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THE APPEARANCE OF ARGININE AND ARGINASE IN PUMPKIN COTYLEDONS. CHARACTERIZATION OF ARGINASE

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Abstract—During germination an increase in free arginine and arginase activity occurs in the cotyledons of pumpkin seedlings grown either in the light or dark, with the increase in enzyme activity paralleling the increase in free arginine content. Arginase was purified 9-fold from etiolated cotyledons and has a pH optimum of 9·5 in pyrophosphate buffer. The velocity of the reaction was linear up to 2 hr with a requirement for manganese. Iron, cobalt and copper gave a slight stimulation while other metals gave no stimulation. Arginase has a K_m of 26 μM with arginine as the substrate.

INTRODUCTION

THE GERMINATION of a seed and the subsequent growth of the resulting seedling produce marked changes in the nitrogenous constituents,[1] one of the most prominent being the breakdown of seed protein. Ashton and Dahmen[2,3] have isolated and characterized several proteolytic enzymes from pumpkin cotyledons which are capable of hydrolyzing these reserves. The reserve protein of pumpkin contains 16 per cent arginine[4] and considerable quantities of arginine would be expected to be liberated during germination. Arginine injected into pumpkin cotyledons was readily degraded to CO_2 through the citric acid cycle.[5] One step of this degradative pathway is the formation of ornithine from arginine mediated by the enzyme arginase (L-arginine amidinohydrolase, E.C.3.5.3.1).

This paper is a report of the appearance of arginine and arginase during the germination of pumpkin seedlings and the characterization and partial purification of arginase from pumpkin cotyledons.

RESULTS

Appearance of Arginine during Germination

The concentration of free argenine in the cotyledons of light- and dark-grown plants increased as germination progressed (Fig. 1). In the light, the maximum arginine concentration was reached 9 days after germination, while in cotyledons of dark-grown plants the maximum concentration was reached 15 days after germination. After reaching this maximum, the arginine concentration in cotyledons grown under both light regimes declined rapidly and approached zero in the cotyledons of plants grown 18 days in the light. The cotyledons of dark-grown plants contained considerably more arginine at all germination

[1] H. S. MCKEE, *Nitrogen Metabolism in Plants*, Clarendon Press, Oxford (1962).
[2] F. M. ASHTON and W. J. DAHMEN, *Phytochem.* 6, 641 (1967).
[3] F. M. ASHTON and W. J. DAHMEN, *Phytochem.* 6, 1215 (1967).
[4] J. BONNER, *Plant Biochemistry*, Academic Press, New York (1950).
[5] W. E. SPLITTSTOESSER, *Plant Cell Physiol.*, in press (1969).

times. The assay method used did not detect significant amounts of free arginine in the axis tissue of seedlings grown in either the light or dark.

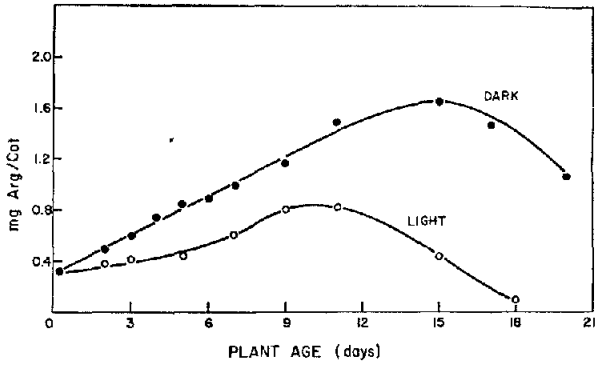

FIG. 1. CHANGE IN FREE ARGININE CONCENTRATION IN COTYLEDONS OF PUMPKIN SEEDS GERMINATED IN THE LIGHT OR DARK.

Arginase Activity during Germination

Arginase activity showed a pattern of development similar to that exhibited by the appearance of arginine (Fig. 2). In the light, enzymatic activity increased rapidly with germination and reached a maximum after 7 days. The activity then declined rapidly and no enzymatic activity was detectable 14 days after germination in the light. In the cotyledons of seedlings grown in the dark, however, the maximum level of arginase activity was not reached until 15 days after germination and this maximum was considerably higher than that produced in the light. Preliminary studies of arginase produced in the light showed that its characteristics were similar to the enzyme produced in the dark. Arginase activity was not detected in the axis tissue of seedlings grown in either light or dark.

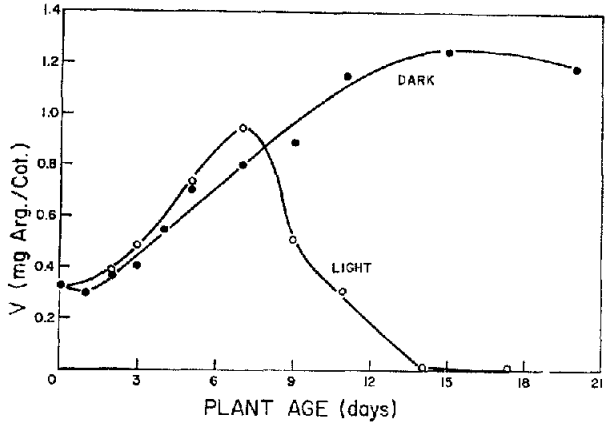

FIG. 2. CHANGE IN ARGINASE ACTIVITY IN COTYLEDONS OF PUMPKIN SEEDS GERMINATED IN THE LIGHT OR DARK.

Partial Purification

Cotyledons of 7-day-old seedlings grown in the dark were used as a source from which to purify the enzyme. Table 1 is a summary of the purification procedure in which arginase was

purified about 9-fold. Early experiments showed that the enzyme was localized in the supernatant solution after centrifugation at 40,000 × *g*. Efforts to purify the enzyme by additional ammonium sulfate fractionations or similar standard procedures resulted in lower specific activities.

TABLE 1. SUMMARY OF ENZYME PURIFICATION

Enzyme fraction	Total activity (units)	Specific activity (units/mg N)	Purification (fold over crude)	Yield (%)
Crude extract	40·2	10·3	1·0	100
Centrifugation I	42·0	20·0	1·9	104
40–60% $(NH_4)_2SO_4$ and dialysis	31·5	78·8	7·7	78
Centrifugation II	16·1	93·6	9·1	40

The reaction mixture was as described under "Experimental".

Incubation Time

The amount of arginine hydrolyzed with time was linear for 120 min (Fig. 3). This is in disagreement with other workers[6,7] who found that a straight line relationship between enzyme activity and time did not exist after 10 min with liver arginase.

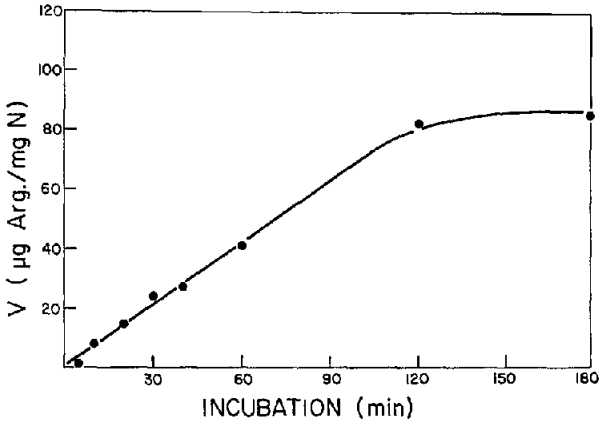

FIG. 3. THE RATE OF ARGININE HYDROLYSIS BY ARGINASE AS A FUNCTION OF TIME.

The Effect of the pH of the Incubation Mixture

The optimum pH of the enzyme, determined by carrying out the reaction in sodium pyrophosphate at various pHs, was found to be approximately 9·5.

The Effect of Protein Concentration or Temperature on Reaction Velocity

When the partially purified enzyme preparation was used, the amount of arginine hydrolyzed was directly proportional to the volume of preparation added over a 40-fold range of

[6] D. D. VANSLYKE and R. M. ARCHIBALD, *J. Biol. Chem.* **165**, 293 (1946).
[7] D. D. GILBOE and J. N. WILLIAMS, JR., *Proc. Soc. Exptl Biol. Med.* **91**, 537 (1956).

concentrations. The crude extract has been kept frozen for several months with negligible loss of activity. At 37° there was little or no inactivation in 2 hr as the amount of arginine hydrolyzed was essentially a straight-line function of time of incubation over this time period. Arginase was quite resistant to heating and could be heated for 5 min at 60° with little loss of activity.

Metal Activation

The activity of the extract was markedly increased by the addition of manganese (Table 2). In some crude extracts no activity could be demonstrated until manganese was added and the activation may be considerably higher than presented in Table 2. There was no difference in activation with $MnSO_4$ or $MnCl_2$ and the latter was routinely included in the reaction mixture. No stimulation was obtained with potassium, sodium, calcium or magnesium. A slight activation was obtained with iron, cobalt and copper although this stimulation was less than 10 per cent.

TABLE 2. THE EFFECT OF MANGANESE ON ENZYMATIC ACTIVITY

Conc. of Mn^{2+} in incubation mixture (μmoles)	Arginine hydrolyzed ($\mu g \times 10^{-2}$)	Activation (%)
0	0·6	—
5	1·8	30
10	2·1	35
20	6·1	101
40	10·1	168
80	11·0	183
100	10·0	167

Substrate Concentration and Arginase Activity

The apparent K_m, determined by the graphical method, is 26 μM which is lower than that of arginase from rat liver.[7]

DISCUSSION

During germination there is an extensive mobilization of reserves from the cotyledons to the growing axis. In pumpkin (Fig. 1) and beans[8] considerable quantities of arginine are released in cotyledons of seedlings grown in the light or dark. Wiley and Ashton[9] have shown that light has no effect upon protein hydrolysis or translocation in pumpkin cotyledons during the first 7 days of germination. The bulk of the protein hydrolyzed during this period was transported to the axis tissue. The findings that only trace amounts of free arginine are present in the axis tissue suggest that the arginine transported to this tissue was used immediately and studies with arginine-^{14}C support this suggestion.[10]

The cotyledons of light-grown plants turned green and expanded several fold and it is probable that, with the onset of photosynthesis, the rate of protein hydrolysis decreased

[8] D. BOULTER and J. T. BARBER, *New Phytol.* **62**, 301 (1963).
[9] L. WILEY and F. M. ASHTON, *Physiol. Plantarum* **20**, 688 (1967).
[10] W. E. SPLITTSTOESSER, *Hort. Sci.* **3**, 123 (1968).

while the rate of arginine utilization in the cotyledons increased. A decline in both arginase activity and arginine concentration occurred although these cotyledons persist as functional leaves. In the cotyledons of etiolated seedlings, photosynthesis would not occur, and previous studies have shown that after 7 days of germination the bulk of the carbon from arginine-^{14}C is released as CO_2 from a large arginine pool.[5] This would account for the differences in arginine concentration shown in Fig. 1.

The demonstration of arginase (Fig. 2) indicates that at least part of the enzymatic machinery for the hydrolysis of arginine is present in the cotyledons of plants grown in the light or dark. It is probable that other enzymes of the ornithine cycle are also present as they are in watermelon seedlings.[11] The relationship between the appearance and decline of free arginine and arginase activity suggest that arginase is intimately associated with arginine degradation. The lack of free arginine and arginase activity in axis tissue indicates that the enzyme is primarily associated with arginine metabolism.

The data do not permit one to establish if arginase was synthesized *de novo* or was present and then activated upon germination. The arginase activity increased 3-fold in cotyledons of light-grown plants while in the cotyledons of dark-grown plants the arginase activity increased 4-fold. If the enzyme were present and only activated upon germination, the same activity maximums should have been reached. However, although a small amount of arginase activity was present initially, it is possible that under the different light regimes, the level of activation upon germination may vary and the question of arginine synthesis or activation in pumpkin is unresolved. Arginase can be extracted readily and is soluble rather than particulate. The straight-line relationship between enzyme activity and time up to 2 hr (Fig. 3) differs from liver arginase which is non-linear after 10 min.[6,7] This suggests that the noted ornithine inhibition with liver arginase[12] may be minimal with the plant enzyme and the addition of equal quantities of arginine and ornithine or lysine had little effect upon the activity of the plant enzyme. However, ornithine inhibition is minimal at the pH optimum although the optimum pH of the pumpkin enzyme was similar to that of the liver enzyme.

The enzyme was activated only slightly by metal ions other than manganese. Partially purified liver arginase was activated by cobalt, manganese and nickel but the highly purified form was activated only by manganese.[13] The explanation for these differences is not known.[13]

EXPERIMENTAL

Plant Material

Pumpkin seeds (*Cucurbita moschata* L. cultivar Dickinson field) were sown in moist vermiculite and maintained in a darkened germinator or sown in soil and maintained in a glasshouse for various times. The seed coats were discarded before use. The enzyme assays were conducted with 7-day-old etiolated cotyledons, unless otherwise specified.

Arginine Assay

Argenine was determined by the colorimetric method of Gilboe and Williams.[14]

Protein Assay

Proteins were determined by the Folin-Lowry method, as described by Layne.[15]

[11] R. KASTING and C. C. DELWICHE, *Plant Physiol.* **33**, 350 (1958).
[12] D. M. GREENBERG, in *Methods of Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 2, p. 368, Academic Press, New York (1955).
[13] M. DIXON and E. C. WEBB, *Enzymes*, Academic Press, New York (1960).
[14] D. D. GILBOE and J. N. WILLIAMS, JR., *Proc. Soc. Exptl Biol. Med.* **91**, 535 (1956).
[15] E. LAYNE, in *Methods of Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 3, p. 448, Academic Press, New York (1957).

Measurement of Enzymatic Activity

The standard 5 ml incubation mixture contained in micromoles:[7] L-arginine, 1; $MnCl_2$, 80; sodium pyrophosphate buffer, pH 9·5, 100; enzymatic preparation. This was incubated at 37° for 30 min unless otherwise specified. After this incubation, 1 ml was removed, added to 4 ml of 0·4% TCA to stop the reaction, and the arginine was assayed as above.[14] Enzymatic activity has been expressed in terms of activity units. One unit is that amount of enzyme responsible for the hydrolysis of 0·1 μmole of arginine in 30 min under standard conditions.

Extraction and Partial Purification of the Enzyme

Throughout the extraction and purification procedures, the temperature was maintained as near 0° as was practical. 1. The crude extract was prepared by grinding the cotyledons in a volume of glass-distilled water equal to 1·5 times their weight. They were ground in a virtis homogenizer for 15-sec intervals for a total of 3 min while maintaining the temperature at 2–5°. The slurry was passed through four layers of cheesecloth. 2. The crude extract was centrifuged at 40,000 x *g* for 20 min. Considerable quantities of fat could now be removed from the top of the tubes. The pale-yellow supernatant contained the enzymatic activity. 3. Enzyme grade $(NH_4)_2SO_4$ was added to give 40 per cent saturation. After stirring for 10 min, the preparation was centrifuged for 10 min at 30,000 x *g*. The precipitate was discarded and additional $(NH_4)_2SO_4$ added to give 60 per cent saturation, stirred for 10 min and centrifuged as before. The precipitate was dissolved in distilled water (1 ml for each 10 ml of crude extract) and dialyzed 18 hr against 100 volumes of distilled water with two changes of dialysis solution. 4. After dialysis the solution was centrifuged at 40,000 x *g* for 10 min. The enzyme activity in the nearly colorless supernatant was stable and was stored frozen.

A summary of the purification procedure, with data from a typical experiment, is presented in Table 1.

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