Phytochemistry, Vol. 27, No. 3, pp. 693–695, 1988.
Printed in Great Britain.

0031-9422/88 $3.00+0.00

ALLANTOINASES OF NODULATED *ARACHIS HYPOGAEA*

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(*Revised received* 22 *July* 1987)

Key Word Index—*Arachis hypogaea*; Leguminosae; groundnut, allantoinases, root and nodule enzymes; parabanic acid and oxaluric acid; inhibition.

Abstract—Nodulated *Arachis hypogaea* plants contain an allantoinase in the nodules that is different from the host tissue enzyme. The root and nodule allantoinases have been purified 10 and 15 fold respectively. They exhibited K_m values of 25 (root) and 8.7 mM (nodule) for allantoin. Nodule allantoinase exhibits two pH optima, a major one at pH 6 and a minor one at pH 4 and is also specifically inhibited by parabanic acid and to a lesser extent by oxaluric acid. *Arachis* root tissue allantoinase, on the other hand, has a single pH optimum at pH 7.2 and is not inhibited by parabanic acid or oxaluric acid.

INTRODUCTION

The ureides (allantoin and allantoic acid) are important forms of fixed nitrogen in nodulated legumes, known to be synthesized within the nodules by conventional metabolic pathways from purines [1–5] and subsequently translocated to the aerial parts [6–8]. Consequently, enzymes involved in the metabolism of ureides are of great importance. Uricases from nodules have been studied [9–13] and there is evidence suggesting that these enzymes, in some systems at least, are different from the uricases of the host plant tissues [14]. Although several legume allantoinases have been studied recently [8, 15–17], very little is known of the allantoinases present in the nodules [11, 14, 18, 19]. We now report on some properties of the allantoinase of *Arachis hypogaea* nodules which reveal that this enzyme differs in certain important respects from the allantoinase of *Arachis* root tissue.

RESULTS AND DISCUSSION

pH activity profiles of nodule and root tissue allantoinases

Although allantoinases from several plant systems have been studied in some detail, there are few studies on nodule allantoinases [10, 14, 18, 19]. Tajima and Yamamoto were among the first to show that the allantoinase activity in nodule extracts from soybean plants had a pH optimum of 5 [14] and more recently Amarjit and Singh [18] found that the allantoinase from pigeon pea nodules is maximally active between pH 7.5 and 7.7.

Based on preliminary data which suggested that, in some respects, the allantoinase of the nodules of *Arachis* plants was different from the corresponding enzyme present in the root tissue, the pH activity profiles of the two enzymes were examined (Fig. 1). The nodule enzyme exhibits a major peak of activity at pH 6 with a minor peak at pH 4. In experiments with six different lots of nodules this pattern was found to occur with the activity at pH 4 being 55% (average: range 53–57%) of that at pH 6. In contrast, the allantoinase of *Arachis* roots has a single pH optimum at 7.2. There are only a few reports of differences in properties between nodule and plant enzymes associated with allantoin metabolism. Tajima and Yamamoto [14] have shown that soybean nodule uricase has a pH optimum of 9.5 and needs no cofactor, whereas soybean radical uricase is maximally active at pH 7 and has an absolute requirement for an uncharacterized low M_r organic cofactor for activity.

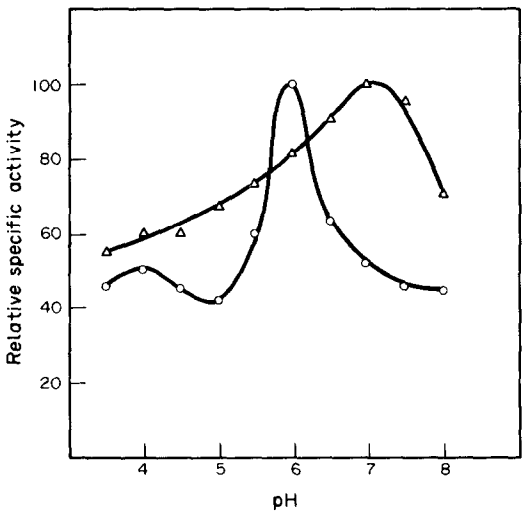

Fig. 1. pH-activity profile of *Arachis hypogaea* allantoinases from nodules (○——○) and roots (Δ——Δ). Relative specific activity (%) has been plotted on the basis of specific activity of allantoinase at pH 6 for the nodule enzyme and pH 7.2 for the root enzyme.

Some properties of nodule and root allantoinases

The root and nodule allantoinases from *Arachis hypogaea* plants were purified 10 and 15-fold respectively. The K_m for the *Arachis* nodule allantoinase was 8.7 mM and that for the enzyme from the root tissue was 25 mM. The lower K_m for the nodule allantoinase as compared with that for the root enzyme is in line with our unpublished observations that non-nodulated young *Arachis* plants

metabolize exogenously supplied allantoin at a very slow rate as compared with several other nodulating legumes.

Neither the root nor the nodule enzyme apparently required any sulfhydryl groups for activity. The partially purified enzymes from both sources were stable for up to 24 hr in the frozen state. One general property of several plant allantoinases would appear to be their tendency to lose activity after partial purification. This has been reported in the case of the allantoinase of soybean shoots [20] and has also been found with pigeon pea nodule allantionase [18]. For this reason, enzymes reported here, as well as several other plant enzymes, have generally been purified *ca* 10- to 15-fold only. The allantoinase of *Lathyrus sativus* (35-fold) and *Dolichos biflorus* (62-fold) had been purified [15, 16] to a slightly greater extent.

Dialysis of the purified enzymes with 1 mM EDTA resulted in little change in allantoinase activity of root extracts. The nodule allantoinase, on the other hand, showed a 58% decrease in activity. Addition of Mn^{2+}, Mg^{2+}, Cu^{2+}, or Zn^{2+} (0.5 mM) to such dialysed extracts resulted in no activation, except for Mg^{2+} which enhanced activity of the nodule enzyme only by 16–20%. No inhibition of activity in either case was obtained by addition of Cu^{2+}, Zn^{2+}, Hg^{2+}, Mn^{2+}, Co^{2+} or Ni^{2+} (1 mM). On a fresh weight basis allantionase activity in nodules averaged 7.34 ± 1.54 units/g fr.wt. and in roots 3.77 ± 0.70 units/g fr.wt. The nodules thus have around two fold higher activity than roots on a tissue fresh weight basis.

In other studies also [21] allantoinase as well as other enzyme activities have been compared on the basis of nodule fresh weight, and this had been used as an index of nodule activity. It may be mentioned, however, that when compared on the basis of protein content, the allantoinase values reported here are higher in the roots by about 80 $\pm$ 10%.

Specific inhibition of nodule allantoinase by parabanic acid

While testing for the effect of a variety of compounds with structural similarities to allantoin, it was found that parabanic acid was specifically inhibitory to the allantoinase of nodules (at both pH 4 and pH 6), but not of the roots. Nodule allantoinase was inhibited 25.2% by parabanic acid (2 mM) and 13% by oxaluric acid (2 mM); corresponding values were 4.8% and 0% for the root enzyme.

The xylem fluid of *Arachis hypogaea*, contains, as for other ureide exporting plants, major quantities of allantoin and allantoate [10], although it has been classified with reservation as an amide exporter [10]. The question of the importance of ureides in *Arachis hypogaea* is therefore still undecided. Our findings of an allantoinase in nodules of *Arachis*, which is distinctly different from the enzyme of the host plant (root) tissue is relevant in this context, because allantoin and allantoate, the product of allantoinase action, are the predominant forms in which nitrogen is transported from the nodule to the plant in legumes with a ureide based metabolism.

The two allantoinases, those from *Arachis* nodules and from the root tissue are clearly different enzymes. The nodule enzyme has its major activity at pH 4 with a minor peak at pH 6 (like the allantoinase of *Dolichos biflorus* [16]) and is specifically inhibited by parabanic acid and to a lesser extent by oxaluric acid, whereas the root allantoinase has a completely different pH activity profile with a single peak at pH 7.2 and is insensitive to inhibition by parabanic acid as well as by oxaluric acid. However, one unusual feature of the inhibition by parabanic acid is that maximal inhibition is obtained at 2.0 mM; higher concentrations were not more inhibitory. This is the first time that parabanic acid has been found to inhibit any allantoinase. The presence of a completely different allantoinase in *Arachis* nodules suggests that this enzyme may have a functional importance in the nitrogen metabolism of *Arachis* nodules.

The results presented here, taken along with those of soybean nodule uricase [14], suggest that some nodulating legumes may synthesize separate uricases and allantoinases in nodules with properties that are different from those of the corresponding enzymes of the host plant tissue.

It is possible that the nodule allantoinase described herein is a rhizobial enzyme. Studies are in progress to examine this. However, current opinion, generally, is not clear regarding the location of nodule allantoinases. According to Herridge *et al.* [7], allantoinase, in cowpea nodules, is equally distributed between bacteria and cortical tissues, with a small amount in insoluble fractions [22]. On the other hand, Hanks *et al.* [8] find that in nodules of *Glycine max*, the allantoinase is associated with the endoplasmic reticulum of the nodule cell.

EXPERIMENTAL

Plant material. Arachis hypogaea seeds inoculated with *Rhizobium* culture (strain NC-92 of ICRISAT, Hyderabad) were sown during winter (November, 1985) and field grown plants were harvested between 65 and 70 days. Nodules were dissected out, freed of root tissue, washed and stored in 20% glycerol at $-10°$. The nodule-free root samples were also stored frozen.

Preparation of enzyme extracts. Washed nodules (2 g fr.wt) were gently blotted dry between folds of filter paper and ground in a glass mortar and pestle with an equal wt of acid washed sand, and ice cold diethanolamine–HCl buffer (0.6 mM, pH 7, 2–3 ml/g fr.wt) containing 1% sodium dodecyl sulphate (SDS). The extract was centrifuged at 2° in a Beckman J2-21 refrigerated centrifuge at 20 000 *g* for 15 min. The residue was re-extracted with 4.5 ml of buffer and centrifuged. The combined supernatant was used as the enzyme source in some experiments. Roots freed of nodules were washed, blotted dry and extracted similarly except that the extraction buffer did not contain SDS.

Under the conditions of extraction, preliminary experiments showed that, in both cases, 80–90% of allantoinase activity was recovered in the supernatant.

Purification of nodule and root allantoinases. Allantoinase activity was extracted separately from *Arachis hypogaea* root tissue (freed of nodules, 6 g fr.wt) and from nodules (free of root tissue, 8 g fr.wt) essentially as described above. Extracts were subjected to $(NH_4)_2SO_4$ fractionation. The bulk of the enzyme activity, in each case, was found in the 0–40% ppt. The ppts were dissolved in minimum vols of 0.6 mM diethanolamine–HCl buffer, pH 6 and dialysed exhaustively against 50 mM dietthanolamine–HCl buffer pH 6 at 2°. The dialysed extracts were concd to small vol. by covering dialysis bags containing enzyme with powdered sucrose (AR) and then applied to DEAE-Sephadex A-50 columns (1.1 × 21 cm), equilibrated with the same buffer. On stepwise development of the column with buffer containing 0.1 M NaCl followed by 0.5 M NaCl, the bulk of the activity eluted in 0.5 M NaCl. Allantoinase from roots was thereby purified 10-fold with 63% recovery and the enzyme from *Arachis* nodules purified 15-fold with 86% recovery. On storage

at 2°, both enzymes tended to lose appreciable activity within 48 hr. Hence, freshly purified preparations were used.

Allantoinase assay. Enzyme extracts were assayed, (except in experiments for determining pH activity profile) in 0.3 mM diethanolamine–HCl buffer at pH6 for the nodule enzyme and pH 7 for the root enzyme. The assay mixture contained 50 μmol allantoin, enzyme extract (0 – 0.5 ml) and buffer, in a total vol of 1.7 ml. After incubation for 30 min at room temp (30 ± 1°), the reaction was terminated by addition of 0.2 ml TCA (50%). The reaction mixture was centrifuged and aliquots from the supernatant used for determination of allantoate as described in ref. [23]. Allantoinase activity has been expressed as μmol allantoate formed/mg protein/30 min under the assay conditions. The specific activities of the nodule and root enzymes employed in testing the effects of parabanic and oxaluric acids were 0.22 and 0.20 respectively. Enzyme activities were invariably compared in diethanolamine–HCl buffers.

Metal ions and other inhibitors, when required, were included in the assay system at levels indicated in the text. When possible activation by metal ions was to be examined, the enzyme extract was dialysed with 1 mM EDTA prior to use and metal ions were included in the assay system as required. In studies dealing with the effect of pH, diethanolamine–HCl buffers of appropriate pH were used.

Chemicals. Parabanic acid and DEAE-Saphadex A50 were from Sigma. Oxaluric acid was prepared from parabanic acid according to ref. [24].

Acknowledgement—The authors wish to record their thanks to the Department of Science and Technology, Government of India, for financial support for the study.

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