Phytochemistry, 1969, Vol. 8, pp. 401 to 404. Pergamon Press. Printed in England

UREIDE METABOLISM IN CASTOR BEANS. EVIDENCE FOR A PARTICLE-BOUND ALLANTOINASE

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(*Received* 5 *June* 1968, *in revised form* 6 *September* 1968)

Abstract—Allantoinase in germinating castor beans has been detected in a solubilized preparation. Unlike the enzyme reported in legumes this one is absent in dormant seed. It appears to be associated with some particulate material and was freed from its bound form with sodium desoxycholate. It has a pH optimum of 7·5–7·6 and a K_m of 13·8 mM (Lineweaver-Burk).

INTRODUCTION

THE PRODUCTION of glyoxylate from fat and carbohydrate in castor beans is well documented.[1–7] Castor beans contain an active lipase[8,9] through the first days of germination suggesting fat as a major source of glyoxylic acid in this seed.

Smaller quantities of glyoxylate (and urea) are also formed as the final products of purine catabolism. These purines, derived from RNA metabolites, give rise to the ureide intermediates allantoin and allantoic acid. Allantoin is converted to allantoic acid by the enzyme allantoinase (allantoin amido-hydrolase, EC 3.5.2.5). Allantoinase has been studied extensively in amphibian and in animal tissues[10–11] and in numerous micro-organisms.[12–15] Florkin and Duchâteau[11] list over 70 references describing investigations of allantoinase in amphibians, birds, and mammals. However, the metabolism of allantoin in higher plants is not as well established. Allantoinase has been reported in ungerminated soybeans,[16–18]

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1 H. BEEVERS, *Biochem. J.* **66**, 23 (1957).
2 G. E. NEAL and H. BEEVERS, *Biochem. J.* **74**, 409 (1960).
3 H. BEEVERS, *Nature* **191**, 433 (1961).
4 D. T. CANVIN and H. BEEVERS, *J. Biol. Chem.* **236**, 988 (1961).
5 H. L. KORNBERG and H. BEEVERS, *Biochem. Biophys. Acta* **26**, 531 (1957).
6 R. W. BREIDENBACH and H. BEEVERS, *Biochem. Biophys. Res. Commun.* **27**, 462 (1967).
7 E. MARRÉ, R. BIANCHETTI and S. COCUCCI, *Ital. J. Biochem.* **15**, 137 (1966).
8 R. L. ORY, A. J. ST. ANGELO and A. M. ALTSCHUL, *J. Lipid Res.* **1**, 208 (1960).
9 R. L. ORY, A. J. ST. ANGELO and A. M. ALTSCHUL, *J. Lipid Res.* **3**, 99 (1962).
10 R. FOSSÉ, A. BRUNEL and P. DE GRAEVE, *Comp. Rend.* **190**, 79 (1930).
11 M. FLORKIN and G. DUCHÂTEAU, *Arch. Intern. Physiol.* **53**, 267 (1943).
12 G. D. VOGELS, *Biochim. Biophys. Acta* **113**, 277 (1966).
13 F. TRIJBELS and G. D. VOGELS, *Biochim. Biophys. Acta* **113**, 292 (1966).
14 G. D. VOGELS, F. TRIJBELS and A. UFFINK, *Biochim. Biophys. Acta* **122**, 482 (1966).
15 G. D. VOGELS and C. VAN DER DRIFT, *Biochim. Biophys. Acta* **122**, 497 (1966).
16 K. W. LEE and A. H. ROUSH, *Arch. Biochem. Biophys.* **108**, 460 (1964).
17 W. FRANKE, A. THIEMANN, C. REMILY, L. MÖCHEL and K. HEYE, *Enzymol. Acta Biocatal.* **29**, 251 (1965).
18 C. VAN DER DRIFT and G. D. VOGELS, *Acta Bot. Neerl.* **15**, 209 (1966).

mung beans[19] and *Phaseolus hysterinus*[18] but, according to Tracy,[20] it is not present in *Ricinus communis*.

Since no other degradative mechanism for allantoin except through allantoic acid has been reported in higher plants, we have examined the castor bean, *R. communis*, before and during germination for allantoinase activity. Unlike the enzyme in the legumes studied, the castor bean allantoinase is absent in dormant seed. It appears on the third day of germination. The enzyme which apparently exists in a particle-bound state, has been solubilized using desoxycholate. Various properties of the soluble system are described.

RESULTS AND DISCUSSION

Appearance of Enzyme After Germination

As shown in Fig. 1, the enzyme is absent in resting seed. It appears on the third day of germination, reaching maximum activity after 6 days. This is a contrast to soybean and mung bean which exhibit allantoinase activity in the dormant seed and might explain why it was not found in the earlier experiments on *Ricinus communis*.[20]

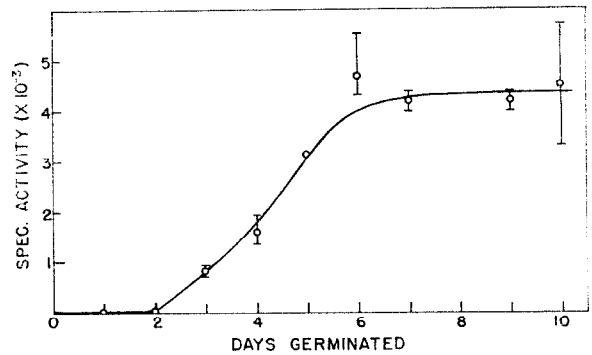


FIG. 1. APPEARANCE OF ALLANTOINASE ACTIVITY UPON GERMINATION OF CASTOR BEANS. GERMINATION CONDITIONS AND ACTIVITY MEASUREMENTS DESCRIBED IN EXPERIMENTAL.

While determining optimum conditions for studying the enzyme, it appeared that the castor bean allantoinase might be either membrane- or particle-bound. Attempts were made to free the enzyme from its bound form with several detergents. Sodium desoxycholate (DOC) not only solubilized the enzyme, but significantly increased the specific activity (Table 1). Kornberg and Beevers[5] have shown that glyoxylate cycle enzymes are present in the mitochondrial fraction obtained from germinating castor beans centrifuged at 25,000 *g* for 15 min. More recently, Beevers and co-workers[6, 21, 22] have found certain specific glyoxylate cycle enzymes associated with a nonmitochondrial subcellular particle, the glyoxysome. Glyoxysomes are obtained by centrifuging a homogenate at 10,000 *g* for 10 min in a sucrose gradient. They sediment differently and do not contain fumarase, NADH oxidase, succinate dehydrogenase, or cytochromes. However, we made no attempts to assay for glyoxylate cycle enzymes in the fractions assayed for allantoinase.

[19] Y. NAGAI and S. FUNAHASHI, *Agri. Biol. Chem.* (*Tokyo*) **25**, 265 (1961).
[20] M. V. TRACEY, *Moderne Methoden der Pflanzenanalyse*, IV, p. 134 (edited by K. PAECH and M. V. TRACEY) Springer-Verlag, Berlin (1955).
[21] R. W. BREIDENBACK, A. KAHN and H. BEEVERS, *Plant Physiol.* **43**, 705 (1968).
[22] T. G. COOPER and H. BEEVERS, *Plant Physiol. Supplement* **43**, S-9 (1968).

TABLE 1. EFFECT OF SODIUM DESOXYCHOLATE ON SOLUBILIZATION OF ALLANTOINASE FROM 6-DAY GERMINATED CASTOR BEAN

Fraction after 25,000 *g* centrifugation	Specific activity ($\times 10^{-3}$)
Fat pad, untreated	0
Ppt., untreated	0·612
Supernatant, untreated	0·107
Ppt., DOC-treated	0·233
Supernatant, DOC-treated	1·280

Homogenate prepared as described in text; substrate concentration, 15 mM. Specific activity expressed as μM glyoxylate formed per mg protein per min.

For the remainder of our investigations, the supernatant obtained after centrifuging the DOC-treated homogenate at 25,000 *g* for 30 min was employed.

pH Optimum

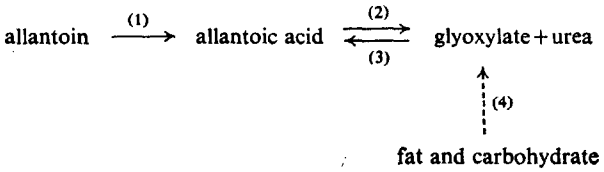
The pH optimum of the germinated castor bean allantoinase is 7·5–7·6. The pH optimum for allantoinase from dormant soybean[16–18] is 7·4–7·7, and from mung bean[19] is 7·5–8·5.

Effect of Substrate Concentration

For these studies K_m was determined by the Lineweaver-Burk[23] and the Hofstee[24] methods. The values of 13·89 mM and 11·9 mM, respectively, for the germinating castor bean allantoinase may be compared to those reported for the soybean variety investigated by Franke *et al.*[17] (6·7 mM), from mung bean[19] (40 mM), *Glycine hispida* L[18] (14·0 mM), and from *Phaseolus hysterinus*[18] (46 mM).

The experiments reported here establish the allantoinase of castor beans as a particle-bound enzyme which is absent in dormant seed but appears after 3 days of germination. The allantoinase appears to be associated with some particulate structure in the plant cell; DOC solubilizes the enzyme. Thus, prior to treatment with DOC most of the activity was located in the sediment after 25,000 *g* centrifugation, but after DOC-treatment allantoinase activity in the supernatant increased significantly. These experiments do not discern the nature of the particulate structure to which the allantoinase is bound. However, Akazawa and Beevers[25] sediment mitochondria from castor bean seedlings at 20,000 *g* for 20 min, Kornberg and Beevers[5] prepare glyoxylate cycle enzymes from castor bean seedlings at 25,000 *g* for 15 min, and Breidenback and Beevers[6] prepare glyoxysomes in a sucrose gradient at 10,000 *g* for 10 min. Finding allantoinase in the 25,000 *g* sediment suggests a possible association of the enzyme with one of these glyoxylate cycle fractions.

Allantoic acid may be derived by the following mechanisms:



[23] H. LINEWEAVER and D. BURK, *J. Am. Chem. Soc.* **56**, 658 (1934).
[24] B. H. J. HOFSTEE, *Science* **116**, 329 (1952).
[25] T. AKAZAWA and H. BEEVERS, *Biochem. J.* **67**, 110 (1957).

Finding such a relatively active allantoinase in the germinating castor bean was unexpected because of the large amount of fat converted to carbohydrate and glyoxylate during germination. However, information available so far on the possible synthesis of allantoic acid from glyoxylate and urea suggests that, though spontaneous combination of glyoxylate and urea can form allantoic acid, they do not combine in plants metabolically.[26] Consequently, suppression of allantoinase from the end products by reaction (3) above does not appear to take place in the castor bean. Furthermore, because of cellular compartmentation in plants, the glyoxylate formed in reaction (4) may not necessarily become part of the ureide system.

Several species of animals have been found in which allantoinase was present but allantoicase, the enzyme which degrades allantoic acid to glyoxylate and urea, was not detectable.[11] Investigations are presently underway to determine if the castor bean contains allantoicase and will be reported later.

EXPERIMENTAL

Material and Methods

Castor beans, *Ricinus communis*, Baker 296 variety, were a generous gift of D. S. Bolley and W. E. Domingo of the Baker Castor Oil Company. Seeds were germinated in vermiculite after prior dusting with Spergon* at 30° in the dark. DL-Allantoin, phenylhydrazine hydrochloride, and the glyoxylate used as the allantoinase were obtained from Sigma Chemical Company.

Preparation of Homogenates

Seeds were shelled and the kernels washed thoroughly in distilled water. The entire kernels (endosperm, cotyledons, and embryo) before or after germination were homogenized in 1·5 volumes of cold 0·1 M K_2HPO_4, pH 8·5, in a Servall Omnimixer for three 3-min periods while immersed in an ice bath. The resulting homogenates had a pH between 7·0 and 7·5. After the homogenization, 0·8% (final concentration by weight) sodium desoxycholate (DLC) was added to the homogenate and mixed well. The DOC-treated homogenate was placed in an ice bath for 1·5–2 hr, then filtered through cheese-cloth to remove larger particles and cell debris. The milky filtrate was centrifuged at 25,000 *g* for 30 min in a refrigerated vacuum centrifuge at 0°. The fat pad, aqueous phase, and the residual precipitate were collected separately for analysis.

Measurement of Allantoinase Activity

Activity was measured by a modification of the method of Lee and Roush.[16] Briefly, 23·7 mg (or 47·4 mg) of allantoin substrate and 9 ml of 0·1 M Tris buffer were adjusted to pH 7·5 with HCl. With the addition of 1 ml of the seed extract to be assayed, this yielded a final substrate concentration of 15 mM (or 30 mM) and did not alter the pH of the mixture. Reaction temperature was 25° (room temperature). After specified reaction times, 2 ml aliquots were removed by pipette and transferred immediately into tubes containing one drop of conc. HCl to stop the reaction. 1 ml of 0·3% phenylhydrazine.HCl was added to each tube; the tubes were placed in boiling water for 2 min, then plunged into ice-water. To each tube was added 1·2 ml of conc. HCl and 1·0 ml of 1·6% potassium ferricyanide; the tubes were mixed well and filtered. The clear, red filtrate was diluted five-fold with water and read at 540 nm in a spectrophotometer after 15 min. Controls for color due to endogenous glyoxylate and other non-specific reactions were obtained by boiling homogenates for 10 min prior to analysis. Allantoinase activity was determined by measuring the glyoxylic acid freed from enzymatically derived allantoic acid using a standard curve prepared from known quantities of glyoxylate. Specific activity is expressed as micromoles of glyoxylic acid formed per mg protein per min. Protein was determined by the method of Lowry *et al.*[27]

Acknowledgement—The authors wish to thank Mr. George I. Pittman for the drawings.

* Use of trade names does not imply recommendation by the Department of the product to the exclusion of others which may be equally suitable.

[26] H. REINBOTHE and K. MOTHES, *Ann. Rev. Plant Physiol.* **13**, 129 (1962).
[27] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).