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PYRROLINE-5-CARBOXYLIC ACID REDUCTASE FROM SOYBEAN LEAVES

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Key Word Index—*Glycine max*; Leguminosae; soybean; pyrroline-5-carboxylic acid reductase; proline.

Abstract—Pyrroline-5-carboxylic acid reductase was purified 40-fold from soybean leaves (*Glycine max* L. var Corsoy). The enzyme was fairly unstable, had a broad pH optimum, and was inactivated by heat and acid; NADH and NADPH both served as cofactors. It had a higher activity with NADH (about 4×) compared to NADPH, but a lower K_m for NADPH. $NADP^+$ inhibited both the NADH- and NADPH-dependent activity. Sulfhydryl group blocking agents reduced the activity as did the carbonyl blocking agent, NH_2OH. Thiazolidine-4-carboxylic acid and phosphate inhibited the enzyme and proline inhibited only at high concentrations. ATP, GTP, and CTP were all effective inhibitors of both the NADH- and NADPH-dependent activity. Phosphorylated nucleotide inhibition was reversed by Mg^{2+} ions.

INTRODUCTION

P5C† reductase (L-proline: NAD(P)-5-oxidoreductase, E.C.1.5.1.2) participates in the synthesis of proline from glutamate. This synthesis is stimulated in leaves under water stress [1–4]. The enzyme has been isolated from turnip leaves [5], pumpkin cotyledons [6,7] and tobacco leaves [8]. This paper reports some new information on the enzyme from soybean leaves, particularly regarding nucleotide inhibition and its reversal by Mg^{2+}

RESULTS AND DISCUSSION

A 40-fold purified preparation (Table 1) was obtained by $(NH_4)_2SO_4$ fractionation and DEAE cellulose chromatography. The enzyme was relatively unstable losing 50% of its activity after 1 and 4 weeks at 4° and −10° respectively. It was sensitive to heat and low pH.

The enzyme had a broad pH optimum from 7.0 to 7.5. The K_m's for L-P5C, NADH, and NADPH were respectively 154 μM, 100 μM, and 50 μM. With the same initial concentrations, the NADH rates were typically four times the NADPH rates (Table 2). NADH added to the NADPH reaction mixture did not change the reaction rate. When NADPH was added to an NADH reaction mixture, the reaction rate decreased to that of an NADPH rate. These results differ from those obtained with the pumpkin enzyme [7] in which the rates were additive. $NADP^+$ was a strong inhibitor, particularly of the NADH activity (Table 3) in contrast to the pumpkin enzyme which was only slightly inhibited.

Our interpretation of these results is that one enzyme uses both cofactors, but has a higher affinity to NADPH. The catalytic capacity for NADPH is lower partly due to production inhibition. NAD^+ does not inhibit (Table 4).

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† Abbreviations: P5C: Δ^1-pyrroline-5-carboxylic acid; T4C: thiazolidine-4-carboxylic acid; PM: 2-pyrrolidine methanol; DTT: dithiothreitol; pCMB: *p*-chloromercuribenzoate.

Table 1. Partial purification of P5C reductase

Fraction	Total volume (ml)	Total protein (mg)	Total enzyme activity (kat)	Specific activity (kat/mg)	Purification factor	Recovery (%)
1000 *g* Supernatant	540	2700	0.069	2.5×10^{-5}	1.0	100
25000 *g* Supernatant	480	1880	0.063	3.33×10^{-5}	1.3	91
0–30% Ammonium sulfate pellet	42	478	0.067	1.4×10^{-4}	5.5	98
Pellet after dialysis and cent.	42	185	0.062	3.35×10^{-4}	15.2	90
Combined DEAE fractions	60	56	0.056	1.00×10^{-3}	39.2	82

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Table 2. Initial NADH and NADPH reaction rates

0.1 mM NADH	NADPH (mM)	Enzyme activity (kat × 10^{-4})
+	0	9.65
+	0.03	3.21
+	0.09	3.21
+	0.15	3.21
−	0.09	3.21
−	0.24	3.21
−	0.30	3.21

Table 3. Effect of $NADP^+$ on P5C reductase with NADH and NADPH as substrates. Initial substrate concentration = 0.1 mM

Substrate	$NADP^+$ concentration (mM)	Relative activity
NADPH	0	100
NADPH	1	63
NADH	0	100
NADH	0.03	60
NADH	0.1	25
NADH	1	7

The reductase was inhibited by Hg^{2+}, pCMB, NH_2OH (Table 4) as previously reported [6,7]. Proline inhibited only at high concentrations. Additionally, it was inhibited by T4C, but not PM, suggesting that the carboxyl group is involved in substrate binding. Phosphate was a competitive inhibitor with NADH ($K_i = 7.7\ \mu M$).

ATP was a competitive inhibitor (Table 4) with NADH ($K_i = 60\ \mu M$) and it inhibited the NADPH activity (Table 5) to a lesser extent. GTP and CTP inhibited as much as ATP, but ADP inhibited less and AMP was without effect. These results suggest the phosphates are more important than the nitrogen base in binding to the enzyme.

Mg^{2+} reversed the inhibition by ATP, ADP, GTP, and CTP (Table 5). At 1 mM ATP, 3 mM Mg^{2+} restored most of the activity and 10 mM restored all the activity. Using the stability constant of 20000 for Mg·ATP, only about 80% of the ATP is complexed in a solution of 1 mM Mg^{2+} and 1 mM ATP. About 97% of the ATP is complexed in a solution of 10 mM Mg^{2+} and 1 mM ATP. At 1 mM ATP and 10 mM Mg^{2+}, all the ATP is complexed. These percentages and the results in Table 5 indicate that free ATP inhibits the reaction and Mg·ATP is not inhibitory. Since Mg^{2+} complexes with the phosphate of phosphorylated nucleosides, these results further implicate phosphate in the binding of phosphorylated nucleosides to the enzyme. Mg^{2+} alone has no effect on the enzyme.

The low K_m for NADPH, the inhibition by phosphorylated nucleosides, the reversal of phosphorylated nucleoside inhibition by Mg^{2+}, and the inhibition by phosphate all support the involvement of phosphate in the binding of the NADH or NADPH to the enzyme.

EXPERIMENTAL

Soybean (*Glycine max* L. var. Corsoy) leaves were blended with 2 vol of 0.5 M KPi buffer pH 7.5 with 1 mM DTT and 1 mM EDTA. Slurry was squeezed through 4 layers of cheesecloth and centrifuged at 1000 *g* for 10 min. Supernatant was centrifuged at 25000 *g* for 30 min. $(NH_4)_2SO_4$ was added to the 25000 *g* supernatant to 30% saturation then centrifuged at 1000 *g* for 10 min. The pellet was resuspended in 5 mM KPi buffer pH 7.5 and dialyzed against 4 hourly changes of 5 mM KPi pH 7.5 buffer. Dialysate was centrifuged at 100000 *g* for 1 hr. Supernatant was added to DEAE cellulose equilibrated with 30 mM KPi buffer pH 7.5. Protein was eluted by a linear salt gradient starting with 30 mM KPi pH 7.5 buffer (100 ml) and ending at 2 M NaCl (100 ml). Protein was determined by the method of ref. [9].

Enzyme assays. P5C reductase reaction mixture contained 0.2 ml enzyme extract, 0.1 mM NAD(P)H, 10 mM DL-P5C, 30 mM HEPES pH 7.5 in 3 ml final vol. The reaction was initiated by adding P5C. Changes in A_{340} were measured at 25°.

P5C synthesis. P5C was prepared either by oxidation of α-amino-δ-hydroxyvaleric acid [5] or synthesized from γ-γ-dicarbethoxy-γ-acetamidobutyraldehyde phenylhydrazone [10]. P5C concentration was determined by its reaction with *o*-aminobenzaldehyde [10].

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Table 4. Inhibition of P-5-C reductase by various compounds

Inhibitor	Concentration (M)	Inhibition (%)
Hg^{2+}	1×10^{-4}	50
pCMB	1×10^{-3}	54
NH_2OH	1×10^{-3}	92
NAD^+	1×10^{-3}	0
L-proline	1×10^{-3}	0
L-proline	1×10^{-2}	0
L-proline	1×10^{-1}	36
Thiazolidine-4-carboxylate	1×10^{-3}	4
Thiazolidine-4-carboxylate	1×10^{-2}	50
2-Pyrrolidine methanol	1×10^{-3}	4
2-Pyrrolidine methanol	1×10^{-2}	8
ATP	5×10^{-5}	29
ATP	1×10^{-4}	58
ATP	1×10^{-3}	96
ADP	1×10^{-3}	75
AMP	1×10^{-3}	0
GTP	1×10^{-3}	91
GTP	1×10^{-2}	100
CTP	1×10^{-3}	91

Table 5. Phosphorylated nucleotise inhibition of P-5-C reductase and its reversal by magnesium ion

Coenzyme	Inhibitor	Inhibitor conc. (mM)	Magnesium conc. (mM)	Inhibition (%)
NADH	ATP	1	0	92
NADH	ADP	1	0	75
NADH	GTP	1	0	91
NADH	CTP	1	0	91
NADPH	ATP	1	0	25
NADPH	ATP	10	0	50
NADH	ATP	1	10	0
NADH	ADP	1	10	0
NADH	GTP	1	10	0
NADH	CTP	1	10	0
NADPH	ATP	10	100	0
NADH	ATP	1	1	80
NADH	ATP	1	3	20

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