Phytochemistry, 1974, Vol. 13, pp. 2081 to 2084. Pergamon Press. Printed in England.

Δ^1-PYRROLINE-5-CARBOXYLATE: THE PRODUCT OF PROLINE DEHYDROGENASE FROM *CUCURBITA MOSCHATA* COTYLEDONS

ALEMAR B. RENA* and WALTER E. SPLITTSTOESSER

Department of Horticulture, University of Illinois, Urbana-Champaign, Urbana, IL 61801, U.S.A.

(*Received* 3 *February* 1974)

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

Abstract—The product of oxidation of proline by pumpkin proline dehydrogenase reacted with *o*-aminobenzaldehyde to give a yellow compound that had an absorption spectrum similar to that obtained from chemically synthesized Δ^1-pyrroline-5-carboxylate. The product of the proline dehydrogenase reaction and synthetic Δ^1-pyrroline-5-carboxylate had identical R_f values. Both authentic Δ^1-pyrroline-5-carboxylate and the product of the enzyme gave a pink colour with acid ninhydrin on paper chromatograms and both had identical elution patterns on Dowex 50(H^+) columns. Neither synthetic Δ^1-pyrroline-5-carboxylate nor the product of proline-dehydrogenase produced γ-amino butyrate with hydrogen peroxide.

INTRODUCTION

α-KETO-δ-AMINOVALERATE is the oxidative product of proline in animal tissues[1,2] which is in equilibrium with Δ^1-pyrroline-2-carboxylate.[3] Proline-[^{15}N-^{2}H] is metabolized to glutamate which contains a large percentage of isotope but the nature of the intermediates was not elucidated.[4] A particulate fraction of rabbit kidney or liver oxidized proline via the cytochrome system[5] and glutamic-semialdehyde (which is in equilibrium with Δ^1-pyrroline-5-carboxylate[6]) was the oxidation product. Following this observation, a large number of animal tissues [7–11] and microorganisms[12–15] were shown to metabolize proline

* Present address: Instituto De Ciencias Biologicas, Universidade Federal De Vicosa, Vicosa, Minas Gerais, Brasil.

[1] KREBS, H. A. (1939) *Enzymologia* **7**, 53.
[2] BLANCHARD, M., GREEN, D. E., NOCITO, V. and RATNER, S. (1944) *J. Biol. Chem.* **155**, 421.
[3] MEISTER, A. (1954) *J. Biol. Chem.* **206**, 577.
[4] STETTEN, M. R. and SCHOENHEIMER, R. (1944) *J. Biol. Chem.* **153**, 113.
[5] TAGGART, J. V. and KRAKAUR, R. B. (1949) *J. Biol. Chem.* **177**, 641.
[6] VOGEL, H. J. and DAVIS, B. D. (1952) *J. Am. Chem. Soc.* **74**, 109.
[7] ADAMS, E. and GOLDSTONE, A. (1960) *J. Biol. Chem.* **235**, 3499.
[8] ERECINSKA, M. (1966) *Acta. Biochem. Polon.* **13**, 209.
[9] JOHNSON, A. B. and STRECKER, H. J. (1962) *J. Biol. Chem.* **237**, 1876.
[10] LANG, K. and LANG, H. (1958) *Biochem. Z.* **329**, 577.
[11] LANG, K. and SCHIMID, G. (1951) *Biochem. Z.* **322**, 1.
[12] COSTILOW, R. N. and LAYCOCK, L. (1969) *J. Bact.* **100**, 662.
[13] FRANK, L. and RANHAND, B. (1964) *Arch. Biochem. Biophys.* **107**, 325.
[14] FRANK, L. and RYBICKI, P. (1961) *Arch. Biochem. Biophys.* **95**, 441.
[15] LING, C. M. and HEDRICK, L. R. (1964) *J. Bact.* **87**, 1462.

via Δ^1-pyrroline-5-carboxylate. However, in plants the catabolism of proline is reported to occur via an unidentified intermediate.[16,17]

The data presented in this paper show that Δ^1-pyrroline-5-carboxylate is produced by pumpkin proline dehydrogenase, and that Δ^1-pyrroline-2-carboxylate is not involved.

RESULTS AND DISCUSSION

Δ^1-Pyrroline-5-carboxylate was produced chemically and purified by ion exchange chromatography. It produced a pink colour with ninhydrin[9,15,18] and reacted with *o*-aminobenzaldehyde. On chromatography, followed by reaction with acid ninhydrin, Δ^1-pyrroline-5-carboxylate could be demonstrated. An aliquot of this fraction was treated with H_2O_2 and then chromatographed; no γ-amino butyrate was formed. After treatment with H_2O_2, Δ^1-pyrroline-5-carboxylic acid is not converted to γ-amino butyrate, whereas Δ^1-pyrroline-2-carboxylic acid is quantitatively converted to γ-amino butyrate.[3,15,18] This shows that Δ^1-pyrroline-5-carboxylate was synthesized and not Δ^1-pyrroline-2-carboxylate. Solutions of Δ^1-pyrroline-5-carboxylate are unstable.[15,18] After chromatography and reaction with ninhydrin, purified Δ^1-pyrroline-5-carboxylate exhibited only one pink spot (R_f 0·11) (Table 1). However, 3 days later, two new compounds (R_f 0·06, 0·09) were observed (Table 2) and the quantity of these new compounds increased as the solution aged. Glutamate has an R_f similar to unknown **1** and unknown **2** may be a polymerization product.[19]

TABLE 1. STABILITY OF Δ^1-PYRROLINE-5-CARBOXYLATE IN ACID SOLUTION AT 4°*

Days after purification	Treatment	Compounds present†	R_f
0	$-H_2O_2$	PCA‡	0·11
0	$+H_2O_2$	Unknown **1**§	0·06
3	$-H_2O_2$	Unknown **1**	0·06
		Unknown **2**	0·09
		PCA‡	0·11
3	$+H_2O_2$	Unknown **1**§	0·06
		Unknown **2**	0·09

* The compounds were separated on DF-81 cellulose paper and identified by reaction with acidninhydrin.
† No γ-aminobutyrate was observed.
‡ PCA: Δ^1-Pyrroline-5-carboxylate.
§ Has the same R_f as glutamate.

Pumpkin proline dehydrogenase is NAD-dependent and the amount of proline-[U-^{14}C] oxidized to Δ^1-pyrroline-5-carboxylate in 1 hr was 4% of the initial amount of proline (3·1 μmol). In rat liver mitochondria[9] high initial concentrations of proline (2·2 mM) are converted to Δ^1-pyrroline-5-carboxylate to only a minor extent (5% in 1 hr). However, when the concentration of proline is low (0·3 mM) about 90% of it is oxidized to Δ^1-pyrroline-5-carboxylate in 3 hr. In pumpkin, proline was always poorly oxidized to Δ^1-pyrroline-5-carboxylate, regardless of the concentration used.

A portion of the complete proline dehydrogenase reaction mixture which contained proline-[U-^{14}C] was chromatographed on Dowex 50 (H^+) columns alone or with an aliquot

[16] MAZELIS, M. and CREVELING, R. K. (1972) *Plant Physiol.* **49**, 26 (suppl.).
[17] MAZELIS, M. and FOWDEN, L. (1971) *J. Expt. Botany* **22**, 137.
[18] STRECKER, H. J. (1960) *J. Biol. Chem.* **235**, 2045.
[19] MAUGER, A. B. and WITKOP, B. (1966) *Chem. Rev.* **66**, 47.

of purified synthetic Δ^1-pyrroline-5-carboxylate (Table 2). Group 2 (Fraction 58–78) gave a positive reaction with *o*-aminobenzaldehyde and was identical with Δ^1-pyrroline-5-carboxylate when purified by paper chromatography. Aliquots of the enzymatically produced Δ^1-pyrroline-5-carboxylate (group 2) were applied to DE-81 paper, treated with 0 or 10% H_2O_2 and then chromatographed. Radioactivity was associated with Δ^1-pyrroline-5-carboxylate and an oxidation product (see Table 1) on the chromatogram that was not treated with H_2O_2. After treatment with H_2O_2, some Δ^1-pyrroline-5-carboxylate was found with a corresponding loss in radioactivity of glutamate and the oxidation product. No radioactivity was associated with γ-amino butyrate. According to Meister[3] and Johnson and Strecker,[9] treatment with H_2O_2 converts small amounts of Δ^1-pyrroline-5-carboxylate to glutamate, but most remains unchanged. However, treatment of Δ^1-pyrroline-2-carboxylate with H_2O_2 completely converts this compound to γ-amino butyrate. The data presented show that Δ^1-pyrroline-5-carboxylate is produced from proline by pumpkin proline dehydrogenase, an observation different from that made with peanut.[17]

TABLE 2. CO-CHROMATOGRAPHY OF SYNTHETIC AND ENZYMATICALLY PRODUCED Δ^1-PYRROLINE-5-CARBOXYLATE

Group no.	Fractions* (tube no.)	cpm × 10^{-3} recovered†	Reaction with *o*-amino-benzaldehyde (A_{443})
1	40 52	20	0·051
2	58–78	165	0·495
3	84–125	3000	0·002

* 7·5-ml Fractions were collected from a Dowex 50 (H^+) column.

† The enzymatic reaction mixture contained 3 μmol of L-proline-^{12}C, 1·8 μCi of L-proline -[U-^{14}C], 0·9 μmol NAD, 500 μg of protein (proline dehydrogenase) and 0·1 M CO_3^--HCO_3^- buffer, pH 10·3, in a final volume of 300 μl.

o-Aminobenzaldehyde was reacted with either synthetic or enzymatically-produced Δ^1-pyrroline-5-carboxylate and the absorption spectra of the yellow products determined. Absorption maxima were observed at 440 and 292 nm. Strecker[18] found absorption maxima at 430 nm, 292 and 232 nm. The products described here did not absorb at 230 nm. The results also show that Δ^1-pyrroline-5-carboxylate was produced by pumpkin proline dehydrogenase, and that Δ^1-pyrroline-2-carboxylate was not involved.

EXPERIMENTAL

Proline dehydrogenase was isolated from 7-day-old pumpkin (*Cucurbita moschata* Poir, cv. Dickinson Field) cotyledons grown in the dark at 28°. Cotyledons were homogenized in 0·1 M phosphate buffer, pH 7·6. The homogenate was filtered through cheesecloth and centrifuged at 31 000 *g* for 15 min. $(NH_4)_2SO_4$ was added to the supernatant to 70% saturation. The suspension was cooled at 0° for 30 min, centrifuged at 31 000 *g* for 15 min, the pellet dissolved in 0·1 M phosphate buffer, pH 7·6, and used as the enzyme source. Δ^1-Pyrroline-5-carboxylate-[^{14}C] was produced by reacting 3 μmol of *L*-proline-[^{12}C], 1·8 μCi *L*-proline-[U-^{14}C] (185 mCi/mmol), 0·9 μmol NAD, proline-dehydrogenase and 0·1 M carbonate-bicarbonate buffer, pH 10·3, in a final vol. of 300 μl. After 1 hr incubation at 30° the reaction was terminated by the addition of 300 μl of EtOH and the precipitated protein removed by centrifugation at 31 000 *g*. Δ^1-Pyrroline-5-carboxylate was synthesized by the method of Jones and Broquist.[20] 8 mg of α-amino-δ-hydroxy-valerate and 40 mg of CrO_3 were dissolved in 10 ml of 4 M HCl and maintained at 40° for 16 hr. The reaction mixture was taken to dryness under vacuum at 40° until most of the HCl was removed. The residue was dissolved in H_2O, neutralized to pH 7 with KOH, and the $Cr(OH)_3$ pt. removed by centrifugation. The supernatant containing Δ^1-pyrroline-5-carboxylate was stored at 4°, at pH 1·5. Δ^1-Pyrroline-5-carboxylate was purified by addition to 0·9 × 50-cm columns of Dowex-50 (H^+) resin at 2°.

[20] JONES, E. E. and BROQUIST, H. P. (1965) *J. Biol. Chem.* **240,** 2531.

The column was washed with 30 ml H_2O and 55 ml 0·1 M HCl and the liquid discarded. Δ^1-Pyrroline-5-carboxylate was then eluted with 0·5 M HCl and 7·5-ml fractions were collected. Δ^1-Pyrroline-5-carboxylate was identified by the colour reaction (pink) with acid ninhydrin[18] and its reaction with *o*-aminobenzaldehyde.[3] Δ^1-Pyrroline-5-carboxylate was applied to Whatman DEAE-cellulose paper (DE-81) and treated with 20 μl of 10% H_2O_2 and the liquid subsequently evaporated. Chromatograms were developed with H_2O for 2 hr. This chromatography separated glu, pro, γ-amino butyrate and Δ^1-pyrroline-5-carboxylate. Radioactivity was determined with a scintillation spectrometer or with a radiochromatogram scanner.

Acknowledgements—This research was funded by the Illinois Agricultural Experiment Station, the United States' Agency for International Development and the Federal University of Vicosa.