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EVIDENCE FOR THE PRESENCE OF ALLANTOICASE IN GERMINATING PEANUTS

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Abstract—Allantoicase activity has been detected in germinating peanut cotyledons. The enzyme is absent in resting seeds but appears in the cotyledons on the fourth day of germination. It then stays at a constant level until the tenth day of germination. The pH maxima of the enzyme is at 7·5 and K_m is 0·847 mM.

INTRODUCTION

THE ENZYME allantoicase (allantoate amidinohydrolyase, EC 3.5.3.4) catalyzes the degradation of allantoate to urea and glyoxylate.[1,2] The presence of this enzyme has been shown to be widespread in animals[3] and microbes.[4] In higher plants, however, in spite of numerous reports regarding the presence of allantoate[5–8] and of evidence that the only pathway of allantoate degradation is to urea and glyoxylate,[9] there has been only one report on the presence of allantoicase,[10] viz., in young seedlings of *Soja hispida*.

We have found evidence for allantoicase activity in germinating peanut cotyledons in this laboratory, and the present communication reports these results. Enzyme activity has been assayed by determining the amount of glyoxylate formed enzymatically from allantoic acid by two methods:

(*a*) Converting the glyoxylate to its phenylhydrazone and measuring the absorption of glyoxylate phenylhydrazone at 324 nm.
(*b*) Reducing the glyoxylate with lactic dehydrogenase in the presence of reduced nicotinamide adenine dinucleotide (NADH) and measuring the extent of reduction by following the decrease in NADH absorbance at 340 nm.

The liberation of urea during the reaction has been established using paper chromatography.

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RESULTS AND DISCUSSION

Presence of Allantoicase in Germinating Peanut Cotyledons

The specific activity of allantoicase at different stages of germination is given in Table 1. The values are averages of determinations made with 0·02 and 0·05 ml of the peanut cotyledon homogenate. Allantoicase activity is absent in resting seeds. It appears on the fourth day of germination and stays constant to the tenth day.

TABLE 1. SPECIFIC ACTIVITY OF PEANUT COTYLEDON ALLANTOICASE AT DIFFERENT STAGES OF GERMINATION

	Specific activity ($\times 10^{-3}$)*	
Days germinated	Method (*a*)	Method (*b*)
0	0	0
1	0	0
2	0	0
3	0	0
4	0·232	0·640
6	0·268	0·675
8	0·370	—
10	0·340	—

* The methods of assaying the enzyme are given under Experimental Section.

The enzyme appears to be completely soluble in aqueous extracts as it is present in the homogenate only, not in the fat pad and the sediment (Table 2). As the enzyme activity of the homogenate does not increase appreciably by recentrifuging at $10{,}000 \times g$ for 60 min, centrifuging directly at $25{,}000 \times g$ for 30 min was considered adequate to remove the inert material.

TABLE 2. DISTRIBUTION OF ALLANTOICASE ACTIVITY IN DIFFERENT FRACTIONS OF PEANUT COTYLEDONS

Fraction*	Specific activity ($\times 10^{-3}$)
Original fat pad ($10{,}000 \times g$; 30 min)	0·00
Original sediment ($10{,}000 \times g$; 30 min)	0·00
Original homogenate ($10{,}000 \times g$; 30 min)	0·38
Recentrifuged sediment ($100{,}000 \times g$; 60 min)	0·00
Recentrifuged homogenate ($100{,}000 \times g$; 60 min)	0·45†

* The method of preparing these fractions is given under the experimental section. The conditions of centrifugation are mentioned in parentheses.

† There was 90 per cent recovery of enzyme activity present in original homogenate.

Linearity of Activity with Enzyme Concentration

The rate of reaction is linear for the first 5 min and is proportional to enzyme concentration. In a typical experiment the absorption due to glyoxylate formed during the first 5 min of the reaction with 0·02, 0·05 and 0·01 ml of homogenate from 6-day cotyledons was 0·0075, 0·0175 and 0·030. For the same conditions the reduction in absorbance of NADH due to oxidation by liberated glyoxylate was 0·005, 0·0125, 0·025.

Progress Curves of Allantoicase Activity

There is an increase in activity up to 7·5 min. The formation of glyoxylate then decreases and comes to a stop at 12·5 min. This may be due to inhibition of the peanut allantoicase by substrate saturation or by the products formed. Substrate saturation has been observed for insect allantoicase by Razet.[11]

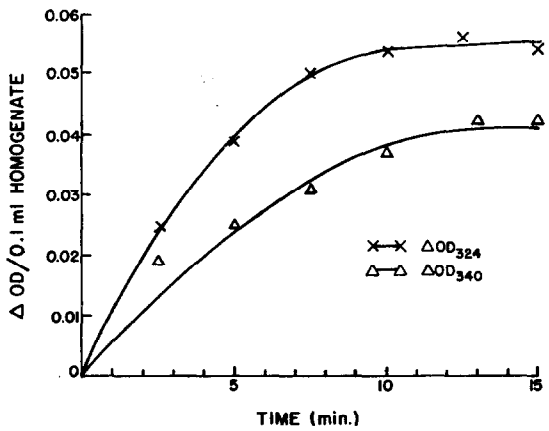

FIG. 1. PROGRESS CURVE OF PEANUT COTYLEDON ALLANTOICASE ACTIVITY USING HOMOGENATE FROM 6-DAY COTYLEDONS.

pH *Optimum*

The pH optimum is between 7·5 and 8·0. In the present report, however, all measurements of allantoicase activity have been carried out at pH 7·5. For determination of the pH optimum, activity was measured by method (*a*) to avoid complications introduced by the lactic dehydrogenase used in method (*b*). In a typical experiment the absorption due to glyoxylate phenylhydrazone formed with 0·1 ml of 6-day cotyledon homogenate was 0·0250, 0·0275, 0·0385, 0·0375 and 0·0200 during the first 5 min of the reaction at pH values of 6·5, 7·0, 7·5, 8·0 and 8·5.

The pH optimum for peanut allantoicase is higher than that reported for microbial allantoicases by Trijbels and Vogels.[12] They found the pH optimum of allantoicase from *Pseudomonas aeruginosa*, *Penicillium citreoviride*, *Pen. notatum* to be 7·2, while that of *Ps. fluorescens* was 6·0.

Reaction Rate of Allantoicase as a Function of Substrate Concentration

Method (*a*) was used for determining allantoicase activity. Employing values of enzyme activity (v) at different substrate concentrations [S], the K_m for allantoicase was determined by plotting $1/[S]$ against $1/v$ as well as by plotting $[S]$ against $[S]/v$. The K_m values by the two methods are 0·895 and 0·800 mM respectively, giving an average of 0·847 mM. This K_m value is lower than the K_m values for microbial allantoicases reported by Trijbels and Vogels.[12] These authors reported values of 17·3, 9·5, 28·6 and 16·7 mM for allantoicases from *Ps. aeruginosa*, *Ps. fluorescens*, *Pen. citreo-viride*, and *Pen. notatum*, respectively. The low K_m value of peanut cotyledon allantoicase, compared to the value of 3·8 mM determined in

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this laboratory for allantoinase from the same cotyledons, however, follows the pattern observed for allantoicase (9·5 mM) and allantoinase (35·0 mM) from *Ps. fluorescens*.[12,13]

Detection of urea as a reaction product. Urea was detected in the reaction mixture using two-phase descending paper chromatography. For this aspect of the studies the protein fraction precipitated between 40–65 per cent $(NH_4)_2SO_4$ saturation from 6-day peanut homogenate was used as the enzyme source. This fraction has been found to contain the allantoicase activity exclusively (Table 3). Furthermore, endogenous allantoic acid and urea

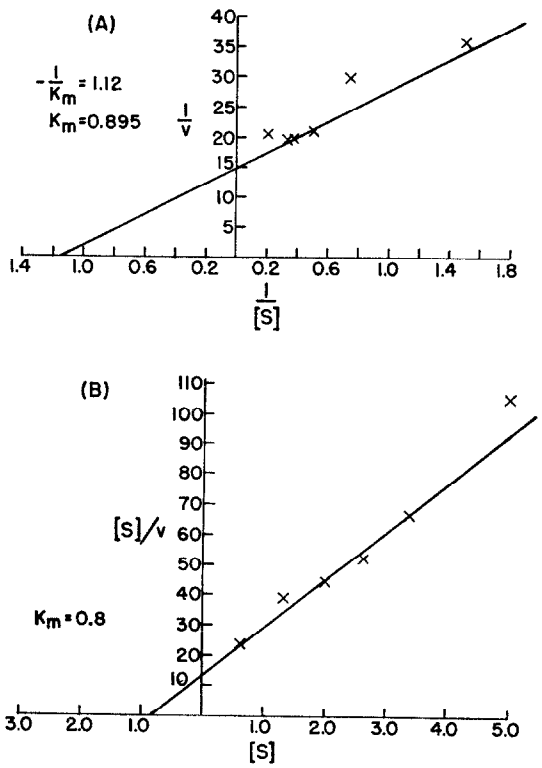

FIG. 2. K_m VALUE OF PEANUT COTYLEDON ALLANTOICASE PLOTTING (A) $1/[S]$ AGAINST $1/v$, (B) $[S]$ AGAINST $[S]/v$. THE CONCENTRATION OF $[S]$ IS EXPRESSED IN mM.

do not interfere with the results as they are completely removed by dialysis during preparation of this fraction. The R_f values of allantoic acid and urea were 0·33 and 0·72 with reference to buffered phenol, and 0·12 and 0·39 with reference to *n*-butanol-acetic acid-water. These compare favorably with the R_f values of authentic allantoic acid and urea carried out under similar conditions in this laboratory as well as those mentioned in the literature.[14,15]

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TABLE 3. DISTRIBUTION OF ALLANTOICASE ACTIVITY IN PROTEIN FRACTIONS OF 6-DAY COTYLEDON HOMOGENATE. THE FRACTIONATION PROCEDURE IS DESCRIBED UNDER EXPERIMENTAL SECTION

$(NH_4)_2SO_4$ saturation %	Protein recovery %	Sp-Act. ($\times 10^{-3}$)	Recovery %	Purification factor
0 (original homogenate)	—	0·258	—	—
0–40	11·6	0	0	0
40–65	38·0	0·495	71·5	*Ca.* 2
65–85	16·0	0	0	0
above 85	10·0	0	0	0
	Total = 75·4		Total = 71·5	

EXPERIMENTAL

Plant Material

Virginia 56-R peanut (*Arachis hypogaea L.*) seeds were germinated in the dark at 30° on paper towels wetted with tap water. The seeds were pre-soaked in 0·5 per cent suspension of Orthocide* (California Chemical Company, Richmond, California) for 3 hr and the water wetting the paper towels contained 0·05 per cent Orthocide to safeguard against fungus infection. Germination was measured from the time the seeds first came in contact with the water to the removal of the cotyledons for experiment. Cotyledons taken from seeds 24 hr after the addition of water have been termed "one-day cotyledons" and so on.

Homogenization of Cotyledons

Cotyledons were removed from the seedlings and washed thoroughly with distilled water. The washed cotyledons were homogenized for 2 min with 1·5 volume their weight of cold (0°) 0·1 M K_2HPO_4 in a Servall Omni-Mixer immersed in ice. In the case of resting seeds, the whole seeds were homogenized after removal of seed testa. The slurry obtained after homogenization was squeezed through two layers of cheesecloth and spun in a Lourdes VA-2 centrifuge at 25,000 × *g* for 30 min at 0°. The supernatant between the fat-pad and the sediment was removed for use in these investigations and is referred to as the "peanut cotyledon homogenate". In some experiments the slurry was first centrifuged at 10,000 × *g* for 30 min at 0° and the supernatant ("original homogenate") between the fat pad ("original fat pad") and the sediment ("original sediment") was recentrifuged at 100,000 × *g* for 60 min in a Beckmann Model L-2 preparative ultracentrifuge to obtain the "recentrifuged sediment" and "recentrifuged homogenate".

Allantoicase Assay

The allantoicase activity was measured by determining the amount of glyoxylate formed from allantoic acid at 30°. The following two methods were used:

Method (a). Glyoxylate was converted to glyoxylate-phenylhydrazone and absorption of the hydrazone was measured at 324 nm. This method has been developed by Lee and Roush[16] and is based on a procedure originally used by Dixon and Kornberg[17] for assaying isocitratase activity spectrophotometrically.

Four matching silica cuvettes were set up as follows:

(1) 2·0 ml of 0·1 M tris buffer, adjusted to pH 7·5 with HCl + 1·0 ml of 24 mM phenylhydrazine, pH 7·0.
(2) 2·0 ml of 0·1 M tris buffer, pH 7·5 + 1·0 ml of 24 mM phenylhydrazine, pH 7·0 + peanut cotyledon homogenate (0·02–0·05 ml).
(3) 2·0 ml of 3 mM allantoic acid (K and K Laboratories, Plainview, New York) dissolved in 0·1 M tris buffer, pH 7·5 + 1·0 ml of 24 mM phenylhydrazine, pH 7·0.
(4) 2·0 ml of 3 mM allantoic acid dissolved in 0·1 M tris buffer, pH 7·5 + 1·0 ml of 24 mM phenylhydrazine, pH 7·0 + peanut cotyledon homogenate (0·02–0·05 ml).

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* Trade names are used to describe exact conditions and do not imply endorsement of one product over another by the Department.

The change in absorptivity (ΔA), keeping cuvette (1) as reference, was recorded in a Beckmann DU Spectrophotometer for 5 min at 324 nm. The change in A effected by the allantoicase present was calculated as ΔA cuvette (4) − [(A cuvette (2) + ΔA cuvette (3))].

A molar extinction coefficient of $1{\cdot}7 \times 10^4$ l/mole/cm for the glyoxylate phenylhydrazone[17] was used in the calculations.

Method (b). The glyoxylate as formed was reduced by lactic dehydrogenase (L-lactate: NAD oxidoreductase, EC 1.1.1.27) in the presence of NADH and the extent of reduction measured by following the decrease in absorbance at 340 nm. Lactic dehydrogenase presumably reduces glyoxylate through one of its isozymic forms which is capable of reducing glyoxylate as well as pyruvate.[18] This method has been used by Brown *et al.*[19] for assaying the activity of African lungfish liver allantoicase. The procedure used in the present study is a modification of their method.

Four matching silica cuvettes were set up as follows:

(1) 3·0 ml of 0·1 M tris buffer, adjusted to pH 7·5 with HCl.
(2) 3·0 ml of 0·1 M tris buffer, pH 7·5, containing 0·3 μmol reduced NADH (Sigma) and 5 μg lactic dehydrogenase (Sigma, Type III) + peanut cotyledon homogenate (0·02–0·05 ml).
(3) 3·0 ml of 2 mM allantoic acid in 0·1 tris buffer, pH 7·5 containing 0·3 μmol reduced NADH and 5 μg lactic dehydrogenase.
(4) 3·0 ml of 2 mM allantoic acid in 0·1 M tris buffer, pH 7·5, containing 0·3 μmol reduced NADH and 5 μg lactic dehydrogenase + peanut cotyledon homogenate (0·02–0·05 ml).

The change in absorptivity (ΔA) at 340 nm, keeping cuvette (1) as reference, was recorded for 5 min. The change in A due to the presence of allantoicase was calculated at A cuvette (4) − [(ΔA cuvette (2) + ΔA cuvette (3))]. A molar extinction coefficient of $6{\cdot}22 \times 10^3$ l/mole/cm for reduced NADH was used in the calculations.

Protein Fractionation

The homogenate was fractionated by adding the required amount of $(NH_4)_2SO_4$ with constant stirring Precipitation was allowed to proceed for 30 min at 0°. The mixture obtained after $(NH_4)_2SO_4$ addition was centrifuged at $25{,}000 \times g$ for 30 min at 0°. The precipitate was resuspended in cold (0°) 0·1 M K_2HPO_4 and the supernatant was used for further fractionations. All fractions were dialyzed overnight against 0·1 M K_2HPO_4 in a cold room (0–5°). The dialysate was changed 2, 4, 8, and 12 hr after the start of dialysis.

Chromatographic Techniques

Two phase descending paper chromatography on Whatman No. 1 paper was employed to confirm the presence of urea in the reaction mixture. The solvents used were buffered phenol, pH 7·5, in one direction and a mixture of *n*-Butanol:acetic acid:water, 9:1:2·9[15], in the other direction. Buffered phenol[20] was prepared by equilibrating phenol (Baker's Analysed Reagent) overnight with 0·067 M Na_2HPO_4—NaOH buffer, pH 12·0. Ehrlich's reagent[15] (2 per cent p-dimethyl-aminobenzaldehyde in 1 N alcoholic HCl) was sprayed on the dried chromatograms to develop the spots. Urea and allantoic acid produced yellow spots upon drying.

Expressing of Enzyme Activities

Specific activities of the enzyme have been expressed as μ moles glyoxylate formed/min/mg protein. Protein was determined by the method of Lowry *et al.*[21]

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