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PROLINE DEHYDROGENASE AND PYRROLINE-5-CARBOXYLATE REDUCTASE FROM PUMPKIN COTYLEDONS

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

Abstract—Activity of proline dehydrogenase and pyrroline-5-carboxylate reductase was greatest after 5 and 7 days germination in green and etiolated cotyledons respectively of pumpkin (*Cucurbita moschata* Poir. cv. Dickinson Field). The ratio of pyrroline-5-carboxylate reductase to proline dehydrogenase activity was constant throughout germination. Both enzymes were purified 30-fold but the ratio pyrroline-5-carboxylate reductase–proline dehydrogenase activity was constant throughout purification. However, this ratio decreased with storage, especially in purified preparations. Both enzymes were stable at high temperature and the ratio pyrroline-5-carboxylate reductase–proline dehydrogenase remained unchanged on heating. Proline dehydrogenase and pyrroline-5-carboxylate reductase were inhibited by sodium bisulfite and cysteine. ATP, ADP and NADP caused inhibition of both enzymes. Proline dehydrogenase utilized NAD but not NADP. Pyrroline-5-carboxylate reductase had a 2·5-fold greater activity with NADH than NADPH. Most of the data presented suggest that proline dehydrogenase and pyrroline-5-carboxylate reductase activities occur on the same protein molecule.

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]. Most of this arginine is metabolized to urea and ornithine [3]. Some ornithine is transported from the cotyledons [1] but a significant portion is converted to PCA† via ornithine transaminase [4]. In pumpkin, PCA is metabolized to proline by PCA-reductase [5]. This enzyme has been described in *Neurospora* [6], liver [7, 8] insects [9] and tobacco [10, 11].

The degradation of proline is accomplished by proline dehydrogenase to produce NADH and PCA [12] in pumpkin [13] and wheat [14]. In animals [7, 15] and microorganisms [16–19], the degradation of proline via PCA is accomplished in the mitochondria, and NAD is not required.

The reaction catalyzed by PCA reductase is reported to be irreversible [14, 15]. However, proline dehydrogenase and PCA reductase may not be separate entities in all organisms, particularly plants. Evidence is presented here that proline dehydrogenase and PCA reductase from pumpkin have many properties in common, and that their enzyme activities may be located on the same entity.

RESULTS AND DISCUSSION

Changes in activity during germination

Over 90% of the proline dehydrogenase and PCA reductase activity were found in the soluble fraction. Both proline dehydrogenase and PCA reductase had an activity maximum after 5 days of germination in green tissues and after 7 days in

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† Abbreviations: PCA-Δ'-pyrroline-5-carboxylate: ASD-enzyme precipitated with ammonium sulfate, dialyzed: ASND-ASD not-dialyzed.

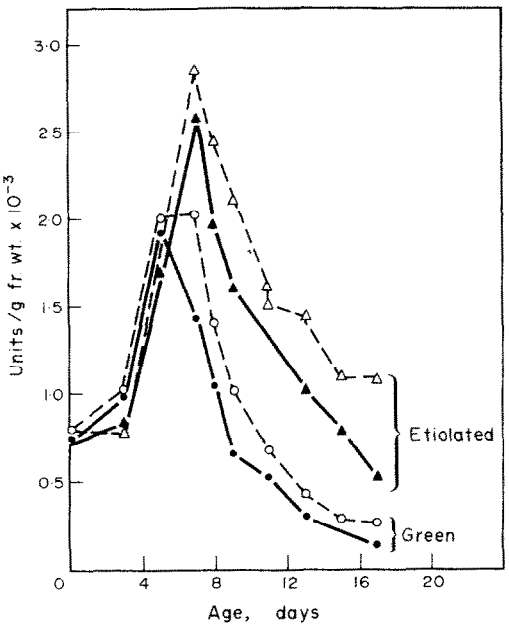

Fig. 1. Changes in soluble proline dehydrogenase and PCA reductase activity in pumpkin cotyledons during germination. (△, ▲) Cotyledons grown in the dark; (○, ●) cotyledons grown in the light; (——) proline dehydrogenase; (– – –) PCA reductase.

etiolated tissues (Fig. 1). The activities of both enzymes followed a similar pattern and were higher in etiolated than in green tissue after 5 days of germination. In pumpkin cotyledons a number of enzymes involved in amino acid metabolism exhibit a similar pattern [2–4]. The ratio of PCA reductase activity to proline dehydrogenase activity remained constant throughout germination.

Changes in activity with pH

PCA reductase showed maximal activity at pH 6·5 and this activity diminished gradually on both sides of the activity optimum with half maximal activity at pH 8. This pH optimum is similar to enzymes obtained from liver [8], insects [9] and tobacco [10, 11]. However, this pH optimum differs from that obtained for enzymes from beef liver [7] and the unpurified enzyme from pumpkin cotyledons [5]. pH 10·3 has been used for the determination of activity of proline dehydrogenases from peanut [20], wheat [14] and algae [21]. In the present study the maximum activity of proline dehydrogenase occurred between pH 9·8 and 10·4. On the alkaline side of the pH optimum, the enzyme activity diminished gradually, while on the acid side, the activity dropped off sharply with half maximal activity at pH 9·2. On treatment at 0° for 10 min at pH 5·5, 32% of the enzyme activity was lost, while at pH 5·0, 96% of the original enzyme activity was lost. However the ratio PCA reductase/proline dehydrogenase remained unchanged. PCA reductase from liver [8] lost activity when precipitated at pH 6·0.

Table 1. Purification of proline dehydrogenase and PCA reductase

Fraction	Proline dehydrogenase			PCA reductase			
	Sp. act. (units/mg protein)	Purification (fold)	Yield (%)	Sp. act. (units per mg protein)	Purification (fold)	Yield (%)	Ratio reductase dehydrogenase
Crude	46	1·0	100	65	1·0	100	1·4
Heated 60°/15 min	88	1·9	104	144	2·2	120	1·6
ASND	609	13·2	114	940	14·4	123	1·5
ASD	670	14·6	121	906	13·9	113	1·4
Sephadex G-100	984	21·4	115	1568	25·5	136	1·6
DEAE	1380	30·0	25	2070	30·0	26	1·5

Partial purification

Both proline dehydrogenase and PCA reductase were relatively stable under a number of conditions used to purify the enzymes (Table 1). An increase in enzyme activity occurred during all steps of purification except fractionation on DEAE columns. Possibly an inhibitor(s) of the enzymes was removed during purification as occurred during purification of PCA reductase from rat liver [22]. Both enzymes were eluted from the Sephadex G-100 column immediately after the void volume and were found in a single activity maximum (Table 1). This indicates that their MW is above 100000. Similar MWs were obtained for proline dehydrogenase from peanut [20], wheat [14] and algae [21]. The MW of proline dehydrogenase of PCA reductase from other tissues has not been reported. When the enzymes were further purified on DEAE-cellulose columns, no enzymatic activity was found in other protein fractions. The fractions containing the enzymatic activity were combined and the enzymes precipitated with ammonium sulfate. More than 75% of the enzymatic activity was lost during this procedure, although the enzymes were purified 30-fold. PCA reductase from liver [7, 8] and tobacco [10] also lost activity on DEAE-cellulose columns.

Table 2. Changes in the ratio PCA reductase–proline dehydrogenase with purification and time after isolation

Enzyme fraction	Days after isolation at −10°	Proline dehydrogenase (units)	PCA reductase (units)	Ratio reductase–dehydrogenase
ASND	3	2440	3760	1·5
	55	2010	2080	1·0
Sephadex	3	980	1570	1·6
G-100	55	210	170	0·8
Sephadex	3	320	470	1·5
G-100ASD	55	170	140	0·8
DEAE	3	1380	2070	1·5
	55	900	300	0·3

Table 3. Effect of SH protective agents on the activity of proline dehydrogenase and PCA reductase

Protective agent	Concentration (mM)	Relative activity: Proline dehydrogenase	Relative activity: PCA reductase
None	—	100	100
$NaHSO_3$	0·5	—	41
	1·0	62	12
	10·0	0	—
Glutathione	1·0	96	105
	10·0	59	107
Cysteine	0·1	115	112
	1·0	92	87
	10·0	51	23

The ratio of PCA reductase to proline dehydrogenase indicates how stable one enzyme is in relation to the other enzyme with changing conditions. This ratio was fairly constant during purification (1·4–1·6) when all fractions were analyzed within 3 days of isolation (Table 1). However, with time, the ratio decreased in all cases and the most purified enzymes experienced the most dramatic change (Table 2). Neither PCA reductase nor proline dehydrogenase lost appreciable activity for at least 7 days at 3° in 0·1 M sodium phosphate buffer pH 7·6, containing 10% $(NH_4)_2SO_4$. However, without ammonium sulfate both proline dehydrogenase and PCA reductase lost activity with time, and at different stages of purification, and this loss was not identical for both enzymes (Table 2).

Proline dehydrogenase and PCA reductase were stable at relatively high temperatures and did not lose activity when heated at 65° for 15 min. Heating at higher temperatures and/or longer times reduced enzyme activity of both enzymes but the ratio PCA reductase–proline dehydrogenase remained unchanged. Addition of the substrates either proline or NAD before the heat treatment decreased the stability of both enzymes, and the addition of both proline and NAD decreased enzyme stability during heating still further. However, the ratio of the enzymes activities remained unchanged.

Inhibitory effects of various compounds

Cysteine, bisulfite and glutathione have a stimulating effect on thiol enzymes by keeping their SH groups in the reduced form. Cysteine and $NaHSO_3$ were very inhibitory to proline dehydrogenase and especially to PCA reductase (Table 3). Glutathione also inhibited proline dehydrogenase but had no effect on PCA reductase even at high concentrations. Rather, glutathione stimulated the PCA reductase activity. Peanut proline dehydrogenase [20] was also inhibited by bisulfite and dithiothreitol, but glutathione was not inhibitory. It was suggested that the peanut enzyme requires disulfide bonds to maintain the proper molecular conformation for activity [20]. It could be postulated that the lack of inhibition of PCA reductase by glutathione was due to its relatively large MW. However, pumpkin proline-dehydrogenase was inhibited by glutathione although a relatively high concentration was required. Heavy metals, *p*-chloromercuribenzoate and iodoacetate inhibited proline dehydrogenase at high concentrations (Table 4). PCA reductase was slightly inhibited by Ag^+ which also inhibited proline dehydrogenase 44%. These results also suggest the SH groups are not required for activity by either enzyme. Rather, disulfide bonds are required. With the exception of the liver PCA reductase [22], the small inhibitions reported in the literature [7, 8, 10, 11] were the consequence of high inhibitor concentrations. Peisach and Strecker [8] concluded that inhibition by

Table 4. Effect of various compounds upon activity of proline dehydrogenase and PCA reductase

Compound	Concentration (mM)	Relative activity: Proline dehydrogenase	Relative activity: PCA reductase
p-chloromercuribenzoate	1·0	70	99
Iodoacetate	1·0	90	102
$ZnCl_2$	0·2	—	94*
	1·0	86	—
$HgCl_2$	0·3	93	40
$CuSO_4$	0·2	105	106*
$AgNO_3$	0·7	56	90
NH_2OH	1·0	93	16
$(NH_4)_2SO_4$	10·0	74	103
ATP	2·0	78	61
ADP	3·0	86	80
L-Proline	20·0	100	74

* 50 mM Tris–Cl, pH 7·2 was used; 0·1 M sodium phosphate buffer, pH 7·2, was used in all other determinations of PCA reductase. The reaction was carried out as described under Experimental.

thiol reagents was insufficient proof to consider SH groups as part of the active site of the PCA reductase in calf liver.

Hydroxylamine, an inhibitor of carbonyl functions, strongly inhibited PCA reductase activity, but had no effect on proline dehydrogenase activity. The inhibition of PCA reductase activity by hydroxylamine was previously observed in liver [8] and in pumpkin cotyledons [5]. Ammonium sulfate inhibited the proline dehydrogenase reaction but had no effect on PCA reductase activity. Both enzymes were inhibited by ATP and ADP. ATP and ADP were competitive inhibitors of PCA reductase in liver [22] and it was suggested that ATP and ADP compete with the point of attachment of both adenine and pyrophosphate of NADH to the enzyme molecule. L-proline inhibited PCA reductase activity by 25% but concentrations as high as 70 mM had no effect on the proline dehydrogenase reaction (Table 4). Proline also inhibited liver proline dehydrogenase [7, 8] but the enzyme from tobacco [10] was not inhibited.

Effects of various pyridine nucleotides

Proline dehydrogenase from pumpkin, like the *Chlorella* enzyme [21], used NAD, andNADP would not substitute for NAD (Table 5). NADP is a non-competitive inhibitor of the pumpkin proline dehydrogenase [13] (Table 5), a competitive inhibitor of the peanut enzyme [20], and does not inhibit the *Chlorella* enzyme [21].

Table 5. Efficiency of pyridine nucleotides as hydrogen donors or acceptors in the proline dehydrogenase and PCA reductase reactions

Hydrogen donor or acceptor	Relative activity	
	Proline dehydrogenase	PCA reductase
NAD	100	—
NAD and NADP	65	—
NADP	5	—
NADH	—	100
NADPH	—	42
NADH and NADPH	—	140
NADH and NADP	—	85
NADH and NAD	—	94

The standard reaction mixture was used as described in Experimental with the addition of the designated pyridine nucleotide to a final concentration of 1 mM. No phosphatase activity was present in the enzyme preparations used.

PCA reductase used NADH and NADPH but showed a 2·5-fold greater activity with NADH (Table 5). The *Clostridium* [19] and liver [8, 22] enzymes had a greater activity with NADH, like the pumpkin enzyme, but the tobacco [10] enzyme had a greater activity with NADPH. In pumpkin, the enzyme activity with NADH plus NADPH was equal to the activities of NADH alone plus NADPH alone (Table 5). This suggests that two PCA reductases are present, one which utilizes NADH and another which utilizes NADPH.

Is proline dehydrogenase identical with PCA reductase? The lack of change in enzyme ratio during purification, heat treatment or pH; the common requirement for disulfide bonds; and the constant enzyme ratio during germination of pumpkin suggest that these two activities are due to the same enzyme. In peanut [20], the reductase utilized NADPH more effectively, and it was suggested [14] that proline dehydrogenase was therefore not a reversal of PCA reductase. In pumpkin however, proline dehydrogenase utilized NAD while PCA reductase catalyzed the reverse reaction more effectively with NADH. The enzyme ratio changed after storage and a number of inhibitors inhibit one reaction but not the other. This suggests that the two activities may be due to two discrete sites or protein entities.

Both proline dehydrogenase and PCA reductase are classified as L-proline-NAD(P)-5-oxidoreductase (E.C.1.5.1.2). Evidence obtained from filtration experiments on Sephadex G-100, G-150 and G-200 suggests that proline dehydrogenase and PCA reductase could occur as two active sites on the same protein. Two active sites on the same protein occurs with oxidation and reduction of cytochrome *C* [23] and recently has been suggested for proline dehydrogenase from wheat [14]. In pumpkin, the change in ratio PCA reductase/proline dehydrogenase with time and purification could indicate instability of the active site responsible for the conversion of PCA to proline. Although most of the data shown here support the suggestion that proline dehydrogenase and PCA reductase are located on the same enzyme protein, additional evidence is still required.

EXPERIMENTAL

Plant material. Pumpkin seedlings (*Cucurbita moschata* Poir. cv. Dickinson Field) were grown either in a darkened germina-

tor at 28° or in a growth chamber with a 24-hr photoperiod (15 klx), at 28° for various times.

Extraction and purification of the enzymes was conducted near 3°. The cotyledons were ground in a VirTis homogenizer with 2 vol. of 0·1 M NaPi buffer, pH 8, 0·1 mM cysteine and 0·1 mM EDTA. The homogenate was filtered through cheesecloth and centrifuged at 31 000 *g* for 15 min. The supernatant (crude enzyme) was heated at 60° for 15 min and then the temp quickly lowered to 5°. After centrifugation, the supernatant was fractionated with $(NH_4)_2SO_4$. The ppt. obtained between 30% and 50% satn was dissolved in isolating medium and dialyzed against 0·1 M NaPi, pH 7·6, for 24 hr. About 0·1 g protein was applied to a Sephadex G-100 column and fractions were collected by elution with a linear gradient of 50 mM NaPi buffer, pH 7·6. The fractions with enzyme activity were combined and precipitated with $(NH_4)_2SO_4$ between 30 and 50% satn. The pellet was dissolved in 50 mM NaPi, pH 7·6, and dialyzed for 20 hr against 10 mM NaPi, pH 8, 1 mM cysteine and 0·1 mM EDTA. DEAE-cellulose (Whatman DE 52) column was equilibrated with 10 mM NaPi, pH 8 and 20 mg of protein from the dialyzed enzyme, eluted from the Sephadex column and precipitated with $(NH_4)_2SO_4$, were applied to this column. The enzyme was removed by gradient elution, with a mixing chamber containing 125 ml of 10 mM NaPi, pH 8, and the reservoir chamber containing 125 ml of 10 mM NaPi, pH 8, and 0·5 M NaCl. The active fractions were combined and the enzyme stabilized by the addition of 10% $(NH_4)_2SO_4$. Protein was determined by biuret [24].

Enzyme assays. (a) *Proline dehydrogenase* reaction mixture contained: enzyme, 20 mM L-proline, 10 mM NAD and 0·1 M Na_2CO_3–$NaHCO_3$ buffer, pH 10·3, in a final vol. of 3 ml. The blank contained everything except NAD. In the colorimetric assay [5] the reaction was initiated by the addition of enzyme and terminated after a 20 min incubation at 30°. The amount of PCA produced was estimated with *o*-aminobenzaldehyde by the method of ref. 15. A unit of enzyme activity is that amount which causes the production of 1 nmol of PCA/hr, at 30°. In the spectrophotometric assay the reaction was started by the addition of L-proline and the increase in A was followed at 340 nm for 3 min, at 32°. In this assay a unit of enzyme activity is that amount that causes an increase in A of 0·001/min at 340 nm.

(b) *PCA reductase* was incubated with 128 μM NADH, 400 μM L-PCA and 0·1 M NaPi buffer, pH 7·4, or 50 mM Tris–Cl, pH 7·2, where specified, in a vol. of 1 ml, at 32°. The reference cuvette contained all the solns except NADH. The reaction was started by the addition of L-PCA and the enzyme activity followed for 3 min by measuring the decrease in A at 340 nm. A unit of enzyme activity is defined as the amount that causes a decrease in A at 340 nm of 0·001/min.

Synthesis of PCA. PCA was prepared from DL-α-amino-δ-hydroxyvaleric acid by a modification [21] of the method of Jones and Broquist [25].

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