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ORNITHINE TRANSAMINASE FROM *CUCURBITA MAXIMA* COTYLEDONS

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Abstract—During germination a marked increase in both soluble and particulate ornithine transaminase occurs in pumpkin cotyledons. Both enzymes had a pH optimum of 8·3 and a requirement for ornithine and α-ketoglutarate. Other keto acids or amino donors showed little activity. The enzymes required an active sulphydryl group for maximum activity. Exogenous pyridoxal phosphate was not required, but hydroxylamine inhibited the reaction and added pyridoxal phosphate overcame this inhibition. Proline inhibited the reaction and may play a role in the fate of ornithine in pumpkin cotyledons.

INTRODUCTION

DURING the germination of pumpkin seeds there is extensive hydrolysis of storage proteins to produce free amino acids which are either metabolized in the cotyledon or directly translocated to the growing axis tissue.[1] This insures a supply of nitrogen for the developing seedling. During the first 8 days of pumpkin germination, 75% of the reserve protein is hydrolyzed[2] and 50% of the total nitrogen in the cotyledon is transported to the axis tissue.[3] Although arginine accounts for 33% of the total nitrogen in pumpkin seeds less than 1% of this is translocated to the axis tissue.[1] Rather, arginine is metabolized[4] via arginase[5] to ornithine and the released nitrogen is transported to the axis tissue as glutamate and its amide.[1,6] Ornithine is further metabolized to glutamate and proline in several species.[3,7,8] Ornithine transaminases have been found in pea,[9] wheat,[9] mung bean,[10] sunflower,[11] peanut,[8] and *Cucurbita pepo*.[12] In mung beans[13] ornithine was shown to be

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converted to glutamic-γ-semialdehyde. This is a report on the localization, partial purification and characterization of L-ornithine: 2-oxoacid aminotransferase (E.C. 2.6.1.13) from pumpkin cotyledon.

RESULTS

Activity Changes During Germination

Ornithine transaminase activity increased during germination (Fig. 1) in both the soluble and particulate fractions. The peak of activity occurred at 5 days of germination in the soluble fraction and at 6 days of germination in the particulate fraction and maximum activity in the particulate fraction was considerably greater than that in the soluble fraction.

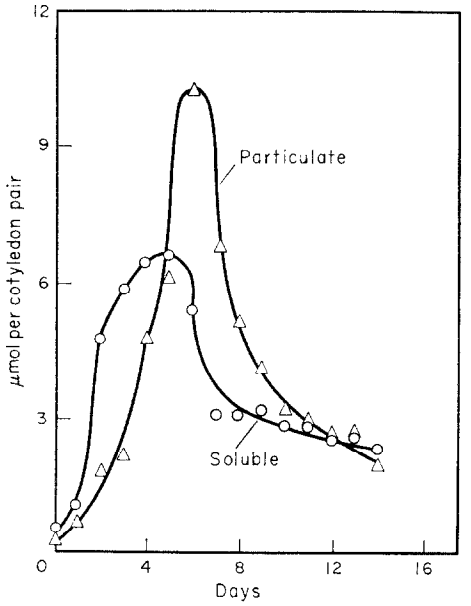

FIG. 1. CHANGE IN ORNITHINE TRANSAMINASE ACTIVITY IN PUMPKIN COTYLEDONS DURING GERMINATION: ACTIVITIES ARE EXPRESSED IN TERMS OF μmol Δ'-PYRROLINE-5-CARBOXYLATE FORMED IN 20 min (see Text).

At 14 days both fractions contained similar levels of activity. To determine if lightly bound transaminase was removed from the particulate fraction during extraction, this fraction was washed repeatedly (Table 1). Each succeeding wash removed less activity from the particulate fraction than the previous wash and although wash 4 removed protein, it did not remove transaminase activity.

Partial Purification

Cotyledons of 5-day-old seedlings grown in the dark were used as a source from which to purify the enzymes. Table 2 is a summary of the purification procedure in which the soluble enzyme was purified 10-fold and the particulate enzyme 15-fold. At this purification the total enzyme yield was 68%. Storage of the enzymes for 2 days at $-4°$ in the grinding media plus 30% glycerol resulted in 50% loss of enzyme activity.

TABLE 1. EFFECT OF REPEATED WASHING ON PARTICULATE ORNITHINE TRANSAMINASE ACTIVITY

Treatment	Activity (μmol product) Particulate	Soluble	Activity in wash (%)
Initial	21·6	—	
Wash 1	19·0	2·1	9·7
Wash 2	17·8	1·6	8·4
Wash 3	17·6	0·1	5·7
Wash 4	17·5	0·0	0

The particulate fraction was isolated from 5-day-old pumpkin cotyledons and washed with the extraction media (see Experimental).

Reaction Requirements

The only requirements for the reaction were enzyme, L-ornithine and α-ketoglutarate (Table 3). The addition of exogenous pyridoxal-5-phosphate, glutathione or EDTA had little effect upon the purified enzymes. However, EDTA stimulated the reaction over 100% when the nonpurified soluble enzyme was used. Other keto acids could not replace α-ketoglutarate. Pyruvate and oxaloacetate gave 5% of the rate measured with α-ketoglutarate. L-α-Acetylornithine and γ-aminobutyrate were ineffective as amino donors.

TABLE 2. SUMMARY OF ENZYME PURIFICATION

Enzyme fraction	Total activity (μmol)	Specific activity (μmol/mg protein)	Purification (fold over crude)	Yield (%)
Soluble enzyme				
Crude	28·0	10·3	1·0	100
Centrifugation I	28·1	19·7	1·9	100
Centrifugation II	14·6	42·6	4·1	52
35–55% $(NH_4)_2SO_4$	9·2	112·6	10·9	33
Particulate enzyme				
Centrifugation II	12·9	105·8	10·3	46
Wash and dialysis	9·8	160·7	15·6	35

The reaction mixture and purification procedures was as described in Experimental. Data is expressed as the amount of pyrroline carboxylate formed.

Kinetic Parameters

The pH optimum for transamination for both the soluble and particulate enzymes was found to be 8·3 using Tris–HCl buffer. The optimum was well defined and activity fell off rapidly on the acid side. When the partially purified enzymes were used, the amount of pyrroline carboxylate produced was directly proportional to the amount of enzyme used and the rate was proportional with time for at least 40 min.

Soluble and particulate transaminase was not inhibited by high concentrations of ornithine or α-ketoglutarate. The reaction showed Michaelis–Menten kinetics and the K_m for ornithine, as estimated from the linear portion of a Lineweaver–Burk plot, was 3·9 mM for the soluble enzyme and 23·6 for the particulate enzyme. The K_m for α-ketoglutarate was 20·2 mM for the soluble enzyme and 23·4 mM for the particulate enzyme.

TABLE 3. ORNITHINE TRANSAMINASE REACTION REQUIREMENTS

Reaction mixture	Pyrroline carboxylate formed (μmol) Soluble enzyme	Particulate enzyme
Complete	900	1900
Minus α-ketoglutarate	0	0
Minus ornithine	10	11
Plus 30 mM EDTA	904	1900
Plus 1 mM glutathione	900	1900
Plus 100 μM pyridoxal phosphate	1000	2000
Plus heat-inactivated enzyme	45	152

The complete reaction mixture was 50 mM ornithine, 50 mM α-ketoglutarate, 200 mM Tris–HCl pH 8·3. Enzyme used was 4·0 mg of soluble and 1·1 mg of particulate from 7-day-old cotyledons.

Effect of Various Amino Acids and Inhibitors

L-Proline, L-canavanine, and L-diaminobutyrate markedly inhibited the enzymatic activity (Table 4). L-Valine, DL-norvaline, δ-aminovalerate and γ-aminobutyrate produced smaller amounts of inhibition. L-Lysine and L-α-acetylornithine had no effect upon enzyme activity.

TABLE 4. INHIBITION OF ORNITHINE TRANSAMINASE BY VARIOUS AMINO ACIDS

Amino acid	Concentration (mM)	Inhibition (%) Soluble enzyme	Particulate enzyme
L-Lysine	30	0	0
L-Proline	30	25	30
L-Valine	30	9	18
L-Canavanine	30	83	57
DL-Norvaline	60	12	12
δ-Aminovalerate	30	0	5
γ-Aminobutyrate	50	7	12
L-α-Acetylornithine	50	0	0
L-Diaminobutyrate	50	36	33

The reaction was conducted as described in the Experimental with 3·8 mg of soluble or 1·9 mg of particulate enzyme.

p-Chloromercuribenzoate, a sulphydryl inhibitor, almost completely inhibited the particulate enzyme activity, while a smaller inhibition was noted with the soluble enzyme (Table 5). With both enzymes, glutathione completely reversed this inhibition. Hydroxylamine inhibited both enzymes and pyridoxal phosphate partially relieved this inhibition. Glutathione or pyridoxal phosphate alone had little effect.

TABLE 5. THE EFFECT OF INHIBITORS UPON THE ACTIVITY OF ORNITHINE TRANSAMINASE

Inhibitor	Subsequent additions	Inhibitor concentration 20 mM	10 mM	0·1 mM	10 μM
		Activity (% of control)			
Soluble enzyme					
*p*CMB				31	93
*p*CMB	Glutathione			80	100
Hydroxylamine		36	70	100	100
Hydroxylamine	Pyridoxal phosphate	66	100		
Particulate enzyme					
*p*CMB				3	14
*p*CMB	Glutathione			82	100
Hydroxylamine		10	72	100	100
Hydroxylamine	Pyridoxal phosphate	31	92	100	

*p*CMB = *p*-Chloromercuribenzoate. Additions were made of 1 mM glutathione and 0·5 mM pyridoxal phosphate.

DISCUSSION

Both soluble and particulate ornithine transaminase were found in pumpkin cotyledons. However, in sunflower,[11] mung bean,[13] and rat liver[14] the enzyme is localized in the mitochondria, with small amounts of activity in the soluble fraction. This soluble activity was presumed to be solubilized mitochondrial enzyme but in the early stages of pumpkin germination, half of the enzyme activity is in the soluble fraction and only small amounts of enzyme could be washed from the particulate fraction (Table 1).

The development of particulate enzyme is correlated with the increase in particulate protein[15] and the development of mitochondria.[16] This may suggest that the soluble enzyme was incorporated into the particulate fraction as organelles developed but the differences between the enzymes argue against this. The particulate enzyme is more sensitive to sulphydryl and carbonyl inhibitors and exhibited a different inhibition pattern with several amino acids. In addition, the development of ornithine transaminase activity parallels the development of arginase,[5] glutamic dehydrogenase,[15] aspartate aminotransferase,[17] and glutamine synthetase[6] which are also entirely or partially found in the soluble fraction in pumpkins.

Many properties of ornithine transaminase from pumpkins are similar to those of the peanut enzyme.[8] Both enzymes are sensitive to hydroxylamine but are not stimulated by pyridoxal phosphate; they have similar pH optima; both were inhibited by L-canavanine, L-valine and DL-norvaline; the K_m for ornithine is similar but that of α-ketoglutarate is somewhat higher for the pumpkin enzymes. The peanut enzyme was not inhibited by proline while the pumpkin enzymes were inhibited. Similar to most tissues, a metabolic interrelationship between arginine, ornithine, glutamate, and proline exists in pumpkin cotyledons.[3,4] Most of the ornithine and glutamate found in the cotyledons is translocated to the axis tissue[1] with small amounts of ornithine being converted to glutamate or proline.[3] Arginine and proline released from the protein reserve accumulate in the cotyledons.[1]

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Arginine elevates ornithine transaminase activity in rat liver[18] and may do so in pumpkin cotyledons as the maximum arginine level precedes the maximum ornithine transaminase activity by 2 days[1] (Fig. 1). However, proline has an inhibitory effect upon ornithine transaminase activity (Table 4) and may be responsible for high levels of ornithine being translocated to the axis tissue.[1] These control mechanisms are presently under investigation.

EXPERIMENTAL

Plant material. Pumpkin seeds (*Cucurbita maxima* L., var. King of the Mammoth) were sown in moist vermiculite and maintained in a darkened germinator until use. Seed coats were discarded before use and enzyme assays were conducted with 5-day-old etiolated cotyledons unless otherwise specified.

Extraction and partial purification of the enzyme. Throughout the extraction and purification procedures, the temp. was maintained near 0°. The crude extract was prepared by grinding the cotyledons in an equal vol. of 0·01 M glutathione, 0·25 M sucrose, 0·1 M K–phosphate, adjusted to pH 7·6 in a virtis homogenizer for 2 min. The slurry was passed through four layers of cheesecloth (crude extract) and then centrifuged at 5000 *g* for 10 min (centrifugation I). Fat was removed from the top of the tubes and the precipitate was discarded. The supernatant was centrifuged at 40 000 *g* for 15 min to yield the soluble and particulate enzyme fractions (centrifugation II). The soluble enzyme was precipitated between 35 and 55% $(NH_4)_2SO_4$ and both enzymes were washed and then dialyzed 4 hr against the extraction medium. The enzymes were stored frozen in 30% glycerol. A summary of the purification procedure with data from a typical experiment is presented in Table 2.

Assay procedures. The standard reaction mixture contained: α-ketoglutarate, 25 mM; L-ornithine, 50 mM; Tris–HCl buffer, pH 8·3, 200 mM; enzyme preparation; final vol. 2 ml. This was incubated at 23° for 20 min. The reaction was then stopped and the amount of pyrroline carboxylate was determined[19–21] using a millimolar extinction coefficient of 2·71.[14] Enzyme activities are expressed as the amount of Δ^1-pyrroline-5-carboxylate formed under the above conditions. Protein was determined by the biuret method.[22]

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