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Purification to Homogeneity of Pyrroline-5-Carboxylate Reductase of Barley

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ABSTRACT

An enzyme has been purified to homogeneity from barley seedlings which has 'proline dehydrogenase' and the pyrroline-5-carboxylic acid reductase activities. The purification achieved is 39,000-fold as calculated from the proline dehydrogenase activity. The subunit molecular weight of the protein is 30 kilodaltons. The native enzyme has molecular weights up to 480 kilodaltons, depending on the buffer environment. From the pH profiles, the specific activities and thermodynamic considerations, it is concluded that the plant proline dehydrogenase functions *in vivo* as a pyrroline-5-carboxylate reductase.

Pyrroline-5-carboxylate reductase (EC 1.5.1.2. L-proline:NAD(P)-5-oxidoreductase)catalyzes the final step in proline biosynthesis. The enzyme has been partially purified and characterized from various animal sources (1, 3, 11, 16, 21, 22, 24), bacteria (1, 2, 10, 11, 15), *Neurospora* (26), and *Saccharomyces* (6, 7). In higher plants (*Pisum* and *Phaseolus*) the reductase was initially detected by Meister *et al.* (11). A partial purification of the enzyme from higher plant sources has been reported subsequently (12, 14, 18, 19, 23).

In this paper we describe a procedure which results in the preparation of P5C[1]-reductase from barley seedlings as a homogeneous single protein. The enzyme is purified 39,000-fold during this process. The purified reductase oxidizes proline under extreme pH conditions (pH 10.3) thus supporting the assumption that in reality the "proline dehydrogenase" (8, 9, 17, 19) of higher plants is a P5C-reductase.

MATERIALS AND METHODS

Barley seedlings (*Hordeum vulgare*, var. Augusta) were grown in a greenhouse (16 h photoperiod, 20°C), watered until the 5th d of growth, and harvested at the age of 10 to 14 d. Six kg of seedlings were frozen in liquid N_2 and ground. The resulting powder was homogenized in a total of 31 L of phosphate buffer (0.1 M phosphate (pH 8.0), 1 mM NaEDTA, and 10 mM β-mercaptoethanol) with an Ultra-Turrax T45 and filtered through cheesecloth. The extract was heated to 60°C for 0.5 to 1 min, immediately cooled, and filtered through Celite 545 (Serva). The filtrate was made 25% saturated with $(NH_4)_2SO_4$ and the resultant precipitate discarded after filtration.

$(NH_4)_2SO_4$ saturation was increased to 50% and the precipitate collected by centrifugation (22,000*g*). The sediment was dissolved in 380 ml of phosphate buffer 2 (50 mM phosphate, pH 7.5, and 10 mM β-mercaptoethanol), dialyzed against buffer 3 (20 mM piperazine-HCl and 1 mM mercaptoethanol, pH 6.3), and applied to a DEAE-Sephacel column (Pharmacia K 50/30, bed volume 470 ml, flow rate 200 ml/h). The column was rinsed with 1 L of buffer 3 and developed in a 0 to 0.3 M KCl gradient in buffer 3 (1600 ml; fractions of 27 ml were collected).

The pooled activity fractions were chromatographed on Amicon Matrex Gel Red A (Pharmacia K 16/20, bed volume 22 ml). After loading and rinsing the column with buffer 4 (15 mM Tris-HCl, pH 8.0, 5% v/v glycerol, 5 mM $MgCl_2$, and 2 mM DTT) it was developed in a 0 to 1.5 M KCl gradient in the same buffer. Matrex gel was prepared and regenerated with 8 M urea and equilibrated with buffer 4.

The final step of the preparation was a chromatography on Amicon Matrex Gel Orange A (bed volume 2.4 ml). The pooled activity fractions from the preceding step were desalted (Sephadex G-25 in column PD-10, Pharmacia) and then applied to the Orange A column. The column was rinsed with 5 ml of buffer 4 and 25 ml of buffer 4 + 0.2 M KCl. the elution of the enzyme was achieved with 0.5 M KCl in buffer 4 (flow rate 30 ml/h).

Enzyme Assay. The 'proline dehydrogenase' activity was assayed during the isolation procedure. The assay system contained 0.7 ml of buffer (7.5 g/L glycine; 5.8 g/L NaCl, 3.0 g solid NaOH resulting in a pH of 10.3); 0.1 ml of proline (0.2 M in water); 0.1 ml of NAD^+ (0.15 M NAD^+ in water, adjusted to pH 5 to 7 with NaOH); and 0.1 ml of test solution. The reference contained buffer instead of proline.

The change of absorbance was measured at 366 nm in an Eppendorf photometer 1101M; 1 unit = 16.7 nkat = 1 μmol NADH/min. The assay for P5C-reductase was conducted by monitoring the loss of the absorbance of NADH at 366 nm. Reaction mixtures of 1 ml contained Tris-phosphate buffer (pH 7.5) (0.04 M Tris), 0.095 M NaCl, 0.08 mM $MgCl_2$, 0.08% (v/v) glycerol, 0.03 mM DTT, 0.19 to 0.76 mM NADH, and 0.225 to 0.9 mM P5C (stock solution: 9 mM L-P5C, 0.6 to 1 M NaCl). The DL-P5C was synthesized by a slightly modified procedure of Williams and Frank (25). The Dowex column was equilibrated with 0.1 M acetic acid instead of 1 M HCl. Protein concentrations in crude extracts were determined by the procedure of Lowry *et al.* (5) and in the final enriched preparations according to the procedure of Schaffner and Weissmann (20).

SDS-PAGE. The SDS-PAGE was carried out in 12.5% polyacrylamide gel according to Laemmli (4). Silver staining of proteins was done as described by Nielsen and Brown (13). Gradient PAGE of native proteins was performed in a gradient

[1] Abbreviations: P5C, pyrroline-5-carboxylate; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide; PMS, phenazine methosulfate.

Table I. *Purification of P5C-Reductase of 14-Day-Old Water-Stressed Barley Seedlings*[a]

PurificationStep	Vol	Activity	Protein	Specific Activity	Enrichment	Yield
	L	*units*	*mg*	*units/mg*	*-fold*	*%*
1. Crude homogenate	31.2	2,053	162,900	0.013	1.0	100
2. Heat precipitation 60°C	25.0	1,373	13,600	0.101	7.8	67
3. $(NH_4)_2SO_4$ precipitation 0–25%, supernatant	26.3	1,348	13,300	0.101	7.8	66
4. $(NH_4)_2SO_4$, precipitate 25–50%	0.379	1,230	7,190	0.171	13.2	60
5. DEAE column	0.193	1,733	122	14.2	1,093	84
6. Affinity chromatography, Red A	0.030	1,217	8.5	143	11,000	59
7. Affinity chromatography, Orange A	0.048	512	1.01	507	39,000	25

[a] For details, see "Materials and Methods."

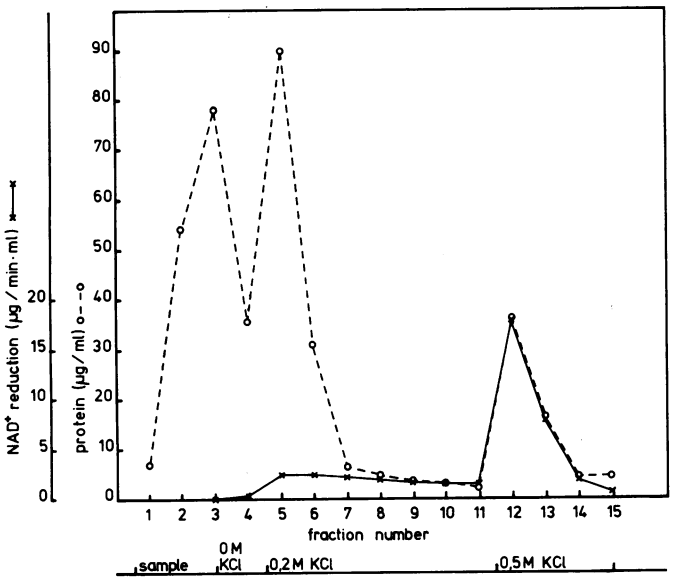

FIG. 1. Affinity chromatography on Matrex Gel Orange A (step 7, Table I) of P5C-reductase. Samples eluting at increasing KCl concentrations were collected. (O- - -O), protein profile; (×——×), activity profile.

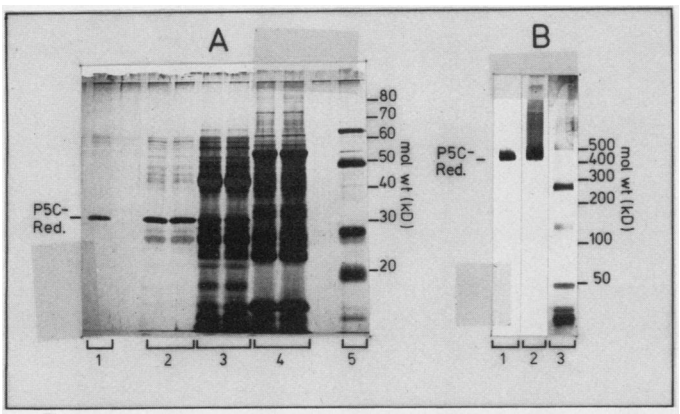

FIG. 2. PAGE of denatured and native P5C reductase. A, SDS-PAGE of purification steps (see Table I). Lane 1, step 7; lane 2, step 6; lane 3, step 5; lane 4, step 3; lane 5, standard proteins. B, Native PAGE of the purified enzyme. Lane 1, substrate staining; lane 2, silver staining; lane 3, standard proteins.

of 5 to 20% acrylamide, 3% bisacrylamide (without stacking gel) at pH 8.9, and buffer concentration (Tris-HCl) of 0.038 M. after 18 h (600 V, 12 cm separating distance), the proteins have reached their pore limit and the approximate mol wt can be determined. Substrate staining for proline dehydrogenase activity was achieved in 50 ml buffer (0.1 M Tris-HCl, pH 7.5) containing 5 mM NAD, 0.2 M Pro, 0.65 mM PMS, and 1.2 mM MTT). Gels and assay systems were sealed in plastic bags and heated for 30 min at 30°C.

RESULTS AND DISCUSSION

The purification steps of P5C-reductase of barley are summarized in Table I. A 39,000-fold enrichment of the enzyme is achieved. Yields up to 25% have been obtained. The specific activity of the "proline dehydrogenase" is about 500 units/mg of protein and thus lower than the specific activity of the reductase (see later). The enzyme of the final purification step is stable for 1 month when stored at −80°C in 5% glycerol and 2 mM DTT. No activity loss is observed.

From Figure 1 it is apparent that the enzymic activity cochromatographs with a protein peak from the Orange A column. Essentially all of the protein (98%) migrated as a single band in SDS-PAGE (Fig. 2A, lane 1) with an apparent mol wt of 30 kD. (The two faint bands of lower mobility in this lane are perhaps polymers of the main protein. These bands are not observed in all preparations.) From this finding we conclude that the P5C-reductase is constituted of one subunit. Native gel electrophoresis of the purified protein (Fig. 2B), stained for proline dehydrogenase, shows a single activity band (lane 1). It coincides with a band stained for protein (lane 2). Comparison to standard proteins of known mol wt (lane 3) reveals a mol wt of the native protein of approximately 420 kD. The native enzyme thus is a homopolymer composed of 12 to 16 subunits. However, the quarternary structure might be changed drastically by the buffer environment. From preliminary results with Sephacryl Gel S-300 runs we know that the protein elutes in a sharp peak of mol wt 480 kD in Tris buffer (0.146 M Tris and 0.1 M HCl [pH 7.8], ionic strength = 0.1). In contrast, with phosphate buffer (50 mM phosphate buffer, pH 7.5) instead of Tris, a fairly broad elution profile with a main peak of mol wt of 220 kD and additional distinct shoulders on both sides of the main peak was observed. As yet it is not known whether aggregation-dissociation reactions of the protein produce these side peaks and whether these changes in quarternary structure are involved in activity regulation. The amino acids Gly, Ala, Cys, Asp, Ser, Met, Thr, His, Val, Ile, Glu, and Orn do not function as substrates. No activity is measured with D-Pro, L-hydroxy-Pro, and L-azatidine-2-carboxylate. Low activities are observed with proline esters. These substrates showed the following activities as compared with proline (100%): L-proline-*t*-butyl ester, 10%; L-proline methyl ester, 16%; and L-proline benzyl ester, 24%. The reductase activity occurs only with P5C. Adenosine, cAMP, and guanosine-5-phosphate do not function as substrates. The enzyme used both NAD^+ and $NADP^+$ as electron acceptors and NADH and NADPH as donors but was most active with the reduced phosphorylated pyridine nucleotide.

This finding might be taken as evidence for the enzyme to

function in an anabolic reaction sequence.

From secondary plots of the P5C-reductase kinetics, the specific activity and the turnover number were calculated. The maximal values determined were 2,500 units/mg (= 41.8 μkat) for the specific activity and 76,000 min^{-1} (per 30 kD protomer) for the turnover number.

The proline dehydrogenase activity of the protein disappears at pH values below 8.5, while the reductase activity has its maximum in the range of pH 6.8 to 7.5. Our results thus agree with the findings of Costilow and Cooper (2) that a single protein carries both the P5C-reductase and the proline dehydrogenase activities.

At pH 7.5 there is no activity measurable with proline in our ordinary assay system. Earlier results show (15) that the equilibrium of the reaction is far on the side of the products NAD^+ and proline. From this and the high specific activity with NADPH we conclude that *in vivo* the enzyme functions as a P5C-reductase. This finding makes the final step in proline biosynthesis in the cytoplasm an irreversible reaction step. A detailed description of the enzymes properties and its proposed function in water stress induced proline biosynthesis is in progress.

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LITERATURE CITED

1. ADAMS E, A GOLDSTONE 1960 Hydroxyproline metabolism. III. Enzymatic synthesis of hydroxyproline from Δ¹-pyrroline-3-hydroxy-5-carboxylate. J Biol Chem 235: 3499–3503
2. COSTILOW RN, D COOPER 1978 Identity of proline dehydrogenase and Δ¹-pyrroline-5-carboxylic acid reductase in *Clostridium sporogenes*. J Bacteriol 134: 139–146
3. GREENBERG DM 1962 Pyrroline-5-carboxylate reductase. Methods Enzymol 5: 959–964
4. LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685
5. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
6. MATZURZAWA T, I ISHIGURO 1980 Δ¹-Pyrroline-5-carboxylate reductase from baker's yeast. Further purification by affinity chromatography with 5'AMP-Sepharose 4B. Biochim Biophys Acta 616: 381–383
7. MATZURZAWA T, I ISHIGURO 1980 Δ¹-Pyrroline-5-carboxylate reductase from baker's yeast. Purification, properties and its application in the assays of L-Δ¹-pyrroline-5-carboxylate and L-ornithine in tissue. Biochim Biophys Acta 613: 318–323
8. MAZELIS M, L FOWDEN 1971 The metabolism of proline in higher plants. II. L-Proline dehydrogenase from cotyledons of germinating peanut (*Arachis hypogea* L.) seedlings. J Exp Bot 22: 137–148
9. MAZELIS M, RK CREVELING 1974 L-Proline dehydrogenase of *Triticum vulgare* germ: purification, properties on cofactor interactions. Phytochemistry 13: 559–565
10. MEILE L, TH LEISINGER 1982 Purification and properties of the bifunctional proline dehydrogenase/Δ¹-pyrroline-5-carboxylate dehydrogenase from *Pseudomonas aeruginosa*. Eur J Biochem 129: 67–75
11. MEISTER A, AN RADHAKRISHNAN, SD BUCKLEY 1957 Enzymatic synthesis of L-pipecolic acid and L-proline. J Biol Chem 229: 789–800
12. MILLER PM, CR STEWART 1976 Pyrroline-5-carboxylic acid reductase from soybean leaves. Phytochemistry 15: 1855–1857
13. NIELSEN B, L BROWN 1984 The basis for colored silver-protein complex formation in stained polyacrylamide gels. Anal Biochem 141: 311–315
14. NOGUCHI M, A KOWAI, E TAMAKI 1966 Studies on nitrogen metabolism in Tobacco plants. VII. Δ¹-Pyrroline-5-carboxylate reductase from tobacco leaves. Agr Biol Chem 30: 452–456
15. PAHLICH E, H -J JÄGER, E KASCHEL 1981 Thermodynamische Betrachtungen über die reversible Reaktionssequenz Glutaminsäure ⇆ Prolin. Z Pflanzenphysiol 101: 137–144
16. PEISACH J, HJ STRECKER 1962 The interconversion of glutamic acid and proline. V. The reduction of Δ¹-pyrroline-5-carboxylic acid to proline. J Biol Chem 237: 2255–2260
17. RENA AB, WE SPLITSTOESSER 1974 Proline dehydrogenase from pumpkin (*Cucurbita moschata*) cotyledons. Physiol Plant 32: 177–181
18. RENA AB, WE SPLITSTOESSER 1974 Δ¹-Pyrroline-5-carboxylate: the product of proline dehydrogenase from *Cucurbita moschata* cotyledons. Phytochemistry 13: 2081–2084
19. RENA AB, WE SPLITSTOESSER 1975 Proline dehydrogenase and pyrroline-5-carboxylate reductase from pumpkin cotyledons. Phytochemistry 14: 657–661
20. SCHAFFNER W, C WEISSMANN 1973 A rapid, sensitive and specific method for the determination of protein in dilute solution. Anal Biochem 56: 502–514
21. SMITH ME, DM GREENBERG 1956 Characterization of an enzyme reducing pyrroline-5-carboxylate to proline. Nature 177: 1130
22. SMITH ME, DM GREENBERG 1957 Preparation and properties of partially purified glutamic semialdehyde reductase. J Biol Chem 226: 317
23. SPLITSTOESSER SA, WE SPLITSTOESSER 1973 Pyrroline-5-carboxylate reductase from *Cucurbita* cotyledons Phytochemistry 12: 1565–1568
24. STRECKER HJ 1971 Δ¹-Pyrroline-5-carboxylate reductase. Methods Enzymol 17: 258–261
25. WILLIAMS I, L FRANK 1975 Improved chemical synthesis and enzymatic assay of Δ¹-pyrroline-5-carboxylic acid. Anal Biochem 64: 85–97
26. YURA T, HJ VOGEL 1959 Pyrroline-5-carboxylate reductase of *Neurospora crassa:* partial purification and some properties. J Biol Chem 234: 335–342