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ALLANTOINASE FROM SHOOT TISSUES OF SOYBEANS

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Abstract—Allantoinase, catalysing the hydrolysis of allantoin to allantoic acid, was isolated from leaves and fruits of soybeans The enzyme was only partially solubilized from 50 000 *g* pellets by detergent treatment and the estimated MW of the soluble enzyme was 50 000. The enzyme was stable to heat treatment up to 70° and had an energy of activation between 20° and 30° of 10.2 kcal/mol. Amino acids, ammonium, nitrate and potential precursors of allantoin such as AMP, ADP, adenine, xanthine, inosine monophosphoric acid and uric acid had no effect on enzyme activity *in vitro*. Urea (20 mM), a product of ureide degradation, had little effect on enzyme activity. The assay pH optimum was between 7.8 and 8.0 and the apparent K_m for allantoin was 10.0 mM.

INTRODUCTION

A number of legumes when relying on nitrogen fixation for their nitrogen requirements transport large amounts of the ureides, allantoin and allantoic acid from the nodules to the shoot [1–5]. Ureides are probably synthesized from recent products of nitrogen fixation via purine synthesis and degradation [6–10] In the shoot ureides are thought to be degraded into glyoxylate and ammonium by the action of allantoinase (EC 3.5.2.5), allantoicase (EC 3.5 3.4) and urease (EC 3.5.1.5.) although the exact pathway in higher plants is not known [10] Allantoinase has been studied in a variety of plants including some legumes [11–22]. Usually the enzyme has been isolated from dry seeds or germinating seedlings. During the life cycle of legumes, however, most of the ureides synthesized in nodules are assimilated in the shoot tissues (leaves and stems) [7]. In addition the effects of potential regulators of allantoinase (e.g. purine precursors and products of ureide assimilation) have been little studied [10]. Here we describe some properties of allantoinase isolated from leaves and fruits of soybeans.

RESULTS

Characterization of allantoinase

Phosphate buffers have been shown to inhibit allantoinase from higher plants [16]. For this reason the effect of different buffers on the pH optimum of allantoinase isolated from soybean leaves was studied. Generally there was little effect of different buffers on enzyme activity at any given pH. With Tris–maleate buffer a single peak in activity was observed at pH 6.7–7.0. With Tricine and Tris–HCl a peak was noted at pH 7.8–8.0. There was little change in activity at pH 6 7–7.9 using HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid) buffer

Allantoinase was remarkably stable to a 10 min heat pretreatment followed by a 10 min assay at the test temperature and increased in activity with increasing temperatures up to 70° In separate experiments two discontinuities were noted in Arrhenius plots (Fig. 1) at 10° and 45°. The energy of activation (E_a) calculated after Segel [23] between 20° and 30° was 10.2 kcal/mol, decreasing to 2.3 kcal/mol above 45° and increasing to

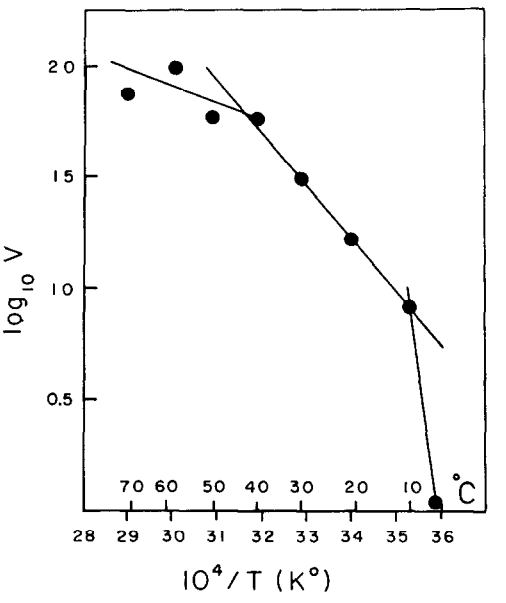

Fig 1 Arrhenius plot of allantoinase activity. Enzyme activity was measured as described in the text using different assay incubation temperatures and method A for the analysis of allantoic acid

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Table 1 Effect of sodium deoxycholate (DOC) on the solubilization of allantoinase from soybean leaf tissue

Fraction*	Total activity (nkat/g fr wt)
Untreated extract	
50 000 *g* supernatant	21
50 000 *g* pellet	10
DOC-treated extract	
50 000 *g* supernatant	29
50 000 *g* pellet	3

*A crude leaf extract was prepared as described in Experimental DOC was added to one half of the extract (final concentration 0 8 % w/v) Extracts were then incubated in ice–water (0) for 2 hr and centrifuged at 50 000 *g* The pellets were resuspended in Tris extraction buffer and used directly in the assay for allantoinase activity Production of allantoic acid was measured by the colourimetric determination of the diphenylformazan derivative of glyoxylate (method A)

32 5 kcal/mol below 10 Such discontinuities in Arrhenius plots are characteristic of membrane-bound enzymes [24] Further evidence that allantoinase may be particulate is presented in Table 1 *Ca* 30% of the total extractable activity was measured in the 50 000 *g* pellet of leaf extracts Incubation of extracts with sodium deoxycholate resulted in a 33% decrease in the activity of the 55 000 *g* pellet Similar treatment of the pellet with Triton X-100 resulted in only small increases in the solubilization of the bound enzyme, suggesting a relatively strong binding of the enzyme to a membrane and/or organelle

The apparent K_m of the soluble enzyme at pH 7 8 in 80 mM triethanolamine buffer was 10 0 mM allantoin From gel permeation data, the estimated MW was *ca* 50 000 To our knowledge this is the first MW estimate for allantoinase isolated from a higher plant.

Effect of reducing agents and inhibitors of sulphydryl-dependent enzymes

Cysteine and other reducing agents have been reported to inhibit allantoinase from soybeans [16, 17]. However, the apparent inhibition by cysteine may be the result of an interference in the procedure used to measure allantoic acid rather than an inhibition of the enzyme *per se* (Fig. 2) When different concentrations of cysteine were added to an assay mixture without enzyme extract, containing 50 nmol allantoic acid (an amount similar to that produced in the control enzyme assay with no cysteine) a decrease in absorbance similar to that observed in the enzyme assay was obtained (Fig 2A, B) Similar results were obtained using either method A or B to measure allantoic acid These results suggest that the effect of cysteine is probably via a complex formation that can occur between glyoxylate and cysteine [25] This complex formation will underestimate the glyoxylate present in the assay mixture thereby indicating an inhibition of enzyme activity Table 2 shows the effects of three other reducing agents on allantoinase activity Only dithiothreitol inhibited the enzyme without also inhibiting the colourimetric reaction Although inhibition by dithiothreitol suggests a dependence on disulphide bridges for maximum activity, PCMBS (*p*-chloromercuribenzene sulphonic acid), an inhibitor of sulphydryl group dependent enzymes, also inhibited enzyme activity. Iodoacetate was slightly inhibitory at 5 mM, but iodoacetamide and iodobenzoic acid had no effect on enzyme activity (Table 2) The addition of sulphydryl protectants to the extraction medium had no effect on enzyme activity

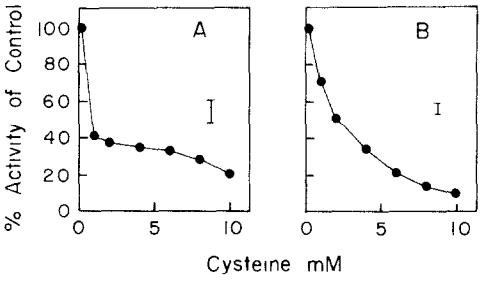

Fig. 2 Effect of cysteine on (A) apparent allantoinase activity and (B) the colourimetric determination of allantoic acid Assays for allantoinase activities were as described in the text Allantoic acid was measured by acid hydrolysis to glyoxylate followed by the colourimetric determination of the diphenylformazan derivative of glyoxylate [31] Bars represent the largest s e

Effect of amino acids, inorganic salts, urea, uric acid and purines

Glutamic acid, asparagine, γ-aminobutyric acid, aspartic acid, serine, alanine, arginine (transport amino acids in soybean leaves) [26], glutamine and glycine, all at a final concentration of 10 mM, had little or no effect on enzyme activity Similarly, ammonium sulphate, potassium nitrate, potassium chloride and dipotassium hydrogen phosphate (all 10 mM) has no effect on enzyme activity. Urea concentrations between 1 and 20 mM inhibited the enzyme by only 30% Using method B to measure allantoic acid, thereby overcoming interference in the assay, xanthine (0 25–2 5 mM), adenine (0 5–5.0 mM), IMP (1 –10 mM), AMP and ADP (1–20 mM) and uric acid (0.16–2.5 mM) were tested but had little effect on enzyme activity

The enzyme isolated from fruit tissues had a similar K_m for allantoin, was also partly particulate and not affected by any of the compounds tested as potential regulators of allantoinase activity

Table 2 Effect of reducing agents and inhibitors of sulphydryl dependent enzymes on allantoinase activity

Effector	Concn range (mM)	Inhibition* (%)
Glutathione	2–6	0
Dithiothreitol	2–10	40–70
Ascorbic acid	1–5	0
PCMBS	0 2 -1	23–57
Iodoacetate	1 -5	3–19
Iodoacetamide	1–5	0
Iodobenzoic acid	0 6–3	0

*Allantoic acid production during the assay was measured by determining the amount of glyoxylate phenylhydrazone formed following acid hydrolysis (method B)

DISCUSSION

The apparent K_m of soluble allantoinase for allantoin reported here and the similar values reported for the seed enzyme [14, 15] are relatively high compared with the concentrations of allantoin in leaves and fruits (0.1–5 mM) [27] Our evidence that the enzyme may be membrane bound is consistent with the recent demonstration that allantoinase activity is present in microsomal bands from soybean leaves [28] and indicates that there may be a compartmentation of the enzyme and substrate *in vivo* as previously suggested [27]. If this is so then the concentration of allantoin at the enzyme site may be high enough to allow the enzyme to function efficiently. Alternatively the K_m of the bound enzyme may be different from that of the soluble enzyme.

The apparently conflicting results with reducing agents and inhibitors of sulphydryl-dependent enzymes indicate that allantoinase requires free thiol groups and disulphide bridges for maximum activity. Similar results were obtained with allantoinase isolated from *Lathyrus sativus* [20] and *Dolichos biflorus* [21].

The lack of any obvious regulatory mechanism on enzyme activity and the high levels of allantoinase reported in leaves, stems and fruits of soybean [10, 27, 29] suggest that allantoinase is unlikely to limit the assimilation of ureides in shoot tissues. The results support the previous suggestion [27] that ureide assimilation in shoot tissues of ureide-producing legumes may be limited by allantoicase (degrading allantoic acid or allantoate).

EXPERIMENTAL

Germination and growth Seeds of soybeans [*Glycine max* (L) Merr cv Wells] were imbibed in H_2O, inoculated with *Rhizobium japonicum* and grown in growth chambers as described previously [27]

Preparation of enzyme extracts and estimation of MW. Mid-rib veins were removed from leaf blades and to *ca* 50 g fr wt lamina was added 350 ml ice-cold 0 05 M Tris–HCl buffer, pH 7.8. Extracts were prepared by homogenization in a Waring blender for 1 min The homogenate was squeezed through four layers of cheesecloth and centrifuged at 50 000 *g* for 30 min at 0° The supernatant was then fractionated by the addition of $(NH_4)_2SO_4$ at 0°. The ppted fractions were collected by centrifugation at 10 000 *g* for 10 min and dissolved in 0.05 M Tris buffer, final pH 7 8. Allantoinase activity was confined to the 40–75% satn fraction This fraction was dissolved in 2–5 ml Tris buffer and applied to a Sephadex G-100 column (1 5 × 80 cm) equilibrated with 0 05 M Tris buffer pH 7 8 The column was eluted with the Tris buffer and the fractions containing allantoinase activity were collected and ppted with $(NH_4)_2SO_4$ (final concn 75% satd) This procedure gave an 11-fold purification with a 32% recovery compared with a crude extract and activities of the order of 10 nkat/mg protein The partially purified extracts were used in all expts Further attempts to purify the enzyme were unsuccessful because enzyme activity was lost after dialysis or elution from a DEAE-cellulose column with Tris buffer.

The MW of allantoinase was estimated from the gel permeation data (Sephadex G-100 column). Ribonuclease-A, α-chymotrypsinogen A, ovalbumin and albumin were used as marker proteins for calibration. Prior to the expts on the properties of allantoinase, the ppted fractions of partially purified enzyme were dissolved in 0.05 M Tris buffer, pH 7.8 and desalted by use of Sephadex G-25 after Feller *et al.* [30].

Measurement of allantoinase activity. Allantoinase activity was measured in extracts using a method similar to that described by van der Drift and Vogels [17] The 1 ml assay mixture contained 80 μmol triethanolamine buffer, pH 7.8, 25 μmol allantoin (Sigma), 0 3 μmol $MnSO_4\ 2H_2O$ Assays were started by the addition of allantoin and tubes were incubated at 30° for 15 min. The reaction was stopped by the addition of 1 drop conc HCl and 0 25 ml aliquots were removed for measurement of the amount of allantoic acid produced. Allantoic acid was measured by two methods *Method A* Acid hydrolysis of allantoic acid to glyoxylate and determination of glyoxylate by measuring its diphenylformazan derivative [31]. In preliminary expts purines and uric acid were found to interfere in the colourimetric procedure used in method A. To overcome this interference a second procedure was used to measure allantoic acid. *Method B* After acid hydrolysis of allantoic acid to glyoxylate as before, the glyoxylate was converted to its phenylhydrazone followed by measurement of glyoxylate phenylhydrazone at 324 nm after Singh [32]. A boiled enzyme and a non-enzyme control were incubated with each assay Time zero controls were inadequate because of non-enzymic breakdown of allantoin into allantoic acid during the assay procedure Protein was measured in extracts by the dye-binding technique of Bradford [33], using bovine serum albumin (Sigma) as a standard Unless otherwise stated all results are means of at least three expts

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