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# $\Delta^1$-Pyrroline-5-Carboxylate Dehydrogenase from Cultured Cells of Potato[1]

## Purification and Properties

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**$\Delta^1$-Pyrroline-5-carboxylate (P5C) dehydrogenase (EC 1.5.1.12), the second enzyme in the proline catabolic pathway and a catalyst for the oxidation of P5C to glutamate, was purified from cultured potato (*Solanum tuberosum* L. var Desiree) cells. Homogeneous enzyme preparations were obtained by a three-step procedure that used anion-exchange, adsorption, and substrate elution chromatography. A 1600-fold purification was achieved, with a recovery of one-third of the initial activity. The purified enzyme was characterized with respect to structural, kinetic, and biochemical properties. It appeared to be an $\alpha$-4 tetramer with subunits of an apparent molecular mass of about 60 kD and had a mildly acidic isoelectric point value. Potato P5C dehydrogenase had Michaelis constant values of 0.11 and 0.46 mM for $NAD^+$ and P5C, respectively. Although $NAD^+$ was the preferred electron acceptor, $NADP^+$ also yielded an unusually high rate, and thus was found to serve as a substrate. Maximal activity was observed at pH values in the 7.3 to 8.3 range, and was progressively inhibited by chloride ions, a finding that strengthens recent suggestions that hyperosmotic stress negatively modulates in vivo proline oxidation.**

In a vast array of organisms ranging from bacteria to mammals, Pro is oxidized to glutamate through just two steps involving catalysis by Pro and P5C dehydrogenases (EC 1.4.3 and EC 1.5.1.12, respectively). In enterobacteria a multifunctional enzyme associated with the cytoplasmic membrane was shown to exert both activities (Brown and Wood, 1992; Ling et al., 1994), whereas for all other species tested so far, the activities depended on two distinct proteins. This discrepancy might reflect differences in Arg catabolism, which may take place via P5C directly from Orn, through pyrroline-2-carboxylate and Pro, or separately through the urea cycle (Fig. 1). Thus, the presence of a distinct P5C dehydrogenase could enable better, simultaneous regulation of the two convergent pathways (Mazelis, 1980; Phang, 1985). Subcellular fractionation studies and sequence analyses accounted for a mitochondrial localization of P5C dehydrogenase in higher plants, yeasts, and mammals (Elthon and Stewart, 1981; Brandriss and Krzywicki, 1986; Small and Jones, 1990). Because the anabolic P5C reductase (EC 1.5.1.2) is cytosolic (Brandriss and Magasanik, 1981) or plastidial (Szoke et al., 1992), the eukaryotic cell is able to avoid futile cycles between glutamate and Pro. Moreover, the interconversion that occurs between Pro and P5C has been proposed as a shuttle mechanism for the transfer of reducing equivalents into mitochondria (Hagedorn and Phang, 1983).

Pro metabolism in plants has attracted great interest because of its involvement in the response to hyperosmotic stress. This amino acid is the most common organic osmoprotectant, one that rapidly accumulates in the cell upon exposure to adverse environmental conditions such as drought and soil salinity (Delauney and Verma, 1993). In transgenic plants in which P5C synthetase, the first enzyme of the biosynthetic route, had been overexpressed, overproduction of Pro was shown to confer increased tolerance to osmotic stress (Kavi Kishor et al., 1995).

Pro-dependent oxygen uptake measurements in mitochondria isolated from water-stressed barley and maize plants suggested that the modulation of its channeling into the respiratory chain could play a role in stress-induced Pro accumulation (Stewart et al., 1977; Sells and Koeppe, 1981). When Pro and P5C dehydrogenase specific activities were measured separately in mitochondria from osmotically stressed maize seedlings, the former were found to be strongly reduced, whereas those of P5C dehydrogenase were unaffected. The occurrence of a stress-driven regulating mechanism(s) was thus inferred exclusively for the first enzyme in the catabolic pathway, which most probably catalyzes the rate-limiting step (Rayapati and Stewart, 1991). In any case, it is now generally accepted that stress-induced Pro accumulation occurs mainly through increased biosynthesis (Delauney and Verma, 1993; Chiang and Dandekar, 1995).

Current strategies aim to obtain salt- and drought-tolerant crops by genetically engineering Pro biosynthesis so as to prevent increases in amino acid production from being annulled by a corresponding increase in catabolic rate. A better knowledge of the regulatory pattern in Pro oxidation is mandatory for this aim. Recent findings have shown that in *Arabidopsis thaliana* Pro dehydrogenase is in fact up-regulated by the exogenous availability of Pro, and

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Abbreviation: P5C, $\Delta^1$-pyrroline-5-carboxylate.

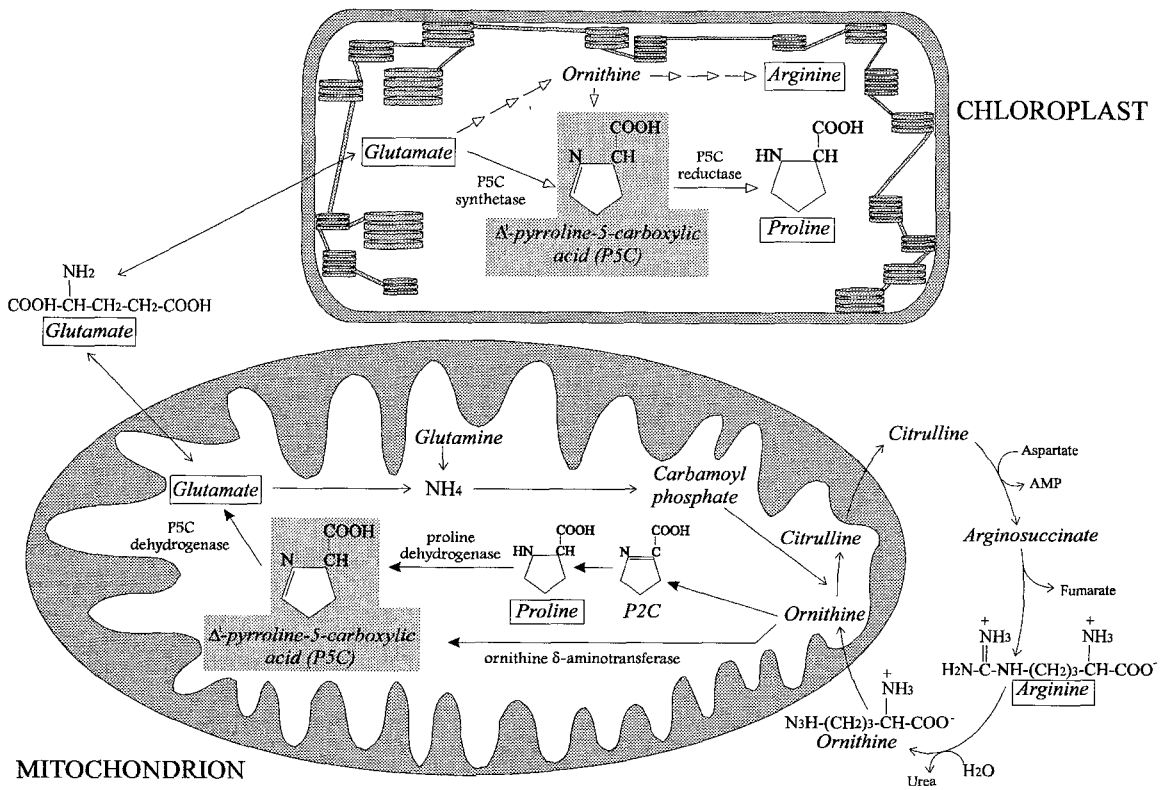


**Figure 1.** Metabolic relationships of P5C.

that its transcript promptly accumulates when dehydrated plants are subsequently rehydrated (Kiyosue et al., 1996; Verbruggen et al., 1996).

In early studies, P5C dehydrogenase activity was detected in crude mitochondrial preparations from several higher plants, in which maximal activity was displayed only after detergent treatment. Biochemical characterization pointed out a clear-cut preference for $NAD^+$ as the electron acceptor and $K_m$ values in the range of $10^{-4}$ to $10^{-3}$ M for both substrates (Stewart and Lai, 1974; Boggess et al., 1975). However, despite the considerable interest in Pro metabolism, P5C dehydrogenase has yet to be purified and/or fully characterized from a plant source. Here we describe the purification to electrophoretic homogeneity and properties of P5C dehydrogenase from cultured cells of potato (*Solanum tuberosum* L.).

## MATERIALS AND METHODS

### Chemicals

Unless otherwise indicated, biochemicals were purchased from Sigma. DEAE-Sephacel, Sephacryl S200, and molecular weight markers were supplied by Pharmacia. Bio-Gel P6DG was from Bio-Rad. DL-P5C was synthesized by the periodate oxidation of δ-*allo*-Hyl, and purified as described previously (Williams and Frank, 1975).

### Cell Culture

Cell-suspension cultures of potato (*Solanum tuberosum* L. var Desiree), kindly provided by Dr. A. Leone (Italian National Research Council, Portici, Italy), were grown in Erlenmeyer flasks in Murashige-Skoog medium (Murashige and Skoog, 1962) containing 0.3% (w/v) Suc and 2 mg $L^{-1}$ 2,4-D. Incubation was in the dark at 26 ± 1°C on a rotary shaker (120 rpm). Cultures were maintained by transfer of 10-mL aliquots to 100 mL of fresh medium every 2 weeks.

### Enzyme Assay

P5C dehydrogenase activity was measured as the P5C-dependent reduction in $NAD^+$. The assay mixture contained 50 mM Hepes-KOH, pH 7.4, 2 mM $NAD^+$, 2 mM DL-P5C, and 10 mM $MgCl_2$ in a final volume of 1 mL. A limiting amount of enzyme (0.05–0.25 nkat) was added to the prewarmed mixture, and the increase in absorbance was determined at 35°C for up to 15 min by continuous monitoring of the sample at 340 nm against blanks from which P5C had been omitted. Activity was determined from the initial linear rate, with the assumption of an extinction coefficient of 6220 $M^{-1}$ $cm^{-1}$. Protein concentration was determined by the method of Bradford (1976), with BSA as the standard.

### Enzyme Purification

*Step 1. Preparation of Crude Extract*

Cultured cells were harvested by vacuum filtration, weighed, powdered in liquid nitrogen, and resuspended in 2 mL $g^{-1}$ of 25 mM Hepes-KOH, pH 7.4, containing 0.5 mM DTT and 1 mM $MgCl_2$, with 1% polyvinyl polypyrrolidone (w/v) added to prevent oxidation of phenolic com-

pounds. All subsequent operations were carried out at 0 to 4°C. The homogenate was centrifuged at 20,000*g* for 30 min, and the resulting supernatant was fractionated with ammonium sulfate. The 0 to 70% saturated fraction was collected by centrifugation at 12,000*g* for 20 min, and pellets were resuspended in extraction buffer and desalted by passage through a Bio-Gel P6DG column.

*Step 2. Anion-Exchange Chromatography*

Crude extracts were loaded at a constant flow of 60 mL $h^{-1}$ onto a DEAE-Sephacel column (2.5 × 10 cm) that had been equilibrated with extraction buffer. After extensive washing, enzyme activity was eluted with a linear gradient that ranged from 0 to 250 mM KCl (500 mL) for the collection of 5-mL fractions.

*Step 3. Adsorption Chromatography*

Active fractions were pooled and applied at a constant flow of 30 mL $h^{-1}$ to a hydroxyapatite column (2.5 × 6 cm) that had been equilibrated with 25 mM $KPO_4$ buffer, pH 7.0, containing 0.5 mM DTT and 1 mM $MgCl_2$. The column was washed with a linear gradient that ranged from 25 to 200 mM $PO_4$ (100 mL), and then eluted with 500 mM $PO_4$ for the collection of 4-mL fractions. Active fractions were buffer-exchanged against extraction buffer as described above.

*Step 4. Substrate Elution Chromatography*

The sample was loaded at a constant flow of 20 mL $h^{-1}$ onto a Blue-2 agarose column (1.6 × 5 cm) that had been equilibrated with extraction buffer. The column was washed until no protein was detected in the eluate. P5C dehydrogenase activity was then eluted with extraction buffer containing 1 mM $NAD^+$ for the collection of 2-mL fractions. Active fractions were pooled, buffer-exchanged (as above) against extraction buffer that had been supplemented with 0.2 M $KPO_4$ buffer (pH 7.4), and subsequently concentrated by centrifugation in a filter unit (30-kD cutoff Ultrafree-CL, Millipore).

### Gel Permeation

Two-milliliter aliquots of a dilution of the purified enzyme were layered onto a Sephacryl S200 (87 × 1.6 cm) column that had been equilibrated with extraction buffer supplemented with 200 mM KCl. Elution proceeded at 4°C at the constant flow of 10 mL $h^{-1}$ for the collection of 1-mL fractions. Molecular mass markers for column calibration were bovine liver catalase (232 kD), rabbit muscle aldolase (158 kD), BSA (66 kD), hen egg ovalbumin (45 kD), bovine pancreas chymotrypsinogen A (25 kD), and bovine pancreas RNase A (13.7 kD).

### Electrophoresis

Discontinuous SDS-PAGE was performed at 25°C with a 4% stacking gel and a 12% separating gel. Nondenaturing electrophoresis was run at 4°C with a 3% stacking gel and a 5% separating gel. Gels were stained for protein with Coomassie blue R-250. Activity stain was by a modification of the procedure described by Chilson et al. (1991) at 35°C in 50 mM Hepes-KOH, pH 7.4, containing 2 mM P5C and $NAD^+$, 5 mM $MgCl_2$, 1 mM phenazine methosulfate, and 0.1 mM nitroblue tetrazolium. Alternatively, lanes were cut into 2.5-mm slices, which were incubated for 20 min at 35°C in 1 mL of reaction mixture, as above. IEF was performed in a vertical gel system (Robertson et al., 1987), with ampholytes within the 3.5 to 10 range. After the run, individual tracks were cut from the gel and either sliced in 2.5-mm segments for the determination of pH, or stained for protein as above.

## RESULTS

### Purification of P5C Dehydrogenase from Potato Cells

A quantitation of P5C dehydrogenase activity in crude extracts from cultured cells of potato was not reliable because of the presence of P5C reductase. When the P5C-dependent reduction in $NAD^+$ was measured, since P5C and NADH are also the substrates for the anabolic enzyme, the result was a futile cycle in which the NADH formed by the dehydrogenase was promptly reoxidized. Thus, after an initial increase, no net change in the absorbance could be observed. However, ion-exchange chromatography easily resolved these two activities (data not shown). Specific activity levels were calculated from total recovery in the DEAE-Sephacel eluate, as inferred from the amount of protein layered onto the column. In extracts from cells harvested at varying stages during the culture growth cycle, the activity levels showed maximal expression for P5C dehydrogenase in the very early exponential phase (data not shown). The enzyme was consequently purified from cells harvested 24 to 48 h after the transfer of cell inocula into fresh medium.

Neither the addition of detergents (0.1% NP-40 or 3-[{cholamidopropyl}dimethyl-ammonio]-1-propanesulfonic acid) nor the presence of protease inhibitors (1 mM PMSF and 1 μM pepstatin A) in the extraction buffer was found to enhance the initial recovery of enzyme activity. The results of the purification procedure are outlined in Table I. The use of adsorption chromatography on a hydroxyapatite column was found to be a high-resolution step, one that allowed the subsequent substrate elution procedure to obtain homogeneous enzyme preparations. Maximal specific activity observed reflected a purification of over 1600-fold, with a yield of more than 30%. We found no evidence to support the presence of P5C dehydrogenase isoforms. At a relatively high ionic strength (0.2 M $KPO_4$ buffer or 0.2 M KCl), purified enzyme preparations were substantially stable; between 70 and 80% of the activity was retained during a 45-d storage at 0°C. More than 90% of the activity was lost after 15 d at 0°C when the protein was stored in extraction buffer.

### Homogeneity of Purified P5C Dehydrogenase

The purified enzyme was electrophoretically homogeneous; when stained for protein, both denaturing and na-

**Table I.** *Purification of P5C dehydrogenase from potato*

The results presented are for a typical purification starting from 500 g (fresh weight) of cultured potato cells.

| Step | Protein | Activity | Specific Activity | Purification | Yield |
|---|---|---|---|---|---|
| | *mg* | *nkat* | *nkat mg$^{-1}$* | *-fold* | *%* |
| Crude extract | 847.5 | 384.6 | 0.454 | 1 | 100 |
| DEAE-Sephacel | 66.4 | 290.4 | 4.37 | 9.6 | 75.5 |
| Hydroxyapatite | 1.18 | 165.0 | 139.8 | 308 | 42.9 |
| Blue-2 agarose | 0.194 | 143.0 | 736.0 | 1621 | 37.2 |

tive polyacrylamide gels showed only a single band (Fig. 2, A and B, respectively). Under denaturing conditions, P5C dehydrogenase had an estimated molecular mass of about 60 kD. Several attempts to stain nondenaturing gels for P5C dehydrogenase activity failed, despite our use of high activity (8 nkat). Only when the lane was immediately sliced and the slices were incubated in the reaction mixture did we observe the very low levels of activity that corresponded to a protein band (Fig. 2B). In this case, total recovery was less than 1% of the loaded activity. The remarkable instability of potato P5C dehydrogenase during native PAGE was less pronounced in partially purified preparations, since faint bands appeared subsequent to activity stain. In any case, the partially purified potato enzyme showed a severe lability compared with the enzyme partially purified from another member of the Solanaceae, *Nicotiana plumbaginifolia* Viviani (Fig. 2C).

### Structural and Functional Properties of Potato P5C Dehydrogenase

The purified protein was thoroughly characterized with respect to physical, kinetic, and biochemical properties

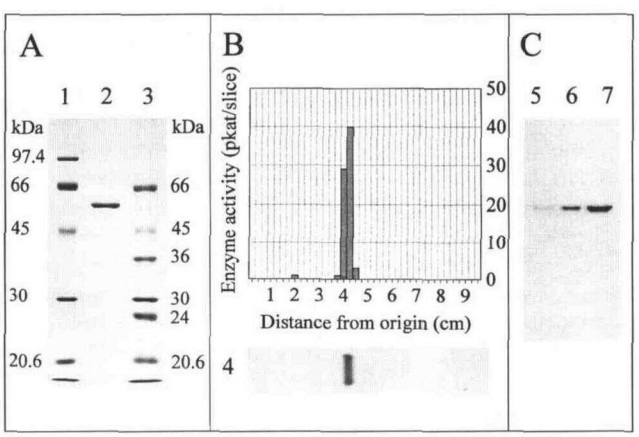


**Figure 2.** Electrophoresis of potato P5C dehydrogenase under native and denaturing conditions. A, SDS-PAGE of the purified enzyme (approximately 4 $\mu$g of protein; lane 2); the molecular masses of protein standards (lanes 1 and 3) run on an identical denaturing gel are indicated. B, Native-PAGE of the same preparation (about 11 $\mu$g, corresponding to 8 nkat, as measured immediately before loading; lane 4). Enzyme activity was measured in slices (2.5 mm) from a parallel lane, and the resulting graph was superimposed on the lane stained for protein. C, Activity stain for P5C dehydrogenase from cultured cells of potato (5 nkat, lane 5) and *N. plumbaginifolia* (1 and 2.5 nkat, lanes 6 and 7, respectively), partially purified by anion-exchange chromatography.

(summarized in Table II). Its behavior upon gel-permeation chromatography was consistent with a native molecular mass of about 240 kD (data not shown). Since the denatured protein migrates as a single band to a position that corresponds to a molecular mass of 60 kD, the native enzyme appears to be a homotetramer. Both IEF and chromatofocusing experiments indicated a pI of about 6.2. Maximal enzyme activity was achieved at 38°C; however, prolonged pretreatments at 45°C in the absence of the two substrates resulted in poor activity loss, a finding that suggests that the catalytically inactive conformation could be more stable than the active one. There were no pronounced effects of pH on potato P5C dehydrogenase activity, which showed a broad optimum for catalysis in the pH range of 7.3 to 8.3. Kinetic analysis enabled us to calculate an affinity constant for P5C of about 0.5 mM. The preferred electron acceptor was, as expected, $NAD^+$, with a $K_m$ value of 0.11 mM. However, $NADP^+$ was also found to be of use, since it yielded an unusually high rate: at 2 mM $NADP^+$ it showed more than 60% of the maximal activity obtainable with $NAD^+$. Since it was possible that a residual presence of $NAD^+$ in pooled fractions from the Blue-2 agarose column would alter the estimate of affinity constants, kinetic experiments were repeated with the enzyme purified by means of an alternative procedure in which substrate elution chromatography had been omitted, and very similar results were obtained (not shown). Enzyme activity was progressively inhibited by chloride ion levels in excess of 0.1 M. NaCl concentrations causing 50, 75, and 90% inhibition of the catalytic rate were 213, 524, and 900 mM, respectively. The same levels of $Na_2SO_4$ were substantially ineffective.

## DISCUSSION

This is the first report to our knowledge to describe the purification of P5C dehydrogenase from a plant species. In early works, the enzyme was detected in crude mitochondrial preparations and the addition of detergents was reported to be required for maximal recovery (Stewart and Lai, 1974; Boggess et al., 1975). In contrast, in extracts from potato cells, detergents were not required and did not improve enzyme yield. The present data are consistent with those found for mammals, in which P5C dehydrogenase was shown to be a soluble protein located in the mitochondrial matrix (Small and Jones, 1990; Hu et al., 1996).

The use of two high-resolution purification steps based on absorption and substrate elution chromatography en-

**Table II.** *Properties of P5C dehydrogenase from cultured cells of potato*

| | |
|---|---|
| Molecular mass | |
| By SDS-PAGE[a] | 59.9 ± 3.5 kD |
| By gel permeation[b] | 237 ± 11 kD |
| pI[c] | 6.2 ± 0.1 |
| Temperature optimum[d] | 38 ± 1°C |
| Activation energy[e] | 40.8 ± 0.9 kJ $mol^{-1}$ |
| Thermal inactivation[f] | |
| Half-life at 45°C | 44.0 ± 11.3 min |
| Half-life at 50°C | 30.2 ± 6.7 min |
| pH optimum[g] | 7.3 → 8.3 |
| $K_{m(app)}$ for P5C[h,i] | 464 ± 23 μM |
| $K_{m(app)}$ for $NAD^+$ [h,i] | 110 ± 7 μM |
| $K_{m(app)}$ for $NADP^+$ [h,j] | 662 ± 36 μM |
| $ID_{50}$ for $Cl^-$ ions[k] | 213 ± 18 mM |

[a] Mean value determined from relative mobility upon SDS-PAGE; four runs of proteins from two different preparations were performed; data for molecular weight markers are shown in Figure 2A. [b] Mean value estimated by comparison with retention patterns of suitable markers upon gel-filtration chromatography, as indicated in "Materials and Methods." [c] Value was estimated from the pH profiles in the gels, measured with an electrode following the re-equilibration of gel slices in water. The same result was obtained by chromatofocusing (not shown). [d] Optimal temperature was defined as the temperature at which maximal activity was obtained during a 5-min incubation. The experiment was repeated on two different enzyme preparations. [e] Result ± SD obtained by the replotting (Arrhenius plot) of data from two independent experiments in which the catalytic rate was measured as a function of temperature in the range of 15 to 37.5°C. [f] Activity of the purified protein was measured under standard assay conditions subsequent both to heat treatment for increasing times (5, 10, 15, 20, 30, 45, and 60 min) and to re-equilibration on ice. Mean half-life was estimated from the equation: $T1/2 = -0.301\ T\ [\log (\text{residual activity/initial activity})]^{-1}$. [g] The pH dependence of enzyme activity was evaluated in the presence of an equimolar mixture of Mes-Hepes-3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid buffers (0.066 M each); pH optimum is defined as the range in which more than 90% of maximal activity is retained. [h] Data obtained with proteins from two independent preparations. The second, nonvariable substrate was fixed at 2 mM. Similar results were obtained with the enzyme purified with an alternative procedure in which the substrate-elution step had been omitted. [i] Concentration for the variable substrate ranged from 50 to 1000 μM. [j] Concentration for the variable substrate ranged from 0.1 to 2.5 mM. [k] Value estimated from the linear regression equation of activity values, expressed as percentage of untreated controls plotted against the logarithm of NaCl concentration.

abled the isolation of a homogeneous enzyme preparation. Measurement of the enzyme's hydrodynamic properties by gel-filtration chromatography indicated a homotetrameric composition of the native protein, with subunits of 60 kD. The same molecular mass has been reported for the subunit of the rat liver enzyme, but in this case the native protein was found to be an $\alpha_2$ dimer (Small and Jones, 1990), as was the bacterial bifunctional enzyme that shows both Pro and P5C dehydrogenase activities (Brown and Wood, 1992). The broad pH optimum found for potato P5C dehydrogenase is inconsistent with previous reports on this plant enzyme, which showed relatively sharp optima around pH 8.0 (Stewart and Lai, 1974; Boggess et al., 1975) or 6.0 (Elthon and Stewart, 1981). However, such behavior in intact or swollen mitochondria might reflect conditions required for in vivo oxidation, and may not occur in purified preparations.

In vitro enzyme activity was found to be reduced markedly in vitro by chlorides. At NaCl concentrations in the 150 to 300 mM range, one in which potato cell viability was progressively reduced (data not shown), a 40 to 60% inhibition of the catalytic rate was observed. These results, which agree with previous data (Boggess et al., 1975), are also consistent with the recent finding of a negative modulation of Pro oxidation under water-stress conditions (Rayapati and Stewart, 1991; Kiyosue et al., 1996; Verbruggen et al., 1996). The mRNA levels of Pro dehydrogenase, the enzyme that catalyzes the first (and possibly the limiting) step in the short catabolic pathway, were found to decline rapidly during dehydration, but to accumulate promptly to high levels during subsequent rehydration (Kiyosue et al., 1996; Verbruggen et al., 1996). Similar data are as yet not available for P5C dehydrogenase, even though enzyme activity level has been found to be unaffected in mitochondria from osmotically stressed maize seedlings (Rayapati and Stewart, 1991). The sensitivity of the enzyme to chlorides might allow the plant cell to reduce P5C oxidation under ionic and osmotic stress conditions as well.

No evidence of multiple enzyme forms was found during the purification and characterization of potato P5C dehydrogenase. However, this result might simply be due to a clear-cut prevalence of an isozyme in the early exponential phase of growth, when the cultured cells were harvested. The existence of distinct enzymes capable of oxidizing P5C was previously suggested on the basis of the differences in pH optima found for P5C respiration in maize mitochondria (Elthon and Stewart, 1982). Two P5C dehydrogenases were indeed resolved in extracts from suspension cultured cells of *N. plumbaginifolia*, another species belonging to the Solanaceae (Forlani et al., 1997).

Experiments are currently in progress to ascertain the possible occurrence of isozymes in potato. Also, polyclonal antibodies raised against the purified enzyme will be used to investigate the expression of P5C dehydrogenase, both in varying tissues during plantlet development, and under water-stress conditions.

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