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ORNITHINE CARBAMYLTRANSFERASE ACTIVITY FROM THE COTYLEDONS OF DEVELOPING AND GERMINATING SEEDS OF *VICIA FABA*

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Key Word Index—*Vicia faba*; Leguminosae; broad bean; ornithine carbamyltransferase activity; maturation; germination; localization; arginase.

Abstract—During maturation the ornithine carbamyltransferase activity from cotyledons of *Vicia faba* sharply decreased. It declined further during subsequent germination. On the other hand, arginase activity was low in mature, air-dry seeds but increased considerably during germination. After centrifugation at 40 000 *g*, more than 90% of the ornithine carbamyltransferase activity remained in the supernatant. The fractions containing tightly coupled mitochondria, showed hardly any ornithine carbamyltransferase activity.

INTRODUCTION

COTYLEDONS of developing *Vicia faba* seeds accumulate large amounts of protein-bound arginine.[1] Experiments with ^{14}C-labelled arginine have shown that during germination it is broken down *in situ*.[2,3] The biosynthesis of arginine in plant tissues occurs by a pathway similar to the Krebs–Henseleit cycle of mammalian tissues.[4] This pathway may be operative in the cotyledons of germinating *V. faba* seeds.[3] The question now arises which enzymes of this cycle are present during the final phases of seed development and during the first few days of seed germination. This paper reports the changes in the activity of ornithine carbamyltransferase (E.C. 2.1.3.3) and its subcellular distribution. The enzyme, which catalyzes the first step of the cycle, has already been demonstrated in extracts from pea seedlings.[5] Some preliminary results are described on changes in the arginase activity (E.C. 3.5.3.1).

RESULTS AND DISCUSSION

Ornithine Carbamyltransferase Assay

Under the conditions employed, the reaction was linear with time, amount of extract added and dependent on the addition of both carbamyl-P and L-ornithine. The optimum pH of the enzyme, determined by carrying out the reaction in phosphate, MES, MOPS, Tris or TES buffer at various pHs, was found to be approx. 8·2 in TES buffer.

Subcellular Distribution

In mammalian liver the carbamylation of ornithine to citrulline, which is catalyzed by ornithine carbamyltransferase, takes place exclusively in the mitochondria.[6] To ascertain

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[2] BOULTER, D. and BARBER, J. T. (1963) *New Phytologist* **62**, 301.
[3] JONES, V. M. and BOULTER, D. (1968) *New Phytologist* **67**, 925.
[4] REINBOTHE, H. and MOTHES, K (1962) *Ann. Rev. Pl. Physiol.* **13**, 129.
[5] KLECZKOWSKI, K. and COHEN, P. P. (1964) *Arch. Biochem Biophys.* **107**, 271.
[6] CARAVACA, J. and GRISOLIA, S. (1960) *J. Biol. Chem.* **235**, 684.

the subcellular distribution of the enzyme in cotyledons of *V. faba*, an extraction and isolation procedure was developed which would separate the mitochondria from other cell organelles. Table 1 presents the average results of three independent experiments. The highest succinate dehydrogenase activity, a mitochondrial marker enzyme, was found to be present in the fractions sedimenting between 2500–20 000 *g* and 20 000–40 000 *g*, whereas more than 90% of the recovered ornithine carbamyltransferase activity—which corresponds with about 80% of the activity of the crude extract—remained in the supernatant after centrifugation at 40 000 *g*. Obviously, ornithine carbamyltransferase activity and mitochondrial activity do not run parallel during differential centrifugation. (a) The possibility that the enzyme is yet present in the mitochondrial fraction but masked, may be excluded since neither freezing and thawing nor a Triton X100 treatment of both the mitochondrial and supernatant fraction resulted in a higher enzyme activity. (b) The oxidation of succinate (+ rotenone) by the mitochondrial fractions from developing seeds and from seeds germinated for 2 or 3 days showed respiratory control (RC ratio 2:3) and oxidative phosphorylation (ADP/O ratio 1·1:1·3). It is unlikely that the high ornithine carbamyltransferase activity, which in rat liver is present in the inner or matrix compartment of the mitochondria,[7] originates from these tightly coupled mitochondria, especially since it is generally known that their capacities to show respiratory control and oxidative phosphorylation is easily lost during an isolation procedure which disintegrates the mitochondrial structure.

TABLE 1. DISTRIBUTION OF THE ORNITHINE CARBAMYLTRANSFERASE ACTIVITY AND THAT OF SOME MARKER ENZYMES AMONG FRACTIONS FROM THE COTYLEDONS OF *Vicia faba* AFTER DIFFERENTIAL CENTRIFUGATION

Centrifugation speed (*g*)	Ornithine carbamyl-transferase*	Succinate dehydro-genase†	*p*-Nitro phenyl-phosphatase‡	Catalase§	Glucose-6-phosphatase‖	Protein (mg)
Crude extract	11 34	—	4 20	852	1 49	56 5
500– 2500	0 13	6	0 13	25	0 04	1 4
2500–20 000	0 25	36	0 16	96	0 04	2 3
20 000–40 000	0 21	25	0 08	133	0 03	1 4
Supernatant after 40 000	9 61	2	3 60	720	1 36	48 1

Each value represents the average result of three independent experiments with cotyledons of seeds germinated for 2–3 days. All enzyme activities are expressed on cotyledons basis.

* μmol citrulline formed/30 min.

† nmol succinate oxidized/min.

‡ μmol *p*-nitrophenyl-P hydrolyzed/10 min.

§ International Units according to Luck.[15]

‖ μmol glucose-6-P hydrolyzed/10 min.

The supernatant after centrifugation at 40 000 *g* (Table 1) contained the bulk of the *p*-nitrophenylphosphatase activity, the catalase activity and the glucose-6-phosphatase activity. So it is not clear whether the ornithine carbamyltransferase activity is a real 'soluble' enzyme or whether it originates from other cell organelles such as respectively lysosomes, microbodies or endoplasmic reticulum. More than 90% of the ornithine carbamyltransferase activity from the cotyledons of peas, roots of peas and beans and shoots of wheat was also found to be present in the supernatant after centrifugation at 40 000 *g*. Thus it is very likely that in plant tissues, in contrast to mammalian tissues, ornithine carbamyltransferase activity is located mainly outside the mitochondria.

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Ornithine Carbamyltransferase and Arginase Activity during Maturation and Germination

Since the dry wt of seeds (24 hr at 105°) hardly changes during the final stages of their development (maturation) whereas their water content sharply decreases, the relative water content is a reliable criterion of physiological age. At a relative water content of 60% (Fig. 1), the cotyledons had almost reached their maximum dry wt. At harvesting the relative water content drops to about 12%. During this maturation phase, the ornithine carbamyltransferase activity on a tissue basis (Fig. 1) and also on a protein basis decreased sharply. A further decrease occurred during subsequent germination.

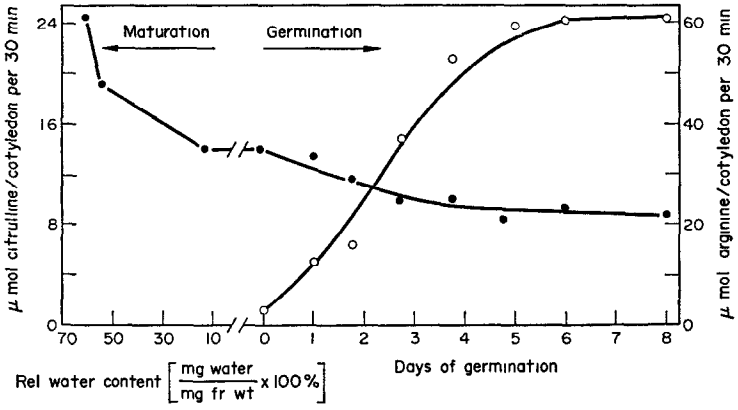

FIG. 1. CHANGES IN ORNITHINE CARBAMYLTRANSFERASE ACTIVITY AND ARGINASE ACTIVITY IN COTYLEDONS OF *Vicia faba* DURING MATURATION AND GERMINATION.

The synthesis of arginine from citrulline via argininosuccinate is catalyzed by argininosuccinate synthetase and argininosuccinate lyase. Both enzymes have been demonstrated in plant tissues.[8–10] The activity of the latter enzyme declined steadily in the cotyledons of germinating peas.[8] Together with the present results this suggests strongly that in the cotyledons of developing seeds which are actively synthesizing arginine, the activity of the enzymes concerned with arginine synthesis is high, but that in the cotyledons of germinating seeds which are actively degrading arginine, the activity of these enzymes steadily decreased. On the other hand, the arginase activity is very low in extracts from mature air-dry seeds (Fig. 1) but increases over 10-fold during a germination period of 8 days. A similar increase in arginase activity has also been found in cotyledons of pumpkin seeds.[11]

EXPERIMENTAL

Plant material. Developing seeds of *Vicia faba* L. cv. R 35, were obtained from plants grown outdoors. Germinating seeds were obtained by soaking air-dry, mature seeds in tap water under aeration for 18–24 hr and transferring them subsequently to moist filter paper in large Petri dishes where they were allowed to germinate further in darkness at 23° for the appropriate time.

Isolation of enzymes. The extracts were obtained by grinding 30 cotyledons (about 20 g fr. wt) in a pestle and mortar with 10 g sand and 10 ml medium containing: 0·4 M mannitol, 50 mM MOPS buffer (pH 7·2) and bovine serum albumine (2·5 mg/ml). The resulting slurry was squeezed through cheesecloth, diluted to about 45 ml with grinding medium and centrifuged at 2500 *g* for 5 min. The supernatant was assayed for

[8] SHARGOOL, P. D. and COSSINS, E. A. (1968) *Can. J. Biochem.* **46**, 393.
[9] SHARGOOL, P. D. and COSSINS, E. A. (1969) *Can. J. Biochem.* **47**, 467.
[10] SHARGOOL, P. D. (1971) *Phytochemistry* **10**, 2029.
[11] SPLITTSTOESSER, W. E. (1969) *Phytochemistry* **8**, 753.

arginase activity. It was recentrifuged at 40 000 *g* for 5 min to obtain the supernatant which was assayed for the ornithine carbamyltransferase activity. The mature, air-dry cotyledons were first pulverized with a 'Multimix' grinder. The powder thus obtained was allowed to imbibe for 30 min at 0–5°. Thereafter it was treated in the same way as the preparations from fresh cotyledons.

Differential centrifugation. The homogenate was centrifuged for 5 min at 500 *g* to remove cell debris. The resulting supernatant was centrifuged successively 5 min at 2500 *g*, 5 min at 20 000 *g* and finally 5 min at 40 000 *g*. The 3 pellets thus obtained were suspended each in 4·5 ml grinding medium. Membranes in the fractions were broken by freezing (in liq. N_2) and thawing them five times or by a treatment with Triton X100 (final concentration 0·5%) just before the enzyme assays were started All steps were performed at 0–5°.

Enzyme assays All enzyme assays were conducted at 25° and corrected for zero time controls. The assay of ornithine carbamyltransferase activity was based on the method of Nakamura and Jones [12] The incubation medium contained: 60 mM TES buffer (pH 8·2); 2 5 mM L-ornithine; 10 mM dilithium carbamyl-P and 0 1 ml enzyme preparation in a final vol of 2 0 ml. The reaction was started by the addition of carbamyl-P and was stopped after 30 min by the addition of 2 ml PCA 8% Protein was removed and the entire 4 ml was assayed for citrulline by a modification of the Archibald procedure.[13] Arginase activity was determined according to the method of Schimke.[14] The incubation medium contained: 0 225 M L-arginine (adjusted to pH 9 7 with KOH) and 3·8 mM $MnCl_2$ (1 8 μmol $MnCl_2$ were added to the arginine immediately prior to use and 2 μmol together with the enzyme preparation) in a final vol. of 1 ml. The reaction was started by the addition of 0·1 ml enzyme preparation (activated by 2 μmol $MnCl_2$ at room temparature for 5 min) and was stopped after 30 min by the addition of 2·5 ml PCA 8% The protein was removed, aliquots (0 5 ml) were taken and the urea formed determined [14] The *p*-nitrophenylphosphatase activity (E C. 3 1 3.2) was determined in a medium containing: 50 mM acetate buffer (pH 5 1); 2 5 mM *p*-nitrophenyl-P and 0 4 ml enzyme preparation in a final vol. of 2 ml. The reaction was started by the addition of the enzyme and terminated by adding 3 ml of 0·5 N NaOH. The *p*-nitrophenyl formed was measured at 405 nm. Catalase (E.C 1 11 1 6) was assayed by the initial rate of 12 5 mM H_2O_2 loss as measured by the decrease of absorbance at 240 nm [15] Glucose-6-phosphatase (E C. 3 1 3.9) activity was measured in a medium containing· 66 mM MOPS buffer (pH 6·5); 20 mM glucose-6-P and 0 4 ml enzyme preparation in a final vol. of 1·5 ml The reaction was started by the addition of the enzyme preparation and stopped after 10 min by the addition of 1 ml TCA 10% Pi was determined according to Fiske and SubbaRow.[16] Details of the succinate dehydrogenase (E.C. 1.3.99.1) assay are reported elsewhere [17] The oxidation of succinate was measured polarographically and the ADP/O ratios and respiratory control ratios calculated as in Estabrook [18] The reaction medium contained: 0·24 M sucrose; 20 mM MOPS buffer (pH 7 4); 0 5 mM EDTA; 5 mM Pi, 10 mM Na-succinate; 5 μg rotenone and 0 3 ml enzyme preparation in a total vol of 3 0 ml ADP (0·2–0 4 μmol) was added several times. Protein was determined by the Lowry procedure.[19]

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