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L-Ornithine: 2-Oxoacid Aminotransferase from Squash (*Cucurbita pepo*, L.) Cotyledons

PURIFICATION AND PROPERTIES[1, 2]

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TI-SHEN LU[3] AND MENDEL MAZELIS[4]
Department of Food Science and Technology, University of California, Davis, California 95616

ABSTRACT

Ornithine: 2-oxoacid aminotransferase (EC 2.6.1.13) has been purified over 400-fold with a total recovery of 14% from acetone powders of cotyledons of germinating squash (*Cucurbita pepo*, L.) seedlings. The pH optimum of the transamination between L-ornithine and α-ketoglutarate is 8 and the Michaelis constants are 4.7 mM and 6.3 mM, respectively. The enzyme has a molecular weight of 48,000 as determined by gel filtration. The reaction is essentially specific for α-ketoglutarate as the amino group acceptor. The enzyme is inhibited very strongly by hydroxylamine, and less severely by NaCN and isonicotinylhydrazide. No inhibition is observed in the presence of 10 mM L-cysteine. The energy of activation is 7.6 kcal/mole. The stability of the enzyme preparation is enhanced by the presence of dithioerythritol and glycerol. The enzyme activity of the most purified fraction is stimulated 30% by the addition of pyridoxal phosphate; however, the evidence for the unequivocal involvement of pyridoxal phosphate was inconclusive.

Proline, glutamic acid, and arginine are readily interconvertible *in vivo* (11). Since the reactions concerned involved either ornithine or its transamination product glutamic-γ-semialdehyde, it is likely that the L-ornithine: 2-oxoacid aminotransferase (EC 2.6.1.13) plays a key role in the necessary metabolic processes.

The presence of this enzyme has been reported in seedlings of pea and wheat (5), mung bean (2), sunflower (13), peanut (8), and pumpkin (14). In the latter two species some characterization studies have been done but with preparations which have been only slightly purified. The present report describes the isolation and properties of a highly purified ornithine aminotransferase from germinating squash (*Cucurbita pepo*) cotyledons.

MATERIALS AND METHODS

Seeds of *Cucurbita pepo* var. Black Zucchini were obtained from the Asgrow Seed Company in Woodland, Calif. Ammonium sulfate was the ultrapure enzyme grade from Mann Research Laboratories. L-Ornithine, α-ketoglutaric acid, pyridoxal-P, dithioerythritol, *o*-aminobenzaldehyde, sodium borohydride, and pronase were all purchased from Sigma Chemical Company. Sephadex and hydroxylapatite gel powders were, respectively, products from Pharmacia Fine Chemicals and Bio-Rad Laboratory. DEAE-cellulose in powder form was from Whatman Co. L-[U-^{14}C]Glutamic acid and DL-[2-^{14}C]ornithine were obtained from International Chemical and Nuclear Corp.

Plant Material. Squash seeds were soaked in running water overnight, then planted in vermiculite in glass trays. The trays were kept in the dark at room temperature. The seeds were given water daily for 6 days.

Preparation of Actone Powder. Cotyledons from 7-day-old seedlings were removed and cooled in ice. They were then homogenized at top speed at 5 C in a Waring Blendor for 30 sec in 5 volumes (v/w) of acetone which had been previously chilled to −15 C. The resulting slurry was filtered under suction and washed thoroughly with chilled acetone on a Büchner funnel. The powder was dried at room temperature, then ground in a mortar and stored at −15 C. It has been stored for at least 3 months without loss of activity.

Assay of Enzyme Activity. The standard reaction mixture contained the following components at the concentrations listed: tris-HCl buffer (pH 8), 125 mM; L-ornithine, 50 mM; α-ketoglutarate, Na salt, 50 mM; pyridoxal-P, 0.05 mM; and enzyme. The final reaction volume was 2 ml. Usually the reaction was begun by the addition of α-ketoglutarate. The incubation was carried out at 30 C for 15 min and assayed for pyrroline-5-carboxylate as previously described (8).

Protein concentrations were determined by the method of Lowry *et al.* (6). Pyridoxal-P was determined colorimetrically by the procedure of Wada and Snell (19).

RESULTS

A steady increase in the specific activity of enzyme preparations from cotyledon acetone powders was observed with increasing age of the seedlings. Seedlings were harvested at the age of 7 days since they were of convenient size and had high enzyme activity at this time.

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[2] These studies form part of a dissertation submitted by T. S. L. to the University of California, Davis, in partial fulfillment of the work requirements for the Ph.D. degree. Portions of this work have been presented at the meeting of the American Society of Plant Physiologists at Asilomar, Calif., in August 1971 and that of the American Society of Biological Chemists in Atlantic City, N. J., April 1973.

[3] Present address: Tobacco Research Institute, Tsau-Hu, Taichung, Taiwan, Republic of China.

[4] To whom requests for reprints should be directed.

Enzyme Purification. All procedures were carried out at 0 to 5 C.

1. The acetone powder was extracted with 20 volumes (v/w) of 10 mM potassium phosphate buffer, pH 7.2, for 30 min with constant gentle stirring. At the end of this time, the residue was removed by centrifugation at 20,000*g* for 20 min and discarded.

2. The volume of supernatant solution was measured. A volume of 1% protamine sulfate solution equal to 50% of the supernatant solution was added slowly with stirring. The resulting dark brown precipitate was removed by centrifugation and discarded.

3. Solid $(NH_4)_2SO_4$ was added slowly to 45% saturation. Constant gentle stirring was applied and the pH was not allowed to drop below 7 by adding dropwise 2 N NH_4OH as necessary. The precipitate obtained at this saturation after stirring 20 min in the cold was removed by centrifugation and discarded. The supernatant solution was then brought to 70% saturation with solid $(NH_4)_2SO_4$, keeping the pH above 7 as before. The protein that precipitated between 45 to 70% ammonium sulfate saturation was collected by centrifugation and dissolved in a minimal volume of 10 mM potassium phosphate buffer, pH 7.2, containing 15% glycerol and 5 mM dithioerythritol. (This buffer was used in all operations hereafter and is called the standard buffer.)

4. The volume of solution was measured and 0.1 ml of 0.5 M Na-α-ketoglutarate was added for each milliliter of solution. The combined solution was then heated to 60 C, held at this temperature for 1 min, and then chilled immediately in a container of ice. After cooling to 10 C or below, any precipitate present was removed by centrifugation and discarded.

5. The supernatant solution from the heat treatment step was passed through a column of Sephadex G-75 (3.5 × 68 cm) which had been equilibrated with the standard buffer. It was then eluted using the same buffer with a flow rate of 15 ml/hr. Five-milliliter fractions were collected. The fractions with higher specific activity than that of the material put on the column originally were collected and pooled.

6. The pooled enzyme solution from the Sephadex column was put on a DEAE-cellulose column (1.8 × 30 cm) which was equilibrated with standard buffer. It was eluted with a linear gradient of 0 to 0.6 M NaCl in standard buffer. The fractions were collected in 3-ml aliquots and those containing the enzyme were pooled.

7. The pooled material from the preceding step was dialyzed overnight against standard buffer and then rechromatographed on the DEAE-cellulose column and eluted as before.

8. The pooled active fractions from the second DEAE-cellulose run were dialyzed against standard buffer and then placed on a hydroxylapatite column (1.2 × 18 cm) which had been equilibrated with standard buffer. The column was then washed with 0.05 M potassium phosphate buffer, pH 7.2, containing 15% glycerol and 5 mM dithioerythritol. The active fractions were then eluted by a solution of 0.15 M potassium phosphate buffer, pH 7.2, containing 15% glycerol and 5 mM dithioerythritol.

The results of a typical purification are summarized in Table I. The purest enzyme preparation (step 8) had three bands examined by standard polyacrylamide disc gel electrophoresis as described by Davis (3).

In the process of developing this protocol for enzyme purification, the following odd features were observed. (*a*) Changing the concentration of the extraction buffer from 0.2 M to 10 mM did not affect the amount of enzyme extracted, but the protamine sulfate step was more effective when 10 mM buffer was used for extraction. (*b*) Much better results were obtained when the heat treatment was done after the ammonium sulfate fractionation rather than before. (*c*) Prior to the heat treatment the enzyme was not retained on the DEAE-cellulose column.

Table I. *Summary of Enzyme Purification*

Step	Fraction	Total Protein	Total Activity	Specific Activity	Yield	Purification
		mg	*IU*	*IU/mg*	%	
1	Acetone powder extract	18,444	105	0.006	100	1.0
2	Protamine sulfate supernate	4,699	89	0.02	85	3.3
3	45–70% $(NH_4)_2SO_4$ precipitate	1,476	85	0.06	80	10
4	Heat treatment	439	72	0.16	68	27
5	Sephadex gel eluate	224	62	0.28	59	47
6	DEAE—1st column	44	33	.75	31	125
7	DEAE—2nd column	26	27	1.0	26	162
8	Hydroxylapatite eluate	5.6	14	2.5	14	417

Products of Reaction. Using essentially the procedure of Seneviratne and Fowden (12) with DL[2-^{14}C]-ornithine as the substrate, it was shown that the enzyme catalyzes the δ-transamination of ornithine to yield glutamic-γ-semialdehyde and its cyclic equilibrium form Δ^1-pyrroline-5-carboxylate.

Michaelis Constants for L-Ornithine and α-Ketoglutarate. The effect of varying substrate concentrations on the rate of the reaction was examined. Each substrate was varied in the presence of a saturating concentration of the other. This allowed each substrate to be determined as a pseudo-first order reaction at initial velocity. The data were fitted to the straight line form of the Michaelis-Menten equation

$$\frac{S}{v} = K_m/V_{max} + \frac{S}{V_{max}}$$

by a linear regression computer program. The correlation coefficient for both the L-ornithine and α-ketoglutarate determinations was 0.999. The intercept and slope for the line obtained from the program was used to calculate K*m* and V_{max}. The K*m* for L-ornithine was 4.7 mM and for α-ketoglutarate 6.3 mM. The V_{max} calculated was 2.65 IU per mg with the ornithine data and 2.45 IU per mg with that from α-ketoglutarate.

Reaction Rate and Enzyme Concentration, Time, and pH. The amount of conversion as a function of time was linear up to at least 40 min under our assay conditions. Within the limits of enzyme concentration tested (up to 90 μg of step 8 enzyme) a linear relationship was observed between activity and amount of enzyme. The pH for maximum activity was determined using both phosphate and tris-HCl buffers. The optimum activity was obtained at pH 8 with both buffers. The optimum was quite marked at this pH.

Stability of Enzyme. The presence of dithioerythritol and glycerol enhanced the stability of storage of the enzyme preparations. With step 3 enzyme preparations 36% of the original activity was lost in 14 days storage at −20 C. Addition of 5 mM dithioerythritol maintained the original activity completely. After gel filtration or dialysis there was a loss of activity immediately, with further loss on storage even in the presence of dithioerythritol. The addition of glycerol to a final concentration of 15% in addition to the 5 mM dithioerythritol stabilized the original activity for weeks at −10 C even with repeated freezing and thawing.

The reaction rate at different temperatures was measured, and by means of an Arrhenius plot (Fig. 1) an activation energy of 7.6 kcal/mole was calculated.

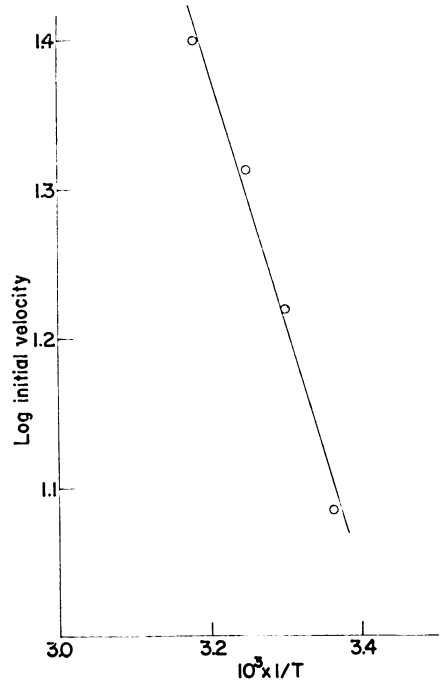

FIG. 1. Effect of temperature on the reaction rate. Standard reaction mixtures as described in the text were used. Reaction mixtures were equilibrated at the desired temperature for 10 min prior to adding 10 μg of step 8 enzyme, specific activity 2.5, to start the reaction. Incubation time was 5 min.

Specificity of α-Ketoglutarate as Amino Group Acceptor. A number of α-keto acids were tested as acceptors of the δ-amino group L-ornithine. Only α-ketoglutarate was found to give significant activity. The results are summarized in Table II.

Activity of Various Amino Acids as Amino Group Donors. The ability of various amino acids to replace L-ornithine in the reaction was investigated. This experiment relied on the formation of glutamic acid by the transamination of the amino acid used with α-ketoglutarate as acceptor. The amino acids examined were: D-ornithine, N-α-acetylornithine, lysine, γ-aminobutyric acid, α-aminobutyric acid, δ-amino valeric acid, α-aminoadipic acid, diaminopimelic acid, and arginine. The reaction mixtures were essentially the same as the standard reaction mixture except L-ornithine was replaced by the amino acid in question. Pyridoxal-P was not included in the reaction mixture because of the possibility of nonenzymatic transamination. The reaction was allowed to proceed for 1 hr at room temperature. A "boiled" enzyme control was included in each case. At the end of the reaction period, the mixtures were heated at 100 C for 2 min and aliquots spotted along with a glutamic acid standard on a filter paper strip. This was then developed one-dimensionally (descending) with 1-butanol-acetic acid-water (4:1:1, v/v) as solvent system. Ninhydrin was used to locate the amino acids formed. Only L-ornithine produced a clear spot of glutamic acid formed by transamination to α-ketoglutarate. D-Ornithine and diaminopimelic acid produced very faint ninhydrin spots corresponding to glutamic acid.

In the case of D-ornithine and N-α-acetylornithine, the *o*-aminobenzaldehyde assay was also done. With N-α-acetylornithine, the assay procedure was modified to that of Südi and Dénes (16). D-Ornithine and N-α-acetylornithine were 8.5% and 1% as active as L-ornithine, respectively.

Inhibition by Various Amino Acids. In the presence of a number of other amino acids, some inhibition of L-ornithine transamination was observed (Table III). Lysine, proline, and glutamine were unusual in having no effect. Leucine, isoleucine, and valine were the most inhibitory giving inhibitions up to 74%.

Determination of Molecular Weight by Gel Filtration. The procedure was that developed by Andrews (1). A column of Sephadex G-75 (3 × 55 cm) was equilibrated with standard buffer before each standard protein or enzyme sample was put on. β-Dextran was used to measure the void volume (V_0) of the column. Proteins of known molecular weight were placed on the column and their elution volume determined. The positions that gave a maximum reading at 280 nm and maximum enzyme activity were adopted as elution volume (V_e) for standards and enzyme, respectively. The ratio of V_e/V_0 of each standard was plotted against log molecular weight to construct the standard curve (Fig. 2). The molecular weight of the enzyme obtained by this method was 48,000 daltons.

Table II. *Relative Activity of Various Keto Acids as Substrates*

All keto acids were used at the same concentration as α-ketoglutarate in the standard reaction mixture. The assay was carried out according to the standard procedure. In the case of glyoxylate, a heat-inactivated enzyme control was included so as to measure the significant nonenzymatic transamination which occurs with this substrate. The difference between the heat-treated control and the active enzyme reaction mixture was used as a measure of the enzymatic conversion. Step 8 enzyme (50 munits) was used in each case.

Keto Acid Used	Relative Activity
	%
α-Ketoglutarate	100
Glyoxylate	2
Pyruvate	4
Oxaloacetate	0
α-Ketoadipate	4

Table III. *Inhibition of Enzyme Activity by Different Amino Acids*

Each of the listed amino acids was added to individual reaction mixtures at a final concentration of 0.1 M, twice that of the L-ornithine present. The composition of the reaction mixture otherwise was that of the standard assay described under "Materials and Methods." Fifty munits of step 8 enzyme were utilized in each reaction vessel.

Amino Acids	Relative Activity
	%
Control	100
+ Leu	26
+ Ile	34
+ Glu	74
+ Gln	95
+ γ-Aminobutyric acid	68
+ Arg	86
+ Pro	97
+ Val	34
+ Diaminopimelic acid	58
+ δ-Amino valeric acid	63
+ D-Orn	82
+ N-α-acetylornithine	82
+ Lys	100

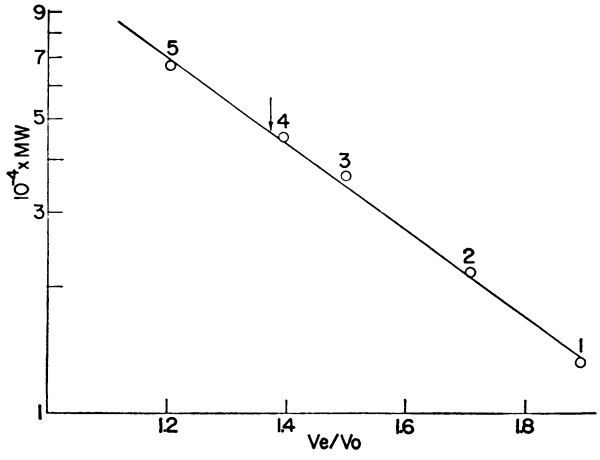

FIG. 2. Determination of the molecular weight of the enzyme by use of a calibrated Sephadex G-75 column using β-dextran to determine V_o. Protein standards used: (1) Cyt *c* (mol wt 13,370); (2) soybean trypsin inhibitor (mol wt 21,500); (3) β-lactoglobulin (mol wt 36,500); (4) ovalbumin (mol wt 45,000); (5) BSA (mol wt 67,000). Eluting solvent was the standard buffer at a flow rate of 0.5 ml/min under constant head pressure and 3-ml fractions collected.

Effect of Exogenous Pyridoxal-P on Enzyme Activity. Added pyridoxal-P stimulated the enzyme activity to some degree at every stage of enzyme purification. However, only after the protamine sulfate step was the stimulation by added pyridoxal-P greater than 50%. The stimulation by exogenous pyridoxal-P did not increase as purification progressed.

Efforts to demonstrate the unequivocal presence or absence of the cofactor pyridoxal-P were inconclusive. It was not possible, by dialysis, to resolve the protein into an apoenzyme completely dependent on added pyridoxal-P. Added L-cysteine had no influence on the activity. After hydrolysis of the enzyme by the proteolytic enzyme pronase, the chemical assay method of Wada and Snell (19) gave a value for pyridoxal-P equivalent to 1 mole of cofactor per 48,000 g of protein.

Inhibitor Studies. The effect of inhibitors which react with carbonyl groups and particularly with pyridoxal-P was investigated. The customary reagents used for such studies are hydroxylamine, CN^-, and isonicotinic acid hydrazide. All demonstrated an inhibitory effect on the enzyme activity. Hydroxylamine was the most effective inhibitor. At 0.12 μM final concentration it produced 52% inhibition which increased to well over 90% at 12.5 μM. The results are summarized in Table IV.

DISCUSSION

Results of the present study allow the comparison of a highly purified plant L-ornithine:2-oxoacid aminotransferase with the same enzyme from a number of other sources. The K*m* values are quite similar to most of the other enzymes from different origins. Strecker (15) reported a considerably lower value for α-ketoglutarate (0.28 mM) with a rat liver enzyme. However, Splittstoesser and Fowden (14) reported much higher values for α-ketoglutarate with enzymes from *Cucurbita maxima*. The pH optimum of this enzyme is also quite similar to values obtained from other organisms. The specific activity of the purest fraction of 2.5 compares not too unfavorably with that of 16.5 of the crystalline transaminase from rat tissue (7).

The present enzyme is very specific toward L-ornithine and α-ketoglutarate as substrates, as are the enzymes from *Neurospora* (18), rat liver (19), and mung bean seedlings (2). D-Ornithine did give a significant activity compared to L-ornithine, but this could be attributed to the possibility that there was a trace of L-ornithine in the commercial D-ornithine preparation, or that some racemase activity might exist in the enzyme preparation. The finding that leucine, isoleucine, and valine were the strongest inhibitors among amino acids for the enzyme is also in good agreement with results obtained with the *Chlamydomonas* enzyme (17). Glutamic acid was slightly inhibitory and did not activate the enzyme as observed by Jenkins and Tsai (4) with a pig kidney enzyme. The stabilization of the enzyme by the presence of dithioerythritol suggests that −SH groups of the enzyme are important.

The molecular weight of 48,000 is much smaller than those previously reported from animal sources. Molecular weights of the enzyme from rat liver (10) and pig kidney (4) were reported to be 132,000 and 248,000, respectively.

The enzyme was inhibited by carbonyl reagents such as hydroxylamine and cyanide, in accord with all aminotransferases so far studied. In those enzymes sudied in detail, pyridoxal-P has invariably been shown to be the coenzyme. Four moles of pyridoxal-P per mole of protein have been found for the homogeneous L-ornithine-aminotransferase from pig kidney (4). Attempts to establish a pyridoxal-P requirement for the present enzyme have yielded equivocal results. The following evidence favors such a requirement for this enzyme. (*a*) Exogenous pyridoxal-P stimulates the enzyme activity. (*b*) The cofactor stabilizes the enzyme activity against extensive dialysis. (*c*) After pronase hydrolysis a positive result was obtained by colorimetric assay for the presence of the cofactor. The enzyme could not be made completely dependent on the addition of pyridoxal-P and the stimulation by exogenous pyridoxal-P did not increase with increasing enzyme purification. Stimulation of enzyme activity by added pyridoxal-P as well as its stabilizing properties could be a result of an indirect effect on the enzyme such as a conformational change when the cofactor is nonspecifically bound to the enzyme protein.

Table IV. *Effects of Common Pyridoxal-P Inhibitors*

Twenty micrograms of step 8 enzyme were incubated with the inhibitor for 10 min at room temperature prior to the addition of the other reagents. Because of a high blank in the presence of isonicotinic hydrazide, a heat-inactivated control was used to correct for nonenzymatically produced absorbance.

Inhibitor	Final Concentration	Relative Activity
	mM	%
None		100
Hydroxylamine	1.2×10^{-4}	48
	2.5×10^{-4}	31
	5×10^{-4}	15
	125×10^{-4}	2
NaCN	0.25	66
	1.25	54
	5.0	28
	10	9
Isonicotinic hydrazide	2.5	42
L-Cysteine	2.5	97
	5.0	91

It will be necessary to obtain a more homogeneous preparation and subject this to sensitive chemical and microbiological assays before this point can be definitively settled.

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