Phytochemistry, 1970, Vol. 9, pp. 1535 to 1538. Pergamon Press. Printed in England.

UNUSUAL HEAT STABILITY OF PEANUT ALLANTOINASE

RATTAN SINGH,* ALLEN J. ST. ANGELO and N. J. NEUCERE

Oilseed Crops Laboratory, Southern Utilization Research and Development Division, New Orleans, Louisiana, U.S.A.†

(*Received* 21 *October* 1969)

Abstract—Allantoinase is present in both germinated and dormant peanut seeds. The enzyme has a pH optimum at 7·5 and is remarkably heat stable. The enzyme is active at 80° and retains considerable part of its activity even at 90°.

INTRODUCTION

THE ENZYME, allantoinase (allantoin amidohydrolase, EC 3.5.2.5), catalyzes the hydrolysis of allantoin to allantoic acid, which is then cleaved to glyoxylic acid and urea by the enzyme, allantoicase. This system is considered a part of the purine catabolic pathway. Recently, allantoinase was shown associated with the amphibian hepatic peroxisomes,[1] and was either membrane or particulate bound in the castor bean.[2] Interestingly, these data suggest that peroxisomes contain enzymes from the ureide metabolic pathway, in addition to those enzymes of the glyoxylate cycle and of peroxidative metabolism. Presence of allantoinase in amphibians, birds and mammals is well documented,[3] but the enzyme has only recently been investigated in the higher plants. In addition to the castor bean investigation reported by Ory *et al.*,[2] allantoinase was reported in ungerminated soybeans,[4–8] mung beans,[9] and in *Phaseolus hysterinus*.[6] Although a second enzyme involved in the ureide metabolic pathway, allantoicase, has been reported in germinating peanut cotyledons,[7] allantoinase has not previously been reported in the peanut. We therefore present in this communication evidence for the presence of allantoinase in the peanut and describe some of its properties including its unusual heat stability.

RESULTS AND DISCUSSION

Specific Activity of Peanut Allantoinase

Allantoinase is present in ungerminated peanut seeds as well as in germinating peanut cotyledons. The activity remains constant until at least the tenth day of germination is reached. Thus, the specific activities ($\times 10^{-3}$) of the enzyme from 0, 1, 2, 3, 4, 6, and 10-day

* Postdoctoral Research Associate, present address: Department of Biochemistry and Nutrition, Punjab Agricultural University, Ludhiana, India.

† One of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture. Requests for reprints should be sent to this Laboratory.

[1] L. P. VISENTIN and J. M. ALLEN, *Science* **163**, 1463 (1969).
[2] R. L. ORY, C. V. GORDON and R. SINGH, *Phytochem.* **8**, 401 (1969).
[3] M. FLORKIN and G. DUCHALEAN, *Arch. Intern. Physiol.* **53**, 267 (1943).
[4] K. W. LEE and A. H. ROUSH, *Arch. Biochem. Biophys.* **108**, 460 (1969).
[5] W. FRANKE, A. THIEMANN, C. REMILY, L. MOCHEL and K. HEYE, *Enzymologia* **29**, 251 (1965).
[6] C. VAN DER DRIFT and G. D. VOGELS, *Acta Botan. Neerl.* **15**, 209 (1966).
[7] G. D. VOGELS, F. TRIJBELS and A. UFFINK, *Biochim. Biophys. Acta* **122**, 482 (1966).
[8] K. RO, *J. Biochem. Tokyo* **14**, 405 (1932).
[9] Y. NAGI and S. FUNAHASHI, *Agri. Biol. Chem.* **25**, 265 (1961).

germinated peanuts were 3·5, 4·4, 4·0, 3·1, 3·8, 3·9 and 5·0 units, respectively. These data serve to illustrate a difference between the activities of peanut and castor bean allantoinase; the latter is absent in the resting seed, appears on the third day of germination, and reaches its maximum activity after 6 days of germination.[2]

Properties of Peanut Allantoinase

The enzyme exhibited a pH optimum of 7·5. Unlike the allantoinase found in the castor bean, the peanut enzyme is associated with the supernatant fraction, i.e. the original homogenate, and not the particulate matter. Fractionation of the supernatant with solid ammonium sulfate showed that the enzymic activity is associated to a major extent with the protein fraction precipitating between 40–60 per cent saturation. This fraction was used for the investigations reported herein. The peanut allantoinase, therefore, resembles the allantoinases from *Phaseolus hysterinus* and *Glycine hispida* studied by Vogels *et al.*[7] in this respect. Similarly, the enzyme, allantoicase, was also reported present in the supernatant fraction of the peanut extracts.[10]

TABLE 1. DISTRIBUTION OF ALLANTOINASE ACTIVITY IN PROTEIN FRACTIONS OF 6-day COTYLEDON HOMOGENATES. THE FRACTIONATION PROCEDURE IS DESCRIBED UNDER EXPERIMENTAL SECTION

$(NH_4)_2SO_4$ saturation (%)	Protein recovery (%)	Sp. act. ($\times 10^{-3}$)	Recovery (%)
(Original homogenate)	—	4·9	—
0–40	11·3	7·0	16·1
40–65	43·0	8·6	75·5
65–85	15·8	2·4	7·6
Total recovery	91·4	—	99·2

Thermal Stability of Peanut Allantoinase

The specific activities of the peanut allantoinase at different temperatures is represented in Fig. 1. The increase in activity is nearly linear between 30–80°, but the activity declines abruptly past 80°. However, even at 90° enzymic activity is still about 71 per cent of the activity found at 30°.

Enzyme-catalyzed reactions generally show an increased rate of activity up to about 45–55°. Normally, at temperatures above this range the catalytic function of the enzyme proteins is destroyed and the activity sharply drops because of thermal denaturation. Plant allantoinase, however, appears to be an exception. Ro[8] found that soybean allantoinase has temperature maxima at 60° and that this enzyme has the same activity at 80° as at 20°. He also found that the enzyme showed only a small loss of activity after heating at 70° for 30 min, and it exhibited a loss of half of its activity at 80° for 30 min. Franke *et al.*[5] also found the soybean allantoinase stable at 70°. The peanut allantoinase preparation described in these studies shows even greater thermal stability than shown by soybean allantoinase preparations reported. Furthermore, the progress curves of enzymic activity at different

[10] R. SINGH, *Phytochem.* **7**, 1503 (1968).

temperatures show that activity follows a straight line during the whole 60 min assay period at 30°, 60° and 80°. An Arrhenius plot of the data shown in Fig. 1, for the temperature range from 30° to 80°, also gives a straight line and the activation energy, determined from the slope, is 5·5 kcal/M.

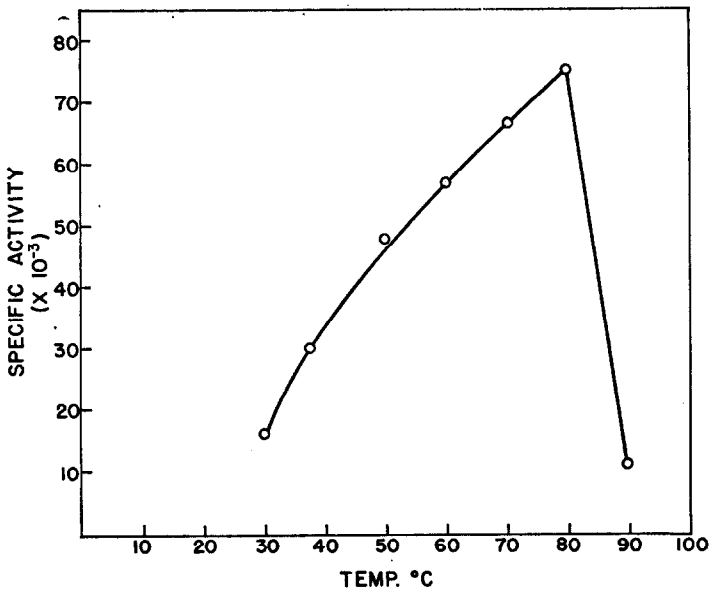

FIG. 1. THERMOSTABILITY OF PEANUT ALLANTOINASE EXTRACTED FROM 6-day GERMINATED PEANUTS.

EXPERIMENTAL

Plant Material

Virginia 56-R peanut (*Arachis hypogaea* L.) seeds were used. Seeds, after prior dusting with Spergon, were germinated in vermiculite at 30° in the dark.

DL-Allantoin, phenylhydrazine hydrochloride, glyoxylate and allantoinase standard were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A.

Preparation of Homogenates

Cotyledons removed from germinated seedlings or whole ungerminated seed after the removal of testa were used as the source of enzyme. Plant materials were thoroughly washed with H_2O and then homogenized for 3 min in 1·5 ml/g of cold 0·1 M K_2HPO_4, pH 8·5, in a Servall Omnimixer immersed in an ice bath. The slurry obtained was strained through two layers of cheese-cloth and centrifuged at 25,000 *g* for 60 min at 0°. The supernatant between the fat-pad and the sediment was removed. It is referred to as the "original homogenate".

Protein Fractionation

The "original homogenate" was fractionated by adding the required amount of solid $(NH_4)_2SO_4$ 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 *g* for 30 min at 0°. The precipitate was resuspended in cold (0°) 0·1 M K_2HPO_4 and the suspension was used for further fractionations. All fractions were dialyzed overnight against 0·1 M K_2HPO_4, 0–5°. The dialysate was changed 2, 4, 8 and 12 hr after the start of dialysis.

Protein Determination

Protein was determined by the method of Lowry *et al.*[11]

[11] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).

Measurement of Allantoinase Activity

Activity was measured by a modification of the method of Lee and Roush.[4] To allantoin (27·7 mg) in 9 ml of 0·1 M Tris buffer (pH 7·5) was added 1 ml of the seed extract giving a substrate concentration of 15 mM. Reaction temperature was 30° unless otherwise stated. After specified times, 2-ml aliquots were removed and added to tubes containing 1 drop of conc. HCl to stop the reaction. One millilitre of 0·3% phenylhydrazine HCl was added to each tube, which were heated to 100° for 2 min, then plunged into ice water. To each tube 1·2 ml of conc. HCl and 1·0 ml of 1·6% $K_3Fe(CN)_6$ was added. The contents were mixed and filtered (Whatman No. 1) and the clear red filtrate diluted 5-fold with H_2O and the absorptivity determined at 540 nm after 15 min. Controls were obtained by boiling enzyme preparations for 10 min prior to analysis. Allantoinase activity was expressed using a standard curve prepared from known quantities of glyoxylate. Specific activity is expressed in moles of glyoxylate formed per mg protein per min. The activities reported are averages of activities determined at 30°.