolerance against zinc in the zinc tolerant ecotypes of this species, and Cox *et al* (1976) who showed that no differences could be found in the *in vitro* zinc inhibition of root acid phosphatases of zinc tolerant and non tolerant *A. odoratum* when the plants were grown in the absence of zinc. This primary observation leads to the suggestion that normal functioning of cells in plants growing in the presence of excessive levels of potentially toxic metals can only be maintained if the enzymes are protected from the levels of metals in the cytoplasm. How this ability to remove excess metals from the cytoplasm is achieved by these plants is not clear. However, there are suggestions that complexing agents such as organic acids (Mathys, 1977; Lee *et al.*, 1977), and proteins (Rauser & Curvetto, 1980) may be involved.

The major kinetic parameter of inportance was K_i as the ES complex had a lesser affinity for the inhibitor. This observation differs from that of Cox (1976), who working with cell wall acid phosphatases, reported K_I to be the major kinetic parameter of importance.

Estimation of inhibitor constants provided no evidence of lesser affinity of inhibitor (zinc) to the enzymes of tolerant clones, an observation also of Wainwright & Woolhouse (1978) for cell wall acid phosphatases of zinc tolerant *A. tenuis*. But

contrary to this in the copper tolerant *A. tenuis* evidence was found of a lesser affinity of the enzyme for the inhibitor (Wainwright & Woolhouse, 1975). Such differences in the behavior of cell wall acid phosphatases in zinc and copper tolerant plants of the same species may be because copper might exert a much greater selection pressure at the cell wall-plasmalemma interface than zinc. Copper is known to be more injurious to the plasmalemma than zinc (Wainwright & Woolhouse, 1975) as it causes a greater leakage of potassium ions from cells than zinc.

The kinetic differences among different enzymes within the tolerant population are not unreasonable. It is likely that enzymes such as cell wall acid phosphatases by virtue of their location come into contact with the metals in the soil more frequently (and at higher levels) than other enzymes like malate or glutamate dehydrogenases which are located away from the cell wall-plasmalemma interface. Therefrore, it seems that the enzymes working close to the plasma membrane are more likely to evolve tolerance against excessive metals than those within the cytoplasm or mitochondria. The probability that toxic metals are complexed and detoxified as soon as they enter the cytoplasm and therefore that cytoplasmic enzymes may never experience high levels of metals (s), and can thus continue to function normally is thus strenghtened by the present findings.

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