Plant Physiol. (1975) 55, 975–977

In Vitro Synthesis of Ureidohomoserine by an Enzyme from Jack Bean (*Canavalia ensiformis*) Leaves

Received for publication December 9, 1974 and in revised form February 19, 1975

THOMAS DENNY O'NEAL
Department of Biology, Rensselaer Polytechnic Institute, Troy, New York 12181

ABSTRACT

An enzyme was extensively purified from jack bean leaves (*Canavalia ensiformis* L.) which produced *o*-ureidohomoserine from L-canaline and carbamyl phosphate. The most highly purified preparations catalyzed both this reaction and citrulline synthesis from ornithine and carbamyl phosphate, and the ratio of the two activities remained nearly constant during purification. When hydrated jack bean seeds were the enzyme source, ornithine carbamyltransferase (EC 2.1.3.3) activity was high but synthesis of ureidohomoserine was barely detectable. Both ornithine carbamyltransferase and the ureidohomoserine synthesizing enzyme had similar Km values for carbamyl phosphate. The purification data suggest that one enzyme may catalyze both reactions in jack bean leaves.

Canavanine, an analog of arginine, is an abundant constituent of the species in the subfamily *Papilinoideae* of the family Leguminosae (1). Although canavanine was first detected in jack bean (*Canavalia ensiformis* L.) seeds in 1929 (6) and has since been the subject of numerous studies dealing with its distribution and action as an antimetabolite in various organisms, the biosynthetic pathway involved in its synthesis remains unknown. A logical hypothesis is that it is synthesized by reactions similar to those involved in arginine biosynthesis. Canavanine appears to be degraded to canaline and urea by the same enzyme that degrades arginine to ornithine and urea (2); *i.e.*, arginase (EC 3.5.3.1). Both arginine and canavanine can condense with fumarate to form arginosuccinate and canavinosuccinate, respectively (11). It seemed likely that, like ornithine, canaline could condense with carbamyl phosphate in a reaction similar to that catalyzed by ornithine carbamyltransferase (EC 2.1.3.3), yielding *o*-ureidohomoserine.

MATERIALS AND METHODS

With the exception of DL-*o*-ureidohomoserine, biochemicals were obtained from Sigma Chemical Co. Hypatite-C was purchased from Clarkson Chemical Co., Williamsport, Pa.

Protein concentration was determined by the procedure of Lowry *et al.* (7), using a BSA standard. Disc gel electrophoresis was performed by the procedure of Davis (3) using 7.5% crosslinkage. Duplicate gel samples were not stained, and were sectioned to correlate enzyme activity with protein bands on the stained gel. Continuous flow electrophoresis was performed on a Brinkmann Model FF electrophoretic separator at pH 8, operated at 75 amp and 2200 v.

Identification of the products employed the use of one-dimensional TLC on Silica Gel G plates, using phenol-H_2O (77:23, v/v) or secbutanol-16% NH_4OH, (3:1, v/v) as the solvents. Citrulline and ureidohomoserine were located by spraying with ninhydrin or Ehrlich's reagent (2% w/v *p*-dimethylaminobenzaldehyde in 5% HCl). Both ureidohomoserine and citrulline had approximately the same R_F value in either solvent, and reacted with both reagents.

The enzymes were assayed at 37 C for 10 min. The assay contained enzyme, 3 mM L-ornithine or L-canaline, 6 mM carbamyl phosphate, 33 mM tris-HCl buffer, pH 7.8 (for CCT[1] or pH 8.3 (for OCT) and H_2O to a final volume of 0.60 ml. The blanks contained no enzyme or no amino acid. Reactions were terminated with 0.1 ml of 3 N HCl, and H_2O was added to a final volume of 1 ml. Turbidity, when present, was removed by centrifuging for 10 min at 12,000*g*. The tubes were kept in crushed ice until assayed.

Both UHS and citrulline were assayed by the procedure of Hunningshake (4), modified slightly. To each cold 1-ml sample above, 1 ml of a cold, freshly prepared solution containing 1.5% diacetyl monoxime and 0.1% semidine was added. After brief mixing, 0.50 ml of cold 75% (v/v) H_2SO_4 was added, followed immediately by thorough mixing on a Vortex mixer. For ureidohomoserine assays, the tubes were heated for 5 min in a 90 C water bath, placed in a test tube rack at room temperature for 5 to 8 min, and then 1 ml of 99% H_2SO_4 containing 10 mM $FeCl_3$ was added, taking care to direct the stream to the center of the tube, followed immediately by thorough mixing. For citrulline assays the tubes were heated for 9 min at 90 C instead of 5 min. UHS gave a ruby red color within 1.5 to 2.5 min of heating, although the color was enhanced by the H_2SO_4-$FeCl_3$ reagent, whereas little color development occurred where citrulline was assayed until the addition of this reagent. The wavelength of greatest absorbance for UHS was 544 to 545 nm, compared to 550 nm for citrulline. The absorbance of the solutions were read 15 to 60 min after the H_2SO_4-$FeCl_3$ reagent was added (the tubes were kept at room temperature). Citrulline and UHS standards gave linear plots up to at least 0.4 μmole. One unit of enzyme activity is defined here as that producing 1 μmole of product (UHS or citrulline)/min at 37 C.

Enzyme Purification. An acetone powder was prepared from 11- to 16-week-old jack bean (*Canavalia ensiformis* L.) leaves (greenhouse-grown) by homogenizing the leaves 1 min in a Waring Blendor at high speed with 8 vol of −40 C redistilled acetone containing 14 mM mercaptoethanol. The filter cake was resuspended in 4 vol of the same acetone-mercaptoethanol solution (−40 C) and blended 1 min at medium speed, followed

[1] Abbreviations: CCT: canaline carbamyltransferase; UHS: ureidohomoserine; OCT: ornithine carbamyltransferase.

Table I. *Purification of Enzyme Catalyzing Ureidohomoserine Synthesis from 15-week-old Jack Bean Leaves*

Fractionation Step	Total Protein	Activity			
		Total	Specific activity	UHS/CIT[1]	Purification
	mg	*units*	*units/mg protein*		*fold*
1. Acetone powder	1395	1526	1.09	0.90	1
2. $(NH_4)_2SO_4$ (45-64%)	298	809	2.71	0.80	2.5
3. Heat (62C)	183	799	4.36	1.0	4
4. $(NH_4)_2SO_4$ elution					
5. G-150 Sephadex	10.7	346	32.3	1.3	29
6. Hydroxylapatite	0.8	146	182.5	1.1	167

[1] Ratio of ureidohomoserine synthetic activity to citrulline synthetic activity (ornithine carbamyltransferase).

by filtration. The filter cake was washed with 2 volumes of −40 C acetone and after further drying under vacuum (at 2–4 C) the powder was removed and spread evenly on paper and allowed to dry at room temperature for several hours, after which it was stored at −20 C.

Twenty grams of the acetone powder were added to 360 ml of 25 mM phosphate buffer, pH 7.1, containing 4 mM mercaptoethanol and 1 mM EDTA. All steps (except the heat step) were performed at 0 to 4 C. After stirring mechanically for 20 to 25 min, the viscous solution was centrifuged for 15 min at 22,000*g*. The supernatant was filtered through three layers of cheesecloth to remove suspended matter. Solid ammonium sulfate was slowly added to 300 ml of the supernatant (4.7 mg protein/ml) to 45% saturation. After 15 min, the solution was centrifuged 15 min at 22,000*g* and the precipitate discarded. The supernatant was brought to 64% saturation with solid ammonium sulfate, and after 10 min was centrifuged as before. The pellet was dissolved in 25 ml of 25 mM phosphate buffer, pH 7.1, containing 2 mM mercaptoethanol and 1 mM EDTA. This solution was placed in a 125-ml thin walled Erlenmeyer flask and the flask was then placed in a 73 to 74 C water bath. The flask was swirled constantly until the temperature of the solution rose to 62 C, at which point the flask was removed and allowed to sit at room temperature for 1 min, and was then immersed in crushed ice. Ten minutes later the solution was centrifuged at 28,000*g* for 8 min. Ammonium sulfate was added to the supernatant to 64% saturation, and after 10 min was centrifuged, discarding the supernatant. Five milliliters of a 55% solution of ammonium sulfate (pH 7.4) were stirred into the pellet and after 15 min were centrifuged for 10 min, and the supernatant was discarded. The pellet was extracted with 2 ml of 50% ammonium sulfate for 15 min, followed by centrifugation. The supernatant was saved, and the pellet was extracted in 2 ml of 45% ammonium sulfate for 15 min. Following centrifugation, this supernatant was added to the 50% supernatant. These pooled extracts were applied to a 2.5 × 39 cm column of G-150 Sephadex equilibrated in 10 mM phosphate buffer, pH 7.1, containing 0.4 mM EDTA. Fractions of approximately 4.1 ml were collected, where the flow rate was 28 to 31 ml/hr. The six fractions of highest specific activity were pooled, and represented about 60% of the total units eluting from the column. The combined fractions were added to a 2.5 × 6 cm column of Hypatite-C (hydroxylapatite), and, after the solution had entered the bed, 65 ml of 0.1 M phosphate buffer, pH 6.8, containing 2 mM mercaptoethanol were applied to the column. The flow rate varied from 45 to 80 ml/hr, and 5-ml fractions were collected. After the first elution, 90 ml of 0.15 M phosphate buffer (pH 6.8) were added, and the enzyme began emerging in a sharp peak corresponding to the 0.15 M buffer, but considerable tailing of the enzyme occurred, with specific activity remaining fairly constant. Fourteen of the fractions of highest specific activity were pooled, and these contained about 75% of the total applied units.

Following the Hypatite-C chromatography the enzyme had been purified 167-fold, as summarized in Table I. The ratio of absorbance at 280:260 nm rose from 0.90 to 0.95 in step I to 1.6 to 1.75 after the Sephadex chromatography.

RESULTS

Stability of Enzyme. The most highly purified preparations lost about 12% of either activity after 8 days at 2 to 4 C, and both stage 3 and 5 enzyme (Table I) were heat stable, losing less than 25% of either activity after heating to 60 to 62 C for 1 min (protein concentration ≥ 1 mg/ml).

Specificity of Enzyme. Jack bean seedlings contained an enzyme or enzymes catalyzing the carbamylation (by carbamyl phosphate) of both ornithine and canaline, yielding citrulline and *o*-ureidohomoserine, respectively. High canaline carbamyltransferase and ornithine carbamyltransferase activities also occurred in acetone powder preparations of immature seed pods of jack bean. It was not possible to separate these two activities during purification; in fact, the ratios remained nearly constant (Table I). In the heat step, however, CCT either lost less than 4% of its activity or actually increased up to 15% in activity, while OCT always lost 10 to 25% of its activity. Following preparative electrophoresis on a Brinkmann Model FF apparatus, using enzyme purified 167-fold, 50% of the CCT activity was recovered in four consecutive fractions with a slight migration towards the anode. The ratio of CCT/OCT (3 mM canaline or ornithine in assay) in each of the fractions was 1.20 to 1.25. Electrophoresis in polyacrylamide gels (pH 8.6) was also performed. On the stained gel (70 μg protein) three distinct bands (two major and one minor) were seen. Elution of the protein from an analogous unstained gel showed that both activities were confined to the lowest of the three bands, and the ratio of activities was 1.01. Only about 6% of the activity of either enzymic activity was recovered.

If jack bean seeds were hydrated for 18 to 22 hr in distilled H_2O before extraction, there was very little CCT activity but

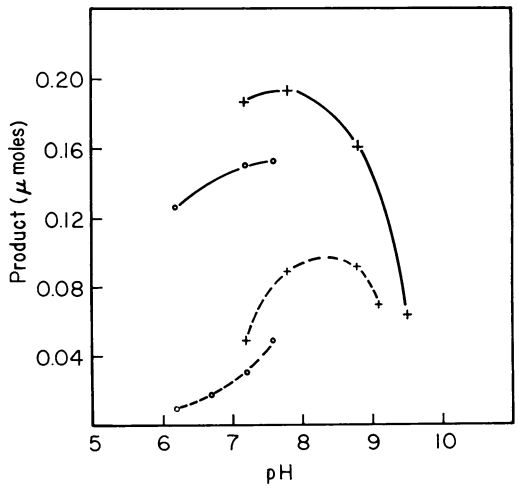

FIG. 1. Determination of the pH optimum for ureidohomoserine and citrulline biosynthesis. Buffer concentrations were 33 mM. The concentration of L-canaline or L-ornithine was 3 mM, and carbamyl phosphate was 4 mM. Tris-HCl buffer, canaline (+——+); phosphate buffer, canaline (○——○); Tris-HCl buffer, ornithine (+-----+); phosphate buffer, ornithine (○-----○). The product was L-citrulline with ornithine and ureidohomoserine with canaline.

high OCT activity. Furthermore, the heat stability of the latter enzyme from this source was quite different (reproducibly) from that of the seedling source. When 45 to 64% ammonium sulfate preparations of the seed homogenate were heated (as described in the purification procedure) 85 to 95% of the OCT activity was lost, compared to 10 to 25% with the seedling enzyme. This was true even though protein concentrations during heating were similar. When young seedlings (12 to 14 days old) were used, the heat step was also very deleterious, causing much inactivation of both activities, again unlike the case with older seedlings. Young seedlings also had much more OCT than CCT.

Linearity. At nearly saturating levels of 12 mM L-canaline, UHS synthesis proceeded linearly for 20 min, and was proportional to enzyme level up to 2 μg of protein (step 6 enzyme).

pH Optimum. The pH optimum for the OCT activity was between 8.2 and 8.6, while that for the CCT activity was between 7.6 to 8 (Fig. 1). The ratio of CCT/OCT at pH 7.8 (tris-HCl) and pH 6.2 (phosphate) was 2.16 and 14.2, respectively.

Effect of Substrate Concentration. The reaction velocities *versus* substrate concentrations all yielded normal hyperbolic plots. The K*m* for L-canaline was 3.8 to 4.1 mM (pH 7.8, 6 mM carbamyl phosphate), while that for L-ornithine was 1.1 mM (pH 8.3, 6 mM carbamyl phosphate). An accurate K*m* for L-canaline could not be determined since that substrate was judged to be only about 80% pure (by TLC). The V_{max} for CCT at nearly saturating (10 mM) levels of L-canaline was from 1.54- to 2.9-fold greater than for OCT (four preparations). The reason for this variation is not known. The K*m* values for carbamyl phosphate using 8 mM L-canaline or 6 mM L-ornithine were 1 mM and 1.1 mM, respectively.

Combinations of 6 mM L-ornithine and 12 mM L-canaline produced no higher absorbance between 545 to 550 nm than either substrate alone (pH 8).

Effect of Sulfhydryl Compounds, Chelators, and Metals. The CCT activity of dialyzed (sulfhydryl-free) enzyme was not significantly affected by 1 to 4 mM mercaptoethanol or 1 mM dithiothreitol, but 4 mM iodoacetate caused 30% inhibition. Appropriate controls were run to correct for the slight effect of sulfhydryl compounds on the colorimetric assay.

EDTA, KCl, and $MgCl_2$ (1 to 5 mM) had no effect, while 2.5 mM $ZnCl_2$ caused 80% inhibition of CCT.

Effect of Analogs and Other Compounds. With 3.33 mM L-canaline, 6 mM carbamyl phosphate, and at pH 7.8, 5 mM N 2-α-acetyl-L-ornithine, L-lysine, or L-arginine caused little or no inhibition of CCT. L-Canavanine (5 mM) or 4.2 mM L-homoserine caused 17% and 39% inhibition, respectively. To test the effect of L-ornithine, advantage was taken of the fact that UHS produces a strong color after heating 2 to 3 min at 90 C (without the H_2SO_4-$FeCl_3$ reagent), while citrulline does not (see "Materials and Methods"). Using this modified assay for UHS, 3 mM L-ornithine caused no inhibition of CCT where the L-canaline concentration was varied between 1 and 4 mM, at pH 7.8. It is also of interest that 1 to 3 mM L-canaline did not inhibit OCT derived from jack bean seeds (L-ornithine was 1.4 mM, pH 8.2).

DISCUSSION

Although an enzyme which catalyzes UHS synthesis efficiently has been isolated and partially purified from jack bean leaves, this does not prove that this pathway is utilized *in vivo*. Warren and Hunt (12) implied that since canaline had never been detected in the free amino acid pool of jack bean it might not, in fact, be synthesized or play a significant role in canavanine biosynthesis. However, not only can canaline be produced from canavanine degradation catalyzed by arginase or a similar enzyme (2) but its presence has recently been reported in jack bean (8) and in *Astragalus sinicus* (5). There are no reports of the presence of UHS in jack bean of any other species, but to my knowledge a search for this compound has not been undertaken. Now that procedures are available for the synthesis of pure L-canaline (9) and L-*o*-ureidohomoserine (10, 13) studies such as the above, coupled with more accurate characterization of the enzyme synthesizing UHS, will be facilitated. An improved method for assay of UHS is now available (10).

As for the mechanism of L-canaline biosynthesis, no data are available. Preliminary studies (O'Neal, D., unpublished data) failed to detect its synthesis in extracts of jack bean leaves (based on detection by TLC) from L-homoserine plus various combinations of Mg^{2+}, ATP, glutamine, asparagine, urea, and ammonia, when assayed at several pH values.

More intensive studies are needed to resolve the question of whether one or more enzymes catalyze both citrulline and UHS synthesis. If there are two distinct enzymes catalyzing these reactions in jack bean leaves, they would have to be very similar in charge and size not to be at least partially separated by the range of techniques employed in this study, but this is, nevertheless, possible.

The fact that extracts from hydrated seeds or young seedlings have an unstable (at 61 C) OCT and synthesize only very low amounts of UHS, while older plants have a stable enzyme(s) efficiently catalyzing both reactions is difficult to interpret without more detailed studies. One interpretation is that one enzyme is modified during jack bean development so that it becomes more stable and less specific, or perhaps a different isoenzyme is produced.

With respect to the pH optimum, it is possible that the much greater decline in activity at pH 6.2 for OCT relative to CCT is due to the relative protonation of the nitrogen atom of canaline *versus* ornithine, since canaline would be (since it is more amide-like) considerably less protonated at pH 6.2 than ornithine (an amine).

Acknowledgments—The author would like to thank Drs. Richard Lowe and Shuh-ji Sheen (University of Kentucky, Lexington, Ky.) who performed the continuous flow electrophoresis and disc gel electrophoresis, respectively. I would also like to thank Dr. Gury Zvilichovsky of the Hebrew University of Jerusalem for his generous gift of L-*o*-ureidohomoserine, which he synthesized in his laboratory (13).

LITERATURE CITED

1. Birdsong, B. A., R. Alston, and B. L. Turner. 1960. Distribution of canavanine in the family *Leguminosae* as related to phylectic groupings. Can. J. Bot. 38: 499–505.
2. Damodaran, M. and K. G. A. Narayanan. 1940. A comparative study of arginase and canavanase. Biochem. J. 34: 1449–1459.
3. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121: 404–427.
4. Hunninghake, D. and S. Grisolia. 1966. A sensitive and convenient micromethod for estimation of urea, citrulline, and carbamyl derivatives. Anal. Biochem. 16: 200–205.
5. Inatoini, H., F. Inugai, and T. Marakami. 1968. Isolierung und Identifizierung von Canalin in den unreifen Samen von *Astragalus sinicus* L. Chem. Pharm. Bull. 16: 2521.
6. Kitagawa, M. and T. Tomiyama. 1929. A new amino compound in the jackbean and a corresponding new enzyme. J. Biochem. 11: 265–271.
7. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.
8. Miersch, J. 1967. Nachweis und isolierung von Canaline (2-Amino-4 aminoxybuttersäure). Naturwissenschaften 54: 169–170.
9. Rosenthal, G. 1973. The preparation and colorimetric analysis of L-canaline. Anal. Biochem. 51: 354–361.
10. Rosenthal, G. 1973. Preparation and colorimetric analysis of *o*-ureido-L-homoserine. Anal. Biochem. 56: 435–439.
11. Walker, J. B. 1953. An enzymatic reaction between canavanine and fumarate. J. Biol. Chem. 204: 139–146.
12. Warren, R. F. and G. E. Hunt. 1971. The biosynthesis of canavanine from $^{14}CO_2$ and its asymmetric labeling in isolated pericarp tissue of *Canavalia ensiformis*. Planta 100: 258–261.
13. Zvilichovsky, G. 1966. Some reactions of aminooxy acids. Synthesis of *o*-ureidohomoserine. Tetrahedron 22: 1445–1451.