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AN UNUSUAL ALLANTOINASE FROM *DOLICHOS BIFLORUS*

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Key Word Index—*Dolichos biflorus*; Leguminosae; allantoinase; enzyme purification and properties.

Abstract—An unusual allantoinase from *Dolichos biflorus* has been purified 62-fold. The purified enzyme has an unusual pH activity profile with a shoulder at pH 4 and a peak at pH 7.5. This is due to a single enzyme which does not need metal ions for activation. In the fully reduced state the enzyme exhibits a single sharp peak at 7.5; when it is not in the sulfhydryl form (in the fully oxidized —S—S— form?) the enzyme shows a single pH optimum at pH 4. K_m values for (±)-allantoin were 5.5 mM at pH 4 and 1.43 mM at pH 7.5. Allantoinase activity has been demonstrated in the resting seed, and increased linearly with time during the first 5 days of seedling growth.

INTRODUCTION

Plant allantoinases have not been extensively studied. Enzymes from soybeans [1] and *Lathyrus sativus* [2] have been purified and their properties are known. In the present study an allantoinase from *Dolichos biflorus* has been purified 62-fold. This allantoinase is unusual in that its catalytic properties depend upon whether it is in the fully reduced (—SH) or the oxidized (—S—S—) form. The pattern of the activity during early germination differs from that observed in *L. sativus* [2] and castor bean [3].

RESULTS

Synthesis of allantoinase

In view of our earlier studies on plant allantoinases [2] the changes in allantoinase levels were followed during germination of *D. biflorus* seeds. Allantoinase activity increased linearly from 38 units for the resting seed to 78 units at 120 hr of germination.

Purification and properties of allantoinase

Seeds were germinated for 18 hr and allantoinase was purified from crude extracts by $(NH_4)_2SO_4$ fractionation, followed by chromatography on DEAE-cellulose. A 62-fold purification was achieved thereby (Table 1). On stepwise elution from DEAE-cellulose, the enzyme emerged as a single, symmetrical peak in 0.2 M NaCl (Fig. 1). The purified enzyme was stable for at least 4 weeks in the frozen state.

Table 1. Purification of *Dolichos biflorus* allantoinase

Fraction	Protein (mg)	Enzyme activity Total units	Specific activity (units/mg)	Enrichment	Yield (%)
Crude extract	1500	690	0.46	1.0	100
40–60% saturated ammonium sulfate ppt.	392	388	0.98	2.1	56
DEAE elute	5.13	146	28.4	62	63*

*Based on 231 units loaded on the column. For experimental details see text.

When the allantoinase activity was studied as a function of pH, a broad peak of activity was measured at pH 4 and a peak at pH 7.5. Hg^{2+} (10^{-6} M) and thioglycollic acid (10^{-4} M) altered the pH-activity profile suggesting the importance of —SH groupings for enzymatic activity. The data are shown in Fig. 2. Hg^{2+} produced a selective inhibition of activity at pH 7.5 and the enzyme then exhibited a single peak at pH 4. Oxidized glutathione (0.1 mM) also stimulated activity at pH 4 by up to 42%. With thioglycollate, on the other hand, activity at pH 4 was almost completely suppressed and a sharp peak remained at pH 7.5. When Hg^{2+} (10^{-6} M) and thioglycollate (10^{-4} M) were both present, the pH-activity profile was identical with that found with thioglycollate alone (not shown in the figure).

The activity of the purified enzyme was unaffected when dialysed exhaustively against 1 mM EDTA. Metal

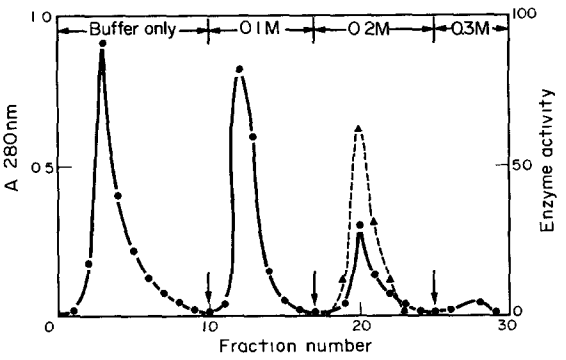

Fig. 1. Elution profile of allantoinase from *Dolichos biflorus*. Column size: 7.5 × 1.5 cm; sample: 231 units of 40–60% $(NH_4)_2SO_4$ fraction in 0.05 M diethanolamine–HCl buffer, pH 7.5 Elution: same buffer containing NaCl in stepwise concentrations of 0–0.3 M as shown. Flow rate 30 ml/hr. (●—●) UV absorbance at 280 nm; (▲--▲) allantoinase activity. For experimental details see text.

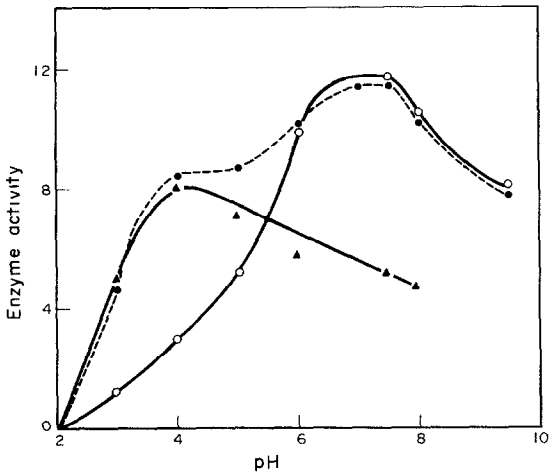

Fig. 2. pH-activity profile of *D. biflorus* allantoinase. (●—●) Activity of DEAE-cellulose purified allantoinase, (▲—▲) with 10^{-6} M Hg^{2+}; (○—○) with 10^{-4} M thioglycollate. For experimental details see text.

ions tested were not stimulatory, and Ni^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+} and Zn^{2+} were not inhibitory at 1 mM; activity at pH 7.5 was reduced by 30% by Cu^{2+}.

The K_m values for (±)-allantoin were determined by conventional methods at pH 4 and 7.5. Lineweaver–Burk plots yielded values of 5.5 mM at pH 4 and 1.43 mM at pH 7.5. In view of the unusual pH-activity profile of purified *D. biflorus* allantoinase, temperature dependent losses of enzyme activities were also studied at pH 4 and pH 7.5. The results obtained are shown in Fig. 3 and indicate that the inactivation process was similar at both pH values.

DISCUSSION

The biosynthesis and properties of plant allantoinases have been studied in relatively few plant species. Soybean allantoinase has been purified about 265-fold [4] and the *L. sativus* enzyme, *ca* 35-fold [2]. Patterns of allantoinase activity have been studied in germinating castor beans [3] and *L. sativus*, and these differ from that now reported during germination of *D. biflorus*.

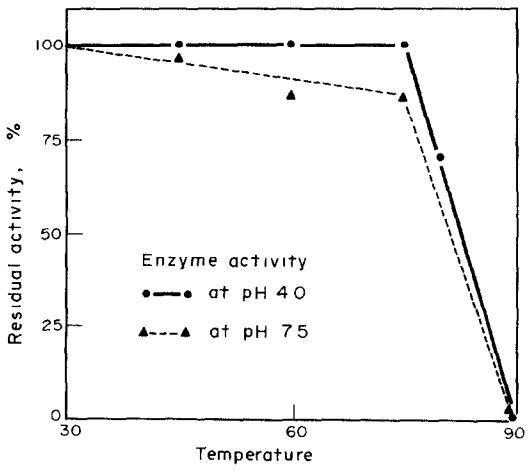

Fig. 3 Thermal inactivation of *D. biflorus* allantoinase activity. For details see Experimental.

Vogels *et al.* have stated [5] that plant allantoinases differ in their response to Mn^{2+} and their dependence on sulfhydryl groups. The present enzyme from *D. biflorus* differs from the known plant allantoinases in its unusual pH activity profile which is critically dependent on —SH and possibly —S—S— groupings. Although the possibility of two separate enzymes having different properties cannot be eliminated, the evidence presented strongly suggests that in *D. biflorus* there is a single enzyme with somewhat unusual properties. Firstly, the elution profile from DEAE-cellulose indicates that the enzyme peak is fairly symmetrical, and closely follows the shape of the protein peak in 0.2 M NaCl. Also, thermal inactivation rates at both pHs of predominant activity are nearly identical. The ratio of the activation determined at pH 4 and 7.5 remains constant during enzyme purification and for enzyme prepared at different stages of germination.

When all groupings susceptible to reduction by thioglycollate are converted to the sulfhydryl form, the *D. biflorus* allantoinase possesses no activity at pH 4 and exhibits a single peak at pH 7.5. The effect of Hg^{2+} suggests that when the enzyme has few (or no) free sulfhydryl groupings it is predominantly active at pH 4 but not at 7.5. While it is not clear whether Hg^{2+} acts by converting —SH to —S—S—, as in the case of oxidation of cysteine [6] or by forming mercaptides, it appears likely that the form of *D. biflorus* allantoinase active at pH 4 is probably an oxidized form of the enzyme. This is supported by the fact that when the enzyme is assayed at pH 4 in the presence of 0.1 mM oxidized glutathione the activity is enhanced by up to 42%. To our knowledge this is the first instance of an allantoinase critically dependent on free sulfhydryl groups for activity at neutral pH and having an acid pH optimum when it is not completely in the reduced —SH form. Alternatively it is possible that when the protein is not fully reduced it exists in a markedly different conformational state characterized by a very different pH-activity profile.

EXPERIMENTAL

Germination. Locally purchased seeds of *D. biflorus* were germinated under sterile conditions as described earlier [2, 9] For enzyme purification, seeds were germinated for 18 hr and used after removing the seed coats.

Enzyme extraction and purification. After germination, seed coats were discarded and the remaining material homogenised with an equal wt of glass powder at 0° with 0.15 M diethanolamine–HCl buffer, pH 7.5 (1 ml/g original seeds) containing 10^{-5} M EDTA. The extract was centrifuged at 6000 *g* for 90 min at 0°. The process was repeated if necessary. The supernatant was the crude extract. The bulk of the enzyme was ppd from the crude extract with $(NH_4)_2SO_4$ at 40–60% satn. This fraction was dissolved in 50 mM diethanolamine–HCl buffer, pH 7.5, and after dialysis a suitable aliquot was applied to a DEAE-cellulose column (1.5 × 7.5 cm). The column was eluted successively with 100 ml of the same buffer and with buffer containing 0.1 M NaCl (70 ml), 0.2 M NaCl (70 ml) and 0.3 M NaCl (30 ml) with an elution rate of 30 ml/hr (Fig. 1). The enzyme eluted in 5 fractions with 0.2 M NaCl (protein content of fractions was monitored by determining *A* at 280 nm and also by the method of ref. [8] as necessary). These were stored frozen and constituted the purified enzyme

Enzyme assay. Reaction mixtures for allantoinase assay were incubated at 30° for 15 min and contained 25 μmol allantoin,

600 μmol diethanolamine–HCl buffer (pH 7.5), other compounds as required, and enzyme in a total vol. of 2 ml. In studies dealing with the effect of pH, 600 μmol diethanolamine–HCl buffer of the desired pH was used. The reaction was terminated with 0.5 ml TCA (50%). Allantoate formed was estimated by a direct procedure [2, 7]. The sp. act. unit was the formation of 1 μmol allantoate/mg protein under the assay conditions. When enzyme stability as a function of temp. was examined, reaction mixtures complete in every respect except for substrate were incubated at the desired temp. for 2 min, chilled rapidly in ice, warmed to 30° and the reaction started off by addition of substrate.

Enzyme formation during germination. Seeds (2 g) were germinated aseptically for varying periods of time, their seed coats removed and enzyme was extracted as described earlier. To ensure complete extraction of the allantoinase (98–100%) two extractions (5 ml buffer each time) were made and the supernatants combined for determination of enzyme and protein content.

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