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THE ALLANTOINASE OF *LATHYRUS SATIVUS*

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Abstract—The presence of a stable allantoinase in *Lathyrus sativus* and its *de novo* synthesis at a maximal rate in the first 48 hr of germination have been demonstrated. The plumule and radicle together exhibited highest enzyme activity. *L. sativus* allantoinase has been purified nearly 35-fold. The purified enzyme was optimally active around pH 7·5, did not require any metal ion for activity and exhibited a K_m of 2·56 mM for (±)-allantoin, and an activation energy of 5·6 kcal/mol. Unlike other plant allantoinases, the *L. sativus* enzyme is highly specific for (±)-allantoin and is shown to be a sulfhydryl enzyme which apparently exists in a stable form *in vivo* obviating the need for added sulfhydryl compounds for maximal activity.

INTRODUCTION

Allantoinase, the enzyme catalyzing the conversion of allantoin to allantoic acid has been extensively studied in microorganisms [1–3]. Though this enzyme was first reported in soyabeans [4] and has been purified about 265-fold from this source [5], little is known about its occurrence and properties in other plants. Vogels and coworkers [3] have classified allantoinases into four categories based on the role of sulfhydryl groups and Mn^{2+} ions in activation and/or inhibition; among these, plant allantoinases had been considered as not being highly stereospecific, dependent on Mn^{2+} for activity and as being strongly inhibited by reducing compounds. We report here some aspects of the *in vivo* synthesis and properties of a partially purified allantoinase from *Lathyrus sativus*.

RESULTS

Synthesis of allantoinase

The pattern of elaboration of plant allantoinases during germination has not so far been examined, except in castor seedlings [6] where allantoinase is absent initially, and is elaborated only after 48 hr of germination. The results obtained in *Lathyrus sativus* indicate that total allantoinase activity increases rapidly and reaches a plateau in the first 48 hr of germination. However, total protein continues to increase up to 6 days; consequently, the specific activity of allantoinase is maximal around 24 hr and decreases thereafter. When allantoinase activity was examined separately in the cotyledons and "seedlings" (i.e. the plumule plus radicle), specific activity is very high in the seedling up to the second day and decreases sharply thereafter. In contrast, allantoinase in the cotyledons is of lower specific activity from the beginning and this further decreases by the second day of germination to an even lower but more or less constant level. To establish that the early increase in activity is due to *de novo* synthesis, the effect of chloramphenicol on protein and enzyme levels was studied in *L. sativus* seeds germinated for 48 hr. The results (Table 1) indicate that when chloramphenicol inhibits protein synthesis, it also concomitantly brings about a decrease in allantoinase content. Thus, the rise in allantoinase level with germination, represents a *de novo* synthesis of the enzyme in *L. sativus*.

Table 1. Effect of chloramphenicol on protein and allantoinase levels in *L. sativus*

Chloramphenicol level (mM)	Fr. wt* (g)	Protein (mg)	Allantoinase activity Total (units)	Allantoinase activity Specific activity (units/mg)
Nil (control)	8·0	448	333	0·74
0·01	8·0	224	165	0·74
0·10	8·0	179	65	0·36
1·00	7·0	106	44	0·42

* All values are for 5 g original seed material, germinated for 48 hr.

Purification and properties of allantoinase

Lathyrus sativus allantoinase was purified from seeds germinated for 48 hr. Since it was fully stable for 2 min at 60°, selective thermal denaturation was carried out with the crude extract. After removal of the denatured protein, allantoinase was purified from the supernatant by $(NH_4)_2SO_4$ fractionation, followed by chromatography on DEAE cellulose; a 35-fold purification was achieved (Table 2). The purified enzyme was quite stable in the frozen state for at least 2 weeks.

The K_m value for the purified enzyme with (±)-allantoin as substrate was found to be 2·56 mM. The *L. sativus* enzyme exhibited less than 5% activity towards (−)-allantoin. The pH optimum was 7·5 in 0·14 M diethanolamine–HCl buffer, the pH activity curve exhibiting a sharp fall in activity on either side of the optimum. Enzyme activities at pH 5·5 and 9·0 were respectively 22·5 and 18·4% of that at pH 7·5. The effect of temperature on enzyme activity was examined in the range 10–60° and from Arrhenius plots of the data the energy of activation was found to be 5·6 kcal/mol.

In view of the fact that allantoinases from some plants and microorganisms are activated by Mn^{2+} ions [1–3], the effect of Mn^{2+} was studied by dialyzing the purified enzyme with added EDTA (final concentration 1 mM) for 6 hr and then exhaustively against 0·05 M diethanolamine–HCl buffer, pH 7·5 at 4°. Added Mn^{2+} had no effect on the activity of the dialyzed enzyme in the range 0·01–1·0 mM Mn^{2+}. The effect of several other metal ions on the activity of purified *L. sativus* allantoinase was also studied at two concentrations, viz. 0·1 mM and 1 mM. Ni^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+} and Cd^{2+} all had no effect. Co^{2+} was slightly stimulatory (137% activity compared to control at 0·1 mM) while Cu^{2+} was slightly inhibitory (80% activity).

In view of the studies of the earlier workers on allantoinases from other sources, the effect of *p*-chloromercuribenzoate and cysteine on *L. sativus* allantoinase were examined. The results show that pCMB (1·0 mM) fully inhibits enzyme activity and the L-cysteine (1·0 mM) reverses this inhibition completely. Hence *L. sativus* allantoinase is dependent on sulfhydryl groups for activity.

DISCUSSION

Plant allantoinases, in general, have been little studied. In germinating castor beans [6], the enzyme is absent from the resting seed and begins

Table 2. Purification of *L. sativus* allantoinase

Fraction	Protein (mg)	Enzyme activity Total units	Enzyme activity Specific activity (units/mg)	Enrichment	Yield (%)
Crude extract	4028	765	0·19	1·0	100
Heat denatured (60°, 2 min)	1960	750	0·38	2·0	98
40–70% saturated ammonium sulfate ppt.	348	320·3	0·92	4·8	41
DEAE eluate	9·15	60·3	6·6	34·7	27·3*

* Based on 92 units loaded on the column.

to be synthesized only after 48 hr. In contrast, in *L. sativus*, there is significant allantoinase activity in resting seeds, and this increases about 3-fold within the first 48 hr. This increase would appear to be due to *de novo* synthesis as indicated by the effect of chloramphenicol (Table 1) and by the fact that the plumule and radicle have much higher levels of activity than cotyledons. *Lathyrus sativus* allantoinase is also somewhat different from plant allantoinases studied so far in other respects. *Lathyrus sativus* allantoinase is active only towards the (+)-isomer and, to our knowledge, this constitutes the first instance of a plant allantoinase with high stereoselectivity; other plant allantoinases are non-specific in this regard [3].

Further, the *L. sativus* enzyme seems to have the lowest K_m value among plant allantoinases, since for (+)-allantoin this turns out to be 1·28 mM. The castor bean enzyme shows a K_m of 13·8 mM [6], that from *P. hysterinum*, 4·6 mM [3], that from soyabeans 6·7 mM [8] and that from mung beans 40 mM [3]. *Lathyrus sativus* allantoinase also shows no metal requirement, but is sulfhydryl dependent, although it does not need any added sulfhydryl compound for maximal activity. Possibly the enzyme exists in a native conformation that ensures that its catalytically active sulfhydryl groups are not readily susceptible to oxidation; alternatively, the enzyme may be active both in the free thiol form and in the disulfide form.

EXPERIMENTAL

Germination. Locally purchased *L. sativus* seeds were sterilized by soaking in 0·1% $HgCl_2$ for 2–3 min, washed repeatedly with sterile dist. H_2O and germinated under aseptic conditions in petri dishes [9]. When needed, chloramphenicol was dissolved separately in sterile H_2O and included.

Enzyme extraction and purification. After germination, seedcoats were discarded and the material homogenized with an equal weight of glass powder at 0° with 0·14 M diethanolamine–HCl buffer, pH 7·5 (5 ml/5 g original seeds), also containing 0·01 mM sodium thioglycollate 0·01 mM sodium azide and 0·1 mM EDTA. The extract was centrifuged at 10000*g* for 15 min at 0°. The residue was reextracted with the same buffer (3 ml). The combined supernatants which showed 95–98% of extractable activity were used for assays. Where enzyme content of cotyledons and seedlings was to be determined separately, the required parts were separated out by dissection and processed as above.

Crude extract (from 40 g seeds) was maintained at 60 ± 1° for 2 min. After rapid chilling, the bulky ppt. formed was centrifuged out (15 min, 10000*g*). The bulk of the enzyme from the supernatant was pptd with $(NH_4)_2SO_4$ at 40–70% saturation. This fraction was dissolved in 0·05 M diethanolamine–HCl buffer pH 7·5 and after dialysis (0°, 72 hr) a suitable aliquot was applied to a DEAE column (1 × 10 cm). After washing with 20 bed vols of the same buffer the enzyme was eluted out with 0·05 M diethanolamine–HCl buffer, pH 7·5 containing 0·16 M NaCl. Fractions 3–5 (5 ml fractions eluted at 10 ml/hr) contained maximal activity and were pooled to constitute the purified enzyme. This was stored frozen.

Enzyme assay. Reaction mixtures for allantoinase assay were incubated at 30° for 15 min and routinely contained 10 μmol allantoin, 266 μmol diethanolamine–HCl buffer (0·14 M, pH 7·5), other compounds as required, and enzyme in a total vol. of 2·0 ml. In studies dealing with the effect of pH, diethanolamine–HCl buffer (0·14 M) of the desired pH was used. The reaction was terminated with 0·5 ml TCA (50%). Allantoate formed was estimated according to a direct procedure [11]. The specific activity unit was the formation of 1 μmol allantoate/mg protein under the assay conditions. Protein was estimated according to the method of Lowry *et al.* [10].

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