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SPECIFIC EFFECT OF MANGANESE ON THE ALLANTOINASE OF *VIGNA RADIATA*

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Key Word Index—*Vigna radiata*; Leguminosae; mung bean; protection of allantoinase; allantoinase; manganese.

Abstract—*Vigna radiata* seedlings germinated in the presence of Mn^{2+} show an unusual increase in allantoinase activity which is proportional to Mn^{2+} concentration up to 5 mM. Though Mn^{2+} is not an activator for *V. radiata* allantoinase, it specifically protects allantoinase against thermal as well as papain-catalysed inactivation. Evidence is presented to show that the primary effect of Mn^{2+} is a protective one, both *in vitro* and *in vivo*, and that this is reflected in the observed enhancement of allantoinase activity in Mn^{2+} grown seedlings. That this unusual effect of Mn^{2+} is a specific one is indicated by the lack of a similar effect with Mg^{2+}. Cu^{2+} is shown to destabilize *V. radiata* allantoinase *in vitro* as well as *in vivo*.

INTRODUCTION

Allantoinase (allantoin amidohydrolase, EC 3.5.2.5) occurs in animals [1–3], higher plants [4, 5], algae [6–8] and micro-organisms [4, 5]. Plant allantoinases are generally fairly stable [9–14]. However, few plant allantoinases have been purified and studied in detail, apart from the enzyme from soybean [9], the stereospecific enzyme of *Lathyrus sativus* [13] and the unusual enzyme from *Dolichos biflorus* [14]. While examining the comparative properties of some plant allantoinases, it was found that *Vigna radiata* allantoinase exhibits a very unusual dependence on Mn^{2+} both *in vivo* and *in vitro*. In this paper it will be shown that Mn^{2+}, though not an activator of *V. radiata* allantoinase, has a marked ability to stabilize this enzyme and that this effect, *in vivo*, is responsible for a Mn^{2+}-dependent increase in enzyme levels in germinating seedlings.

RESULTS

Preliminary experiments

In initial studies, some of the basic properties of *V. radiata* allantoinase were studied in enzyme extracts obtained from seedlings germinated for 3–5 days. Enzyme activity was present throughout the pH range 4–9, with a peak at pH 7.5. Lineweaver–Burk plots, gave a K_m value of 6.6 mM.

One of the peculiar features of *V. radiata* allantoinase was its instability on purification. The enzyme lost activity on dialysis as well as on storage in the cold. Though *V. radiata* allantoinase was eluted from DEAE-cellulose columns by 50 mM diethanolamine–HCl buffer, pH 7.5, immediately after the void volume, with a resultant, five-fold increase in sp. act., the eluted enzyme was highly unstable on storage in the cold. Hence all studies were conducted with fresh extracts obtained from seedlings germinated for 72 hr except where otherwise specified.

Elaboration of allantoinase during germination

On germination of *V. radiata* in water, allantoinase sp. act. increased rapidly and linearly during 1–5 days, activity increasing 11-fold in 5 days. Analysis of 4-day-old seedlings showed that increase over the resting seed level was as follows: whole seedlings 8-fold; cotyledons, 6.2-fold; and seedling (plumule plus radicle), 11.5-fold. These data suggest that there is greater synthesis in the shoot, and indicate that allantoinase is newly synthesized during growth in seedlings.

Effect of metal ions on allantoinase levels in germinating seedlings

On the basis of preliminary experiments which showed that germination of *V. radiata* seeds in $MnSO_4$ resulted in an increase in allantoinase levels, experiments were conducted with increasing concentrations of Mn^{2+}. The results (Fig. 1) show that there is an unusual and linear

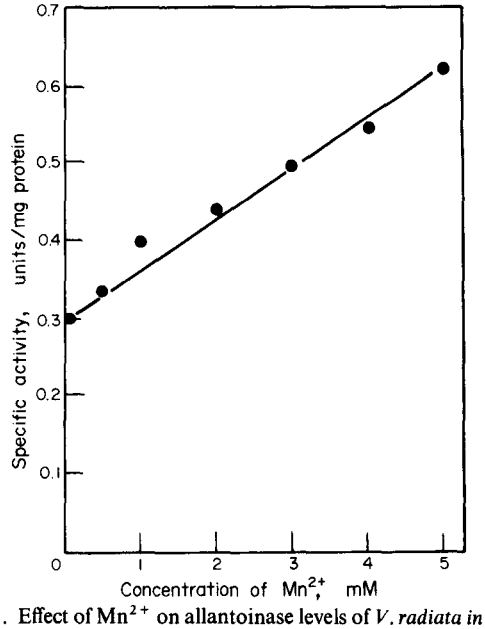

Fig. 1. Effect of Mn^{2+} on allantoinase levels of *V. radiata in vivo*. *V. radiata* seeds (2 g) germinated with different concentrations of Mn^{2+} for 72 hr. For experimental details, see text.

increase in allantoinase over the range 0–5 mM Mn^{2+}. In other experiments, it was found that Mg^{2+} under the same condition did not have the same effect. With 0–10 mM, Cu^{2+}, on the other hand, there was a decrease in allantoinase levels. The data shown in Fig. 2 indicate that there is also an inhibition of seedling growth at the higher levels of Cu^{2+}. However, if the relative inhibition of activity to growth, expressed as percentage of the control value (in water alone) is examined, it is seen that growth is inhibited more than allantoinase activity, suggesting that Cu^{2+} more specifically affects allantoinase than growth.

That the effect of Mn^{2+} in increasing allantoinase levels during germination is not due to its role as an activator of *Vigna* allantoinase was evident since addition of Mn^{2+} to extracts from control seedlings (i.e. those germinated in water alone) did not result in enhanced activity. Also, extracts dialysed against EDTA lost activity which could not be restored by added Mn^{2+} subsequently.

Effect of Mn^{2+} *and* Cu^{2+} *on thermal inactivation of* V. radiata *allantoinase*

On the basis of preliminary experiments, it was thought that the unusual effect of Mn^{2+} observed herein could be a reflection of its ability to stabilize *V. radiata* allantoinase. To examine this possibility, initially, thermal inactivation of allantoinase activity in control extracts (from seedlings germinated in water alone) was studied at 70° with and without added Mn^{2+}, Cu^{2+} and Mg^{2+}, all metal ions at a final concentration of 5 mM. The results (Fig. 3) show that Mn^{2+} is unique in that it affords nearly complete protection to *V. radiata* allantoinase against thermal inactivation. The specificity of this effect is emphasized by the lack of a similar response with Mg^{2+}. In contrast, *V. radiata* allantoinase is inhibited by Cu^{2+} and its thermal inactivation rate is enhanced further by added Cu^{2+}.

The relevance of the above *in vitro* effects of metal ions to their influence *in vivo* is shown by the data of Fig. 4, which depicts thermal inactivation of allantoinase activity in extracts from seedlings germinated with 5 mM Mn^{2+} and Cu^{2+}. Again, it can be seen that allantoinase activity in extracts derived from seedlings germinated in Mn^{2+} is stable to thermal inactivation in contrast with control and

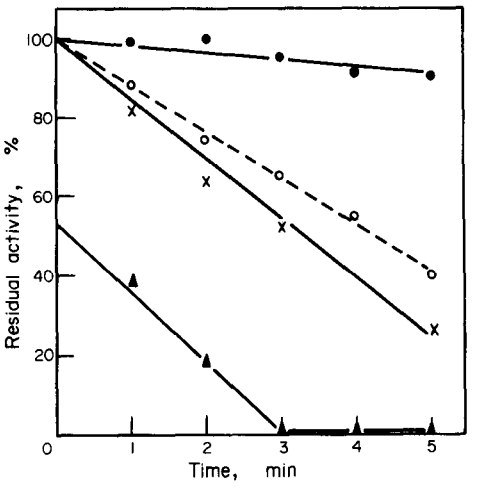

Fig. 3. Effect of metal ions *in vitro* on thermal inactivation of *V. radiata* allantoinase. Enzyme extracts subjected to thermal inactivation with added metal ions as indicated at pH 7.5 and 70°, for different time periods, and then assayed. For experimental details, see text. ○, Control; ●, 5 mM Mn^{2+}; ▲, mM Cu^{2+}; ×, 5 mM Mg^{2+}.

that germination in Cu^{2+} results in an enzyme which is more susceptible to thermal inactivation.

Moreover, when extracts from Mn^{2+}-grown seedlings were treated with EDTA and then thermally denatured, allantoinase inactivation was progressive with EDTA concentration. Activity decreased sharply and linearly up to 2 mM. EDTA and more slowly thereafter, up to 5 mM EDTA. Residual allantoinase activity was 40% at 2 mM and 30% at 5 mM EDTA, respectively. This shows clearly that the stability of enzyme activity in these extracts is due to protection afforded by association of Mn^{2+} with *V. radiata* allantoinase.

Papain inactivation of V. radiata *allantoinase*

The results presented above show that Mn^{2+} has a protective effect on *V. radiata* allantoinase, both *in vivo* and *in vitro*. To obtain further support for the above

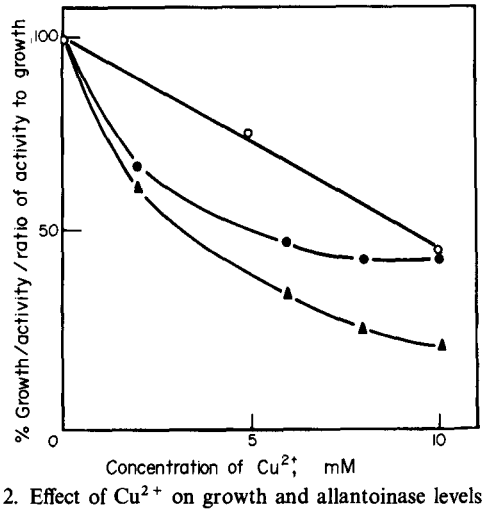

Fig. 2. Effect of Cu^{2+} on growth and allantoinase levels of *V. radiata in vivo*. *V. radiata* seeds (2 g) germinated with different concns of Cu^{2+} for 72 hr. For experimental details, see text. ○, Growth; ▲, allantoinase; ●, ratio of activity to growth.

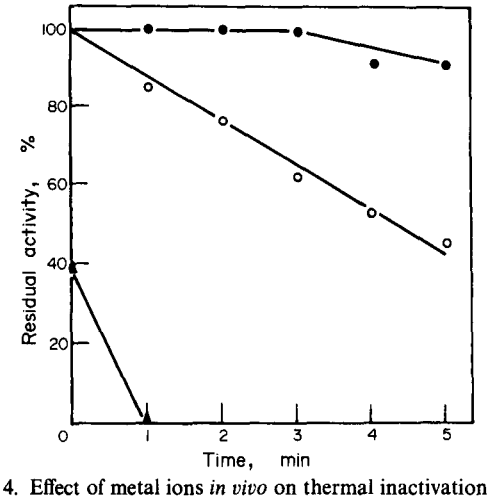

Fig. 4. Effect of metal ions *in vivo* on thermal inactivation of *V. radiata* allantoinase. Enzyme extracts from *V. radiata* seedlings germinated for 72 hr in water only (○), 5 mM Mn^{2+} (●) or 5 mM Cu^{2+} (▲) subjected to thermal inactivation at pH 7.5 and 70°. For experimental details, see text.

hypothesis, allantoinase from control seedlings was subjected to inactivation with papain with (experiment A) and without 5 mM added Mn^{2+} (experiment B); for comparison, enzyme extracts from Mn^{2+}-grown seedlings were also similarly treated with papain without further addition of Mn^{2+} (experiment C). The fall in allantoinase activity was linear with time in all cases and residual activities after 3 min treatment with papain were (as per cent control) as follows: A, 97; B, 44; and C, 92. These data show that under the experimental conditions employed herein, addition of Mn^{2+} *in vitro* to control extracts, as well as germination of seedlings in Mn^{2+} provides protection to allantoinase against papain inactivation.

Uptake of Mn^{2+} *by* V. radiata *seedlings during germination*

Seedlings grown with 5 mM Mn^{2+} were analysed for their Mn^{2+} content, after removal of seed coats and extensive washing. Typical values for 2-g seeds, after 72 hr germination were as follows: fr. wt of seedlings 7.38 g; dry wt 1.38 g; Mn^{2+} content 909 μg. Assuming, as a first approximation, that the Mn^{2+} is uniformly distributed over the entire water space, this results in a content of 2.24 mM Mn^{2+} for the entire seedling material. Repeat analyses showed that the above values were reproducible to within $\pm$10–15%.

DISCUSSION

A highly unusual interaction between Mn^{2+} and *V. radiata* allantoinase is brought out by the results of the present study. While Mn^{2+} is not an activator of this allantoinase, it nevertheless specifically stabilizes the enzyme against thermal as well as proteolytic (papain) inactivation. Protection is seen both when Mn^{2+} is added *in vitro* to enzyme extracts as well as when Mn^{2+} is apparently incorporated into *Vigna* allantoinase during elaboration of this enzyme in the presence of Mn^{2+}. The latter results in a Mn^{2+}-dependent increase in enzyme activity which would appear to be a reflection of a slower rate of breakdown of the allantoinase *in vivo* under the influence of intracellular Mn^{2+}. The specificity of Mn^{2+} is emphasized by the absence of such effects with Mg^{2+} and the opposite influence exerted by Cu^{2+}.

While it is difficult to assess whether Mn^{2+} exhibits quantitative differences *in vivo* and *in vitro*, it is nevertheless evident that the range of concentrations in which Mn^{2+} exerts its stabilizing effect *in vivo* are probably not very different from those that are required *in vitro*. This is suggested by the fact that overall, Mn^{2+} concentration of Mn^{2+} grown seedlings is around 2.24 mM, which is quite comparable with the 5 mM that is needed for maximal protection of *V. radiata* allantoinase *in vitro*. However, the calculated value is only indicative of the order of magnitude of Mn^{2+} concentrations *in vivo*, since it is based on the entire water space of germinating seedlings; if Mn^{2+} has greater affinity to *V. radiata* allantoinase than to other protein(s) then the value could be quite different and probably much closer to that needed for stabilizing the allantoinase *in vitro*. This is further emphasized by the fact that EDTA annulment of Mn^{2+} protection does need concentrations of EDTA in the range 2–5 mM.

This unusual role of Mn^{2+} in relation to *V. radiata* allantoinase is quite novel and unexpected. Vogels *et al.* [4] had earlier classified allantoinases into several groups based on their dependence on Mn^{2+}. Allantoinases of *Streptococcus allantoicus* and *Arthobactor allantoicus* are activated by Mn^{2+} and reducing agents. *Pseudomonas* allantoinases are inhibited by Mn^{2+}; some plant enzymes studied by them are activated by Mn^{2+}. *V. radiata* allantoinase seems to exhibit a completely different kind of interaction with Mn^{2+}.

An effect of Mn^{2+} somewhat reminiscent of its influence on *V. radiata* allantoinase has been described by Van der Drift and Vogels [15] but with *Pseudomonas aeruginosa* allantoicase; here also, Mn^{2+} is not an activator but protects the enzyme against thermal inactivation. However, in the case of this allantoicase the authors had suggested than Mn^{2+} is probably tightly bound to the enzyme and cannot be detached from it easily. On the other hand, Mn^{2+} which is incorporated in *V. radiata* allantoinase is not firmly bound since it is apparently easily removed from it by EDTA.

There are very few recorded instances of a metal ion which is not an activator for an enzyme affecting its stability *in vivo* as well as *in vitro*. A curious instance where Mn^{2+}, while it does not affect K_m or V_{max} and has no apparent catalytic role, does nevertheless drastically affect stability is that reported by Coleman and Weiner for a manganese-containing alcohol dehydrogenase of a strain of *Saccharomyces cerevisiae* [16]. However, in this case, Mn^{2+} makes the enzyme markedly unstable. *V. radiata* allantoinase is unique in that Mn^{2+} has a vital stabilizing function.

EXPERIMENTAL

Germination. Locally purchased seeds of *V. radiata* (L.) Wilczek were germinated under sterile conditions as described earlier [13, 17] with 10 ml sterile H_2O alone or in sterile solns of $MnSO_4$, $CuSO_4$ or $MgSO_4$ of desired concns. Except where otherwise specified, germination was for 72 hr at 30 $\pm$ 1°.

Enzyme extraction. Freshly germinated seedlings were freed of seed coats, washed extensively with sterile dist. H_2O and homogenized with an equal wt of acid washed sand and 0.6 mM diethanolamine–HCl buffer, pH 7.5 (1 ml/g fr. wt) in a glass mortar at 0°. The extract was centrifuged in the cold for 10 min at 15000 *g* and the supernatant used as the enzyme extract. In some expts, after germination, cotyledons were dissected out, and the cotyledons and seedlings extracted separately to prepare enzyme extracts.

Assays. Allantoinase activity was determined at pH 7.5 (except where otherwise specified) in 0.1–0.5 ml aliquots of extract incubated in a total vol. of 2 ml with allantoin (30 μmol) and diethanolamine–HCl buffer (0.3 M, pH 7.5, 1200 μmol) for 5 min at 30°. The reaction was stopped by 50% TCA (0.5 ml), following which the reaction mixture was centrifuged and allantoate formed estimated in aliquots as described earlier [18]; sp. act. was expressed as μmol allantoate formed/mg protein under the conditions of the assay. Protein content of extracts was determined by a slight modification of the Lowry procedure [19].

Thermal inactivation. Enzyme extract (0.5 ml) was incubated at 70° in small-capped vials for the desired time, and then chilled rapidly to 0°. Allantoinase activity was then assayed as above. When effects of metal ions on thermal inactivation were being studied, Mn^{2+}, Cu^{2+} or Mg^{2+} was added to the enzyme extract to provide the required concn prior to incubation at 70°.

Papain inactivation. Enzyme extract (1 ml) was mixed with a freshly made soln of papain [2 mg; crystalline papain in $(NH_4)_2SO_4$ suspension PAP 7EA, Worthington Chemicals], 0.2 ml of freshly made cysteine (1 M, pH 7.5) in a total of 2 ml and

incubated at 37° for the required time (0–3 min). Aliquots (0.1 ml) were then withdrawn and allantoinase activity estimated as before. Where the effect of Mn^{2+} was studied, enzyme extract was preincubated with Mn^{2+} (5 mM) for 1 min and papain inactivation conducted. A papain control (no enzyme) was also run. Also Mn^{2+} (up to 5 mM) did not inhibit papain activity under our experimental conditions.

Treatment of enzyme with EDTA. EDTA was added to enzyme extracts as required and assays performed after dialysis against 0.6 M diethanolamine–HCl buffer pH 7.5. In some expts EDTA (adjusted to pH 7.5) was added to enzyme extracts and allantoinase activity assayed.

Assay of Mn^{2+} in seedlings. Seedlings germinated for 72 hr in 5 mM Mn^{2+} were freed of seed coats, washed exhaustively with glass-dist. H_2O until free of Mn^{2+} and digested with 5 ml of a 1:1 mixture of 15.7 M HNO_3 and 10 M HCl on a sand-bath. The contents were extracted with 5 ml H_2O by boiling, cooled neutralized and manganese determined by a permanganate procedure [20].

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