Phytochemistry, 1973, Vol. 12, pp. 1565 to 1568. Pergamon Press. Printed in England.

PYRROLINE-5-CARBOXYLATE REDUCTASE FROM *CUCURBITA* COTYLEDONS

SHIRLEY A. SPLITTSTOESSER and WALTER E. SPLITTSTOESSER
Department of Horticulture, University of Illinois, at Urbana-Champaign Urbana, IL 61801, U.S.A.

(*Received* 14 *December* 1972. *Accepted* 21 *December* 1972)

Key Word Index—*Cucurbita maxima*; *C. moschata*; Cucurbitaceae; pumpkin; pyrroline-5-carboxylate reductase; proline.

Abstract—Pyrroline-5-carboxylate reductase, which required reduced pyridine nucleotide and Δ'-pyrroline-5-carboxylate for proline synthesis, was isolated from pumpkin cotyledons. The enzyme was found in the soluble fraction and had a 4·5-fold greater activity with NADH than NADPH. The enzyme was inhibited by NH_2OH, NADP, ATP and slightly by proline. Glutathione or pyridoxal-5-phosphate had little effect on enzyme activity. The enzyme had a pH optimum between 7·0 and 7·6 and was not inhibited by high concentrations of NADH or Δ'-pyrroline-5-carboxylate.

INTRODUCTION

THE RESERVE proteins of pumpkin seeds contain a high percentage of arginine which constitutes one-third of the total nitrogen in the cotyledons.[1,2] During the first 8 days of germination, 75% of the reserve protein is hydrolyzed[3] and 50% of the total nitrogen in the cotyledon is transported to the axis tissue.[2] However, less than 1% of the nitrogen is translocated to the axis tissue as arginine.[1] Instead, arginine is metabolized[4] via arginase[5] to ornithine and urea[2,4] and the urea molecule, which contains 50% of the arginine nitrogen is further metabolized by urease.[2] Much of the resulting ammonia is utilized in the formation of glutamate and its amide[6] which are then transported to the axis tissue.[1] Ornithine is also transported from the cotyledons but a significant part of the ornithine is converted to glutamate and proline, and labelling studies have shown that this conversion occurs readily in a number of plants.[2,7-9] Although this conversion is established in mammals and microorganisms,[10] less is known about these enzymes in plants.[11] Ornithine transaminase is present in pumpkin cotyledons[12] and a pyrroline-5-carboxylate reductase which used NADPH for proline synthesis in the light has been found in tobacco leaves, but no kinetic parameters were determined.[13] This is a report on pyrroline-5-carboxylate reductase (L-proline: NAD(P)-5-oxidoreductase, E.C. 1.5.1.2) from etiolated pumpkin cotyledons.

[1] CHOU, K. H. and SPLITTSTOESSER, W. E. (1972) *Physiol. Plant.* **26**, 110.
[2] LIGNOWSKI, E. M. and SPLITTSTOESSER, W. E. (1971) *Physiol. Plant.* **25**, 225.
[3] SPLITTSTOESSER, W. E. and STEWART, S. A. (1970) *Physiol. Plant.* **23**, 1119.
[4] SPLITTSTOESSER, W. E. (1969) *Plant Physiol.* **44**, 361.
[5] SPLITTSTOESSER, W. E. (1969) *Phytochemistry* **8**, 753.
[6] LIGNOWSKI, E. M., SPLITTSTOESSER, W. E. and CHOU, K. H. (1971) *Plant Cell Physiol.* **12**, 733.
[7] MORRIS, C. J., THOMPSON, J. F. and JOHNSON, C. M. (1969) *Plant Physiol.* **44**, 1023.
[8] MAZELIS, M and FOWDEN, L. (1969) *Phytochemistry* **8**, 801.
[9] NAYLOR, A. W. (1959) *Symp. Soc. Exp. Biol.* **13**, 193.
[10] GREENBERG, D. M. (1961) *Metabolic Pathways*, Vol. 2, p. 191, Academic Press, New York.
[11] FOWDEN, L. (1965) in *Biosynthetic Pathways in Higher Plants* (PRIDHAM, J. B. and SWAIN, T., eds.), p. 87, Academic Press, New York.
[12] SPLITTSTOESSER, W. E. and FOWDEN, L. (1973) *Phytochemistry* **12**, 785.
[13] NOGUCHI, M., KOIWAI, A. and TAMAKI, E. (1966) *Agr. Biol. Chem.* **30**, 452.

RESULTS

Partial Purification

Cotyledons from 7-day-old seedlings were used as the source of the enzyme (Table 1). The crude enzyme fraction often contained less total activity than subsequent fractions and the soluble enzyme was purified 12-fold with a yield of 44%. About 3% of the total enzyme activity was found in the particulate fraction when standard mitochondrial isolation techniques were used. The enzyme was stored at −4° in 0·01 M phosphate buffer (pH 7·4) containing 0·01 M glutathione and 50% glycerol for 2 months, or 1 month in 0·1 M phosphate buffer (pH 7·4) with 10% loss of enzyme activity.

TABLE 1. SUMMARY OF ENZYME PURIFICATION

Enzyme fraction	Total activity (units)	Specific activity (units/mg protein)	Purification (fold over crude)	Yield (%)
Crude	7500	0·09	—	100
Centrifugation	7330	0·19	2·1	98
30–40% $(NH_4)_2SO_4$	4000	0·44	4·9	53
Dialysis & centrifugation	3330	1·08	12·0	44

The reaction mixture and purification procedures were as described in Experimental.

Reaction Requirements

The reaction required Δ′-pyrroline-5-carboxylate, enzyme and a reduced nucleotide. The addition of pyridoxal-5-phosphate, glutathione or EDTA had little effect upon the purified enzyme. However, the undialyzed enzyme was stimulated over 200% with 30 mM EDTA. The enzyme used NADH and NADPH but showed a 4·5-fold greater activity with NADH. With purification, and under storage conditions in which 70% of the total enzyme activity was lost, this 4·5-fold greater activity with NADH over NADPH remained constant. After completion of the enzymatic reaction, the reaction mixture was separated on paper chromatograms and proline was detected with ninhydrin or isatin.

The reaction was slightly reversible at pH 7·4 but was readily reversible at pH 10·3 and required enzyme, NAD and proline. After incubation, a new compound (Δ′-pyrroline-5-carboxylate) was formed which reacted with *o*-aminobenzaldehyde.

Kinetic Parameters

A broad pH optimum for reduction was noted between 7·0 and 7·6 and pH 7·4 was used in the assay. Enzyme activity declined rapidly on both sides of this optimum. The amount of Δ′-pyrroline-5-carboxylate reduced was directly proportional to the amount of enzyme used over a 5-fold range and the rate was proportional with time for 10 min.

The enzyme was not inhibited by high concentrations of NADH or Δ′-pyrroline-5-carboxylate. The reaction showed Michaelis–Menten kinetics and the K_m for NADH was 60 μM and the K_m for Δ′-pyrroline-5-carboxylate was 90 μM.

Effect of Various Inhibitors

A number of compounds inhibited the enzymatic activity (Table 2). NH_4 ion was slightly inhibitory. Heavy metals (Ag and Hg) strongly inhibited the reaction while another sulfhydryl inhibitor, *p*-chloromercuribenzoate, did not inhibit the reaction. Hydroxylamine, an inhibitor of carbonyl functions, strongly inhibited the reaction but the addition of 0·5 mM pyridoxal-5-phosphate had no effect on this inhibition. Glutamate or proline at 20 mM also did not affect activity but high concentrations of proline gave some inhibition. ADP had no effect on activity but similar concentrations of NADP or ATP inhibited the reaction about 40%.

TABLE 2. INHIBITION OF PYRROLINE-5-CARBOXYLATE REDUCTASE BY VARIOUS COMPOUNDS

Inhibitor	Concn (mM)	Activity (% of control)	Inhibitor	Concn (mM)	Activity (% of control)	Inhibitor	Concn (mM)	Activity (% of control)
NH_4Cl	10·0	90	NH_2OH	0·5	36	NAD	1·0	94
$AgNO_3$	0·3	35	NH_2OH	1·0	8	NADP	1·0	57
$HgCl_2$	0·1	44	Proline	50·0	88	ATP	1·0	59

The reaction was carried out as described under Experimental.

DISCUSSION

The results of the present study allow the comparison of a higher plant pyrroline-5-carboxylate reductase with similar enzymes from different organisms. The pumpkin enzyme was soluble, similar to the enzyme from liver[14,15] and microorganisms,[16,17] and in contrast to the enzyme from tobacco leaves which was isolated from the chloroplast fraction.[13]

The enzyme from *Neurospora crassa* had a pH optimum of 6·0,[17] that from *Tetrahymena pyriformis* was pH 9·1[16] while the liver[14,15] and tobacco enzymes[13] were near pH 7·0, similar to the pumpkin enzyme. The *Clostridia*[18] and liver[14,15,19] enzymes had a greater activity with NADH, similar to the pumpkin enzyme, but enzymes from tobacco[13] and *T. pyriformis*[16] were more active with NADPH. There is also a large variation in Michaelis constants for enzymes isolated from various sources. The K_m for NADH for the enzyme from pumpkin was 60 μM, from rat liver 250 μM[14] and from calf liver 840 μM.[15] The K_m for Δ'-pyrroline-5-carboxylate was 90 μM for the enzyme in pumpkin, 1·8 mM in *T. pyriformis*,[16] and about 0·3 mM in liver.[14,15] The inhibition pattern of the pumpkin enzyme was similar to the enzymes isolated from liver[14,15] and tobacco.[13] They require an active sulfhydryl group for activity, are inhibited by NH_2OH, ATP, NADP and proline. The pumpkin enzyme was readily inhibited by heavy metals and ammonia. The inhibition due to the ammonium ion was relieved by dialysis (Table 1).

Proline oxidases isolated from *E. coli*[20] and liver[21,22] were particulate and did not require

[14] SMITH, M. E. and GREENBERG, D. M. (1957) *J. Biol. Chem.* **226,** 317.
[15] PEISACH, J. and STRECKER, H. J. (1962) *J. Biol. Chem.* **237,** 2255.
[16] HILL, D. L. and CHAMBERS, P. (1967) *Biochem. Biophys. Acta* **148,** 435.
[17] YURA, T. and VOGEL, H. J. (1959) *J. Biol. Chem.* **234,** 335.
[18] COSTILOW, R. N. and LAYCOCK, L. (1969) *J. Bacteriol.* **100,** 662.
[19] SMITH, M. E. and GREENBERG, O. E. (1956) *Nature* **177,** 1130.
[20] FRANK, L. and RANHAND, B. (1964) *Arch. Biochem. Biophys.* **107,** 325.
[21] STRECKER, H. J. (1960) *J. Biol. Chem.* **235,** 3218.
[22] JOHNSON, A. B. and STRECKER, H. J. (1962) *J. Biol. Chem.* **237,** 1876.

a pyridine nucleotide. The reverse reaction demonstrated for the pyrroline carboxylate reductase from pumpkin is similar to that shown for the yeast[23] and peanut[24] enzymes, although the peanut enzyme did not produce Δ′-pyrroline-5-carboxylate from proline. In animals, *E. coli*, and probably peanut, proline oxidase is not the same enzyme as pyrroline-5-carboxylate reductase. However, proof that different enzymes in pumpkin catalyze proline formation and oxidation can only be obtained when pure enzymes are obtained and it is shown that the preparation which catalyzes proline formation does not catalyze its oxidation.

EXPERIMENTAL

Plant material. Pumpkin seeds (*Cucurbita maxima* L., cv. King of the Mammoth or *C. moschata* Poir. cv. Dickinson Field) were sown in moist vermiculite in a darkened germinator. After 5–7 days the seed coats were removed from the cotyledons.

Extraction and partial purification of the enzyme. Throughout the extraction and purification procedures, the temperature was maintained near 0°. The crude extract was prepared by grinding 40 cotyledon pairs in an equal vol. of 0·1 M $KHPO_4$, pH 7·4 in a VirTis homogenizer for 2 min. The slurry was passed through 4 layers of cheesecloth and centrifuged at 1000 *g* for 10 min (crude extract). The crude extract was centrifuged at 25 000 *g* for 10 min and fat which floated was removed. The ppt. was discarded as little enzyme activity was found in this fraction when it was isolated in 0·01 M glutathione, 0·25 M sucrose and 0·1 M $KHPO_4$, pH 7·6 (isolation media for mitochondria). The soluble enzyme was precipitated between 30 to 40% $(NH_4)_2SO_4$, dissolved in 0·01 M glutathione, 0·01 M $KHPO_4$ (pH 7·4) and 50% glycerol, dialyzed 3·5 hr against this solution, and then centrifuged at 25 000 *g* for 10 min and the ppt. discarded. A summary of the purification procedure with data from a typical experiment is presented in Table 1.

Assay procedures. The Δ′-pyrroline-5-carboxylate was synthesized as follows:[25] 40 mg of DL-α-amino-Δ-hydroxyvaleric acid (Cyclo Chemical Corp., P.O. Box 71557, Los Angeles, Calif.) and 20 mg of CrO_3 were dissolved in 5 ml of 4 N HCl and maintained at 40° for 18 hr. This mixture was then taken to near dryness at 40° under reduced pressure, dissolved in H_2O, neutralized to pH 7·0 with KOH, centrifuged to remove the precipitated $Cr(OH)_3$ and this process repeated. The supernatant containing the Δ′-pyrroline-5-carboxylate was then stored at 4° at pH 2·0. The pH was adjusted to 7·4 before use. This procedure produces DL-Δ′-pyrroline-5-carboxylate and the results are expressed on the basis of the active L form.[14,15]

The standard reaction mixture contained 25 mM Δ′-pyrroline-5-carboxylate; 100 μM NADH; 200 mM Tris–HCl buffer, pH 7·4; enzyme preparation, 0·1 mg protein to a final vol. of 1·1 ml. The change in absorbance of the reaction mixtures at 340 nm were followed at 23° in a double-beam spectrophotometer equipped with a recorder. The reference cuvette contained all the reactants except Δ′-pyrroline-5-carboxylate. The changes in absorbance were followed and the initial reaction velocities determined. The amount of Δ′-pyrroline-5-carboxylate was determined using a millimolar extinction coefficient of 2·71.[26] One unit of enzyme activity equals an optical density change (at 340 nm) of 0·01 per min under the above conditions. Protein was determined by the biuret method.[27]

Acknowledgements—The authors gratefully acknowledge the aid of Professor L. Fowden, University College, London where this work was initiated while the authors were on study leave. This research was funded in part by the Illinois Agricultural Experiment Station.

[23] LING, C. M. and HEDRICK, L. R. (1964) *J. Bacteriol.* **87**, 1462.
[24] MAZELIS, M. and FOWDEN, L. (1971) *J. Exp. Botany* **70**, 137.
[25] STEWART, C. R. (1967) Ph.D. Thesis, p. 30, Cornell University, Ithaca, New York.
[26] STRECKER, H. J. (1971) in *Methods of Enzymology* (TABOR, H. and TABOR, C. W., eds.), Vol. 17B, p. 251, Academic Press, New York.
[27] LAYNE, E. (1957) in *Methods of Enzymology* (COLOWICK, S. P. and KAPLAN, N. O., eds.), Vol. 3, p. 450, Academic Press, New York.