

Proline Oxidase and Water Stress-induced Proline Accumulation in Spinach Leaves¹

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ABSTRACT

Spinach (*Spinacia oleracea* L.) leaf discs accumulated free proline when exposed to polyethylene glycol solutions of water potential less than -10 bars. At -20 bars, the accumulation was 11 micromoles per gram original fresh weight in a 24-hour period.

When the leaf organelles were separated on a sucrose gradient, a proline oxidase was detected in the mitochondrial fraction. Isolated mitochondria were used for the study of the properties of the enzyme which was assayed by both oxygen uptake measurement and reduction of 2,6-dichlorophenol-indophenol in the presence of phenazine methosulfate. There was a stoichiometry of one-half mole of oxygen uptake per mole of Δ^1 -pyrroline-5-carboxylate production in the enzymic reaction. The enzyme had an optimal activity at pH 8.0 to 8.5 and an apparent K_m value of 0.028 molar for proline. $MgCl_2$ and flavin adenine dinucleotide were required for maximal activity. Addition of sucrose, mannitol, or polyethylene glycol to reduce the water potential of the reaction mixture to as low as -20 bars resulted in little inhibition. The enzyme preparation was unable to reduce NAD to NADH, and NAD did not inhibit the enzyme activity. The enzyme preparation reduced cytochrome *c* in the presence of KCN. Triton X-100 at low concentration strongly inhibited the enzyme activity. The enzyme was apparently linked to the mitochondrial electron transport system. The *in vitro* activity of the enzyme under optimal assay conditions was high enough to prevent proline accumulation under water stress condition; presumably this activity was restrained *in vivo*.

reductase and P5C dehydrogenase have been studied and found to be in the cytosol and mitochondria, respectively (4, 25). The kinase-dehydrogenase for the conversion of glutamate to P5C was detected in extracts of green leaves (17). The only enzyme that has not been reported is the O_2 -dependent proline oxidase. In animal tissues, this enzyme is localized on the inner mitochondrial membrane and requires O_2 and not pyridine nucleotide as the electron acceptor. In higher plants, a NAD-dependent proline dehydrogenase has been demonstrated (14-16, 18, 25). Since NAD-proline dehydrogenase and P5C reductase catalyze reactions with the same reactants and coenzyme operating in opposite directions, they may actually be the same enzyme. In one report (18), evidence was presented that the two enzyme activities are catalyzed by the same protein molecule.

In a recent report (3), mitochondria from etiolated plant tissues were shown to possess proline-dependent O_2 uptake ability. The main reaction products were glutamate and organic acids, and P5C appeared to be a transient intermediate. The report suggests that O_2 -dependent proline oxidase is present in plant mitochondria. The direct detection of the enzyme and the study of its properties, especially in a tissue that can actively accumulate proline under water stress, are essential in our understanding of the mechanism of proline metabolism during water stress.

In this report, we demonstrate the existence of O_2 -dependent proline oxidase in the mitochondria of spinach leaves. Its enzymic properties are reported. Spinach leaf discs are shown to accumulate proline under water stress although the *in vitro* proline oxidase activity is high enough to prevent the proline accumulation.

MATERIALS AND METHODS

Plant Materials. Spinach (*Spinacia oleracea* L.) leaves were obtained locally. Leaf discs of 1 cm in diameter were cut with a cork borer and allowed to float in Petri dishes containing PEG (4000) solution. The Petri dishes were placed in an environmental chamber, model 1-E-30 (Percival Co., Des Moines, Iowa), with continuous illumination under white fluorescent lamps (intensity 9.6×10^3 erg $cm^{-2} s^{-1}$) at 25 C. At time intervals, the discs were removed, blotted dry, frozen in liquid N_2 , and stored in a freezer until use.

Organelle Preparation. The preparation of a total organelle profile in a sucrose gradient followed that described previously (12). The leaves were deribbed, chopped into small pieces with a razor blade in a Petri dish containing grinding medium, and then ground gently with a mortar and pestle. The homogenate was filtered through a Nitex cloth with pore size $35 \mu m^2$. The filtrate was layered directly onto a sucrose gradient which was composed of a 30-ml gradient from 30% (w/w) to 60% sucrose. The gradient was centrifuged at 10,000 rpm for 10 min in a Beckman L2-65B ultracentrifuge using rotor SW 27, and fractionated.

Higher plants accumulate free proline in response to external water and salt stresses. Tracer experiments using radioactive glutamate established that the accumulation is due to an increase in proline synthesis from glutamate as well as a decrease in proline oxidation (23, 24). The physiological significance of proline accumulation is not known. While some workers maintain that it is a pathological consequence (9), others suggest that proline at high concentration acts as an osmoticum (26), a protective agent of enzymes and cellular structures (21), and a storage compound of reducing equivalent and nitrogen for rapid growth after the stress (2).

To elucidate the mechanism of proline metabolism during water stress as well as under normal conditions, the enzymes of the metabolic reactions between proline and its precursor, glutamate, have been investigated. From the information obtained through animal and bacterial systems (1, 8, 20, 27, 28), the following reactions could be involved (Fig. 1). In higher plants, P5C³

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³ Abbreviations: P5C: Δ^1 -pyrroline-5-carboxylate; P2C: Δ^1 -pyrroline-2-

carboxylate; DCPIP: 2,6-dichlorophenol-indophenol; FAD: flavin adenine dinucleotide.

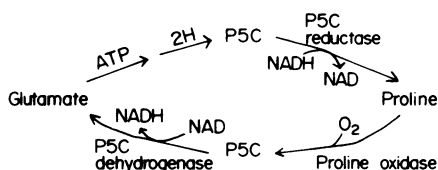


FIG. 1. Enzymic reactions between glutamate and proline.

For the preparation of mitochondria free of contaminating intact chloroplasts, the homogenate was obtained similarly using an onion chopper instead of a razor blade and a Petri dish. The homogenate was filtered through eight layers of cheesecloth. All of the intact chloroplasts and a fraction of the broken chloroplasts in the homogenate were removed by centrifugation at 500g for 10 min. The resulting supernatant fraction was recentrifuged at 10,000g for 30 min to yield a particulate fraction which was subjected to equilibrium sucrose gradient centrifugation (11). After 4 h of centrifugation, the visible mitochondrial band was collected and pooled from each of six gradients. The mitochondrial preparation was mixed with 2 volumes of 0.05 M Tris-HCl (pH 8.5), and centrifuged at 10,000g for 30 min. The pellet was resuspended in Tricine-KOH (pH 7.5) containing 0.6 M sucrose.

Assays. Free proline content in the leaf discs was assayed by the method described earlier (22). P5C was synthesized according to Strecker (28) using a P5C precursor obtained from Calbiochem. The quantitative analysis of P5C in the reaction mixture followed that described by Strecker (28) using *o*-aminobenzaldehyde.

Proline oxidase was assayed by either O₂ uptake measurement or spectrophotometry. In the O₂ uptake assay, the 3-ml reaction mixture contained 0.05 M Tris-HCl (pH 8.5), 5 mM MgCl₂, 0.5 mM FAD, 0.25 M sucrose, 0.1 M proline, and mitochondrial preparation. The reaction was monitored in a Clark O₂ electrode at 29 C using proline to initiate the reaction. In the spectrophotometric assay, the 1-ml reaction mixture contained 0.05 M Tris-HCl (pH 8.5), 5 mM MgCl₂, 0.5 mM FAD, 1 mM KCN, 1 mM phenazine methosulfate, 0.06 mM DCPIP, 0.1 M proline, and mitochondrial preparation. The reaction was monitored at 600 nm at 25 C using proline to initiate the reaction. The assay of Chl, catalase, and Cyt oxidase followed the methods described earlier (10, 11). Proline-dependent Cyt *c* reduction was measured in a 1-ml reaction mixture containing 0.05 M Tris-HCl (pH 8.5), 5 mM MgCl₂, 0.5 mM FAD, 5 mM KCN, 0.5 mg Cyt *c*, 0.1 M proline, and mitochondrial preparation. The reaction was monitored at 550 nm at 25 C.

Paper Chromatography. Paper chromatography was used to identify the reaction product qualitatively. The reaction mixture was shaken in air for 2 h. The amount of proline in the reaction mixture was reduced to 25 mM in order to minimize the proline spot on the paper chromatograph. Before and after the reaction, 10 μl of the reaction mixture was spotted on a Whatman chromatography paper (No. 1). Synthetic P5C was also spotted separately with and without proline for reference. Descending chromatography was run in 1-butanol-acetic acid-water (4:1:5) (16). After 24 h, the chromatographic paper was dried and sprayed with 0.2% ninhydrin in acetone.

RESULTS

Accumulation of Proline by Spinach Leaf Discs under Water Stress. Spinach leaves contained a negligible amount of free proline under normal conditions. When leaf discs were floated on a solution of PEG at -20 bars, they accumulated proline to more than 10 μmol/g fresh weight over a period of 24 h (Fig. 2A). This amount is equivalent to more than 0.1% of the total fresh weight. After the leaf discs had floated on PEG solution of different concentrations for 24 h, only those floated at -15 and -20 bars accumulated proline (Fig. 2B). Thus, the leaf discs did not accumulate proline until after an external threshold level of water stress at -10 bars.

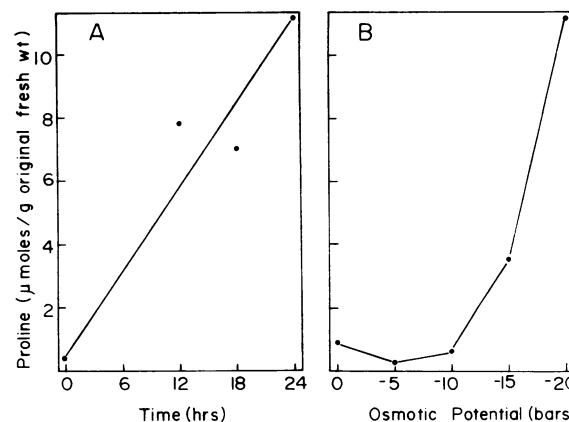


FIG. 2. Amount of free proline accumulated by spinach leaf discs. A: leaf discs were floated on PEG solution of -20 bars for various lengths of time; B: leaf discs were floated in PEG solution of indicated water potential for 24 h.

Subcellular Localization of Proline Oxidase. The subcellular localization of proline oxidase was studied using DCPIP as the electron acceptor. The total leaf extract was resolved into various organelle fractions using rate sucrose gradient centrifugation (Fig. 3). The intact chloroplasts, peroxisomes (catalase as the marker), broken chloroplasts, and mitochondria (Cyt oxidase as the marker) migrated to densities 1.20, 1.17, 1.16, and 1.14 g/cm³, respectively. Soluble enzymes of the cytosol and of broken organelles appeared at the top of the gradient. Proline oxidase was located together with Cyt oxidase at density 1.14 g/cm³ and thus represented a mitochondrial enzyme.

The mitochondrial fraction free of contaminating chloroplasts was obtained from equilibrium sucrose gradient centrifugation and used in subsequent study of proline oxidase.

Stoichiometry of Reaction Catalyzed by Proline Oxidase. The production of P5C and the uptake of O₂ were monitored in the reaction catalyzed by proline oxidase. There was a 1:1 stoichiometry of O₂ atom uptake and P5C production (Fig. 4).

In the quantitative assay of P5C, we employed the method of Strecker (28) using *o*-aminobenzaldehyde which reacted with both P5C and P2C. In order to see if indeed P5C was the reaction product, descending paper chromatography (16) of the reaction mixture was carried out. Before the enzymic reaction, only one yellow spot of ninhydrin-reactive compound was detected which was identified as the substrate proline. After the reaction, an additional pink spot was observed further away from the origin than the proline spot; it was identified as P5C. In the present solvent system of paper chromatography, P2C migrates slower than proline and P5C (16). We conclude that P5C was the reaction product that reacted quantitatively with *o*-aminobenzaldehyde (Fig. 4).

Properties of Proline Oxidase. In the DCPIP assay, the enzyme had its maximal activity at pH 8.0 to 8.5 (Fig. 5) and exhibited an apparent *K_m* value of 0.028 M for proline (Fig. 6). In our further study, we employed both the DCPIP assay and O₂ uptake assays. The O₂ uptake ability of the enzyme preparation was more sensitive to inhibition due to omission of assay components or addition of detergents (Table I). The enzyme required MgCl₂ and FAD for maximal activity, and FMN could not substitute for FAD. D-Proline was not a reactive substrate, and at a similar concentration as L-proline, it inhibited the enzyme activity partially. Triton X-100 at low concentration drastically reduced the enzyme activity. NAD exhibited no appreciable inhibition. The O₂ uptake ability of the enzyme preparation was greatly reduced by 5 mM KCN. NaCl was inhibitory to the O₂ uptake activity but had little effect, even at a concentration as high as 1 M, on the DCPIP reduction activity. Several compounds commonly used in osmotic studies were tested for their ability to inhibit the O₂

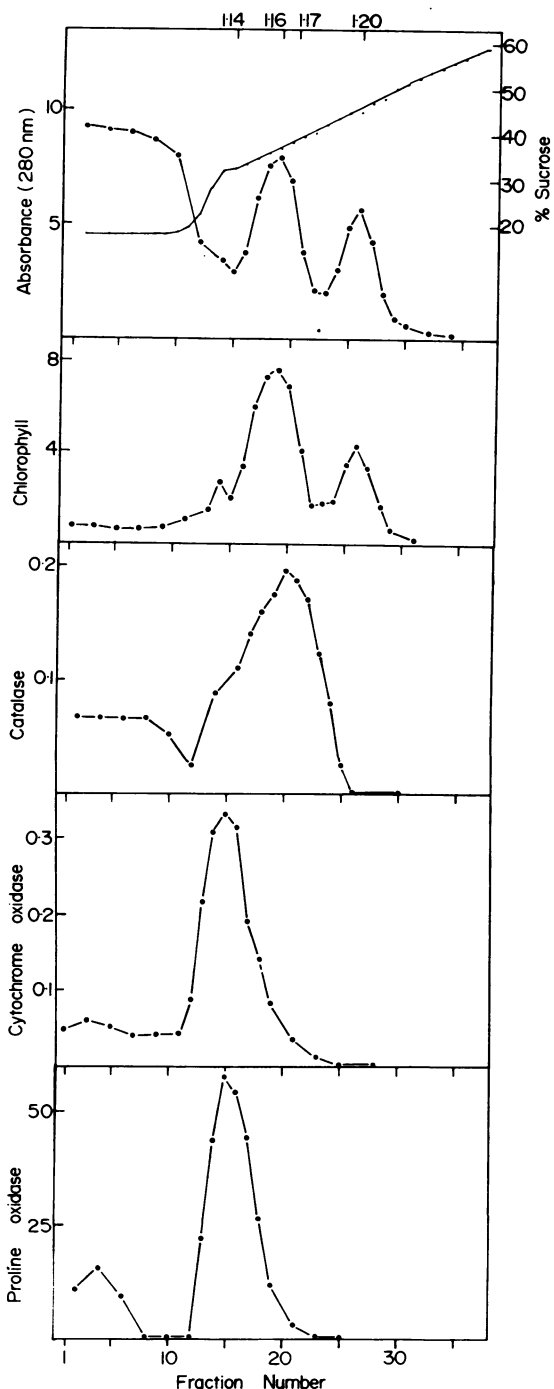


FIG. 3. Separation of organelles from total extract of spinach leaves. Total extract was applied directly onto sucrose gradient and centrifuged at 10,000 rpm for 10 min. Units/gradient fraction: protein, relative *A* at 280 nm; Chl, relative *A* at 652 nm; all enzymes, $\mu\text{mol}/\text{min}$.

uptake activity *in vitro*. Addition of sucrose and mannitol to reduce the water potential of the reaction mixture to as low as -20 bars resulted in little inhibition. Similarly, addition of PEG to reduce the water potential to -15 bars also had negligible effect. In a PEG solution of -20 bars, the observed O_2 uptake was reduced to 50%; we are not sure whether or not this reduction was a result of the interference of the assay due to the high viscosity of the reaction mixture.

In the presence of proline, the mitochondrial preparation was unable to reduce added NAD to NADH, but reduced exogenous Cyt *c*.

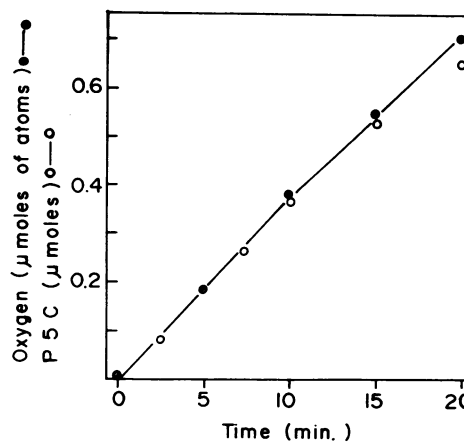


FIG. 4. Time course of O_2 uptake and P5C production in enzymic reaction catalyzed by proline oxidase.

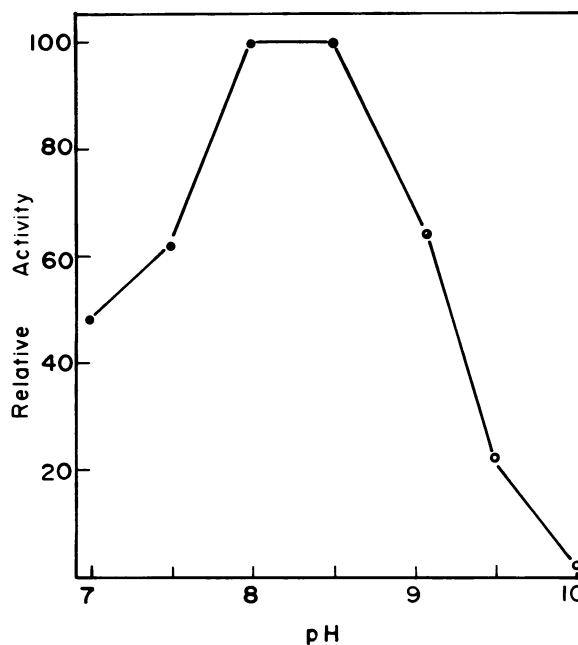


FIG. 5. Effect of pH on activity of proline oxidase. (●): Tris-HCl buffer; (○): cyclohexylaminoethanesulfonic acid-NaOH buffer. DCPIP reduction assay was used; 100% relative activity represents 8 nmol/min of enzyme activity in 21 μg of mitochondrial preparation.

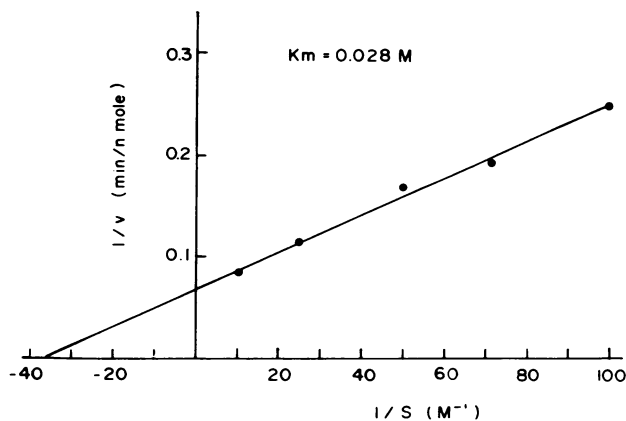


FIG. 6. Double reciprocal plot of activity of proline oxidase. DCPIP reduction assay was used.

Table 1. Effect of various compounds on the activity of proline oxidase. The enzyme activity was assayed by monitoring DCPIP reduction or oxygen uptake. Relative activity of 100 represented 8 nmoles/min in the DCPIP assay and 20 nmoles/min in the O₂ uptake assay using, respectively, 21 mg and 422 mg of mitochondrial preparation.

Addition to or deletion from the complete reaction mixture	Relative activity	
	DCPIP reduction	oxygen uptake
Nothing	100	100
- MgCl ₂	82	73
- FAD	73	14
- FAD, + FMN	73	-*
- L-proline, + D-proline	9	0
+ D-proline	90	39
+ triton-X-100 (0.1%)	43	17
(0.5%)	14	-
+ NAD (0.14 mM)	100	100
(0.28 mM)	100	-
+ KCN (5 mM)	-	7
+ NaCl (0.24 M)	100	78
(0.48 M)	97	33
(1.0 M)	86	-
+ Sucrose (- 5 bars)	-	92
(-10 bars)	-	99
(-15 bars)	-	78
(-20 bars)	-	92
+ Mannitol (- 5 bars)	-	96
(-10 bars)	-	89
(-15 bars)	-	100
(-20 bars)	-	100
+ PEG (- 5 bars)	-	100
(-10 bars)	-	94
(-15 bars)	-	88
(-20 bars)	-	47

* not tested

The failure of NAD to inhibit the enzyme activity and the inability of the enzyme preparation to reduce NAD to NADH indicate that NAD cannot serve as an electron acceptor. The enzyme is apparently not a NAD-proline dehydrogenase. The localization in the mitochondria, the relatively high K_m value for proline, the possible membrane association, the requirement of Mg²⁺ and FAD for maximal activity, and the linkage to Cyt and O₂ all indicate that the properties of the spinach leaf enzyme resemble those of a similar enzyme described in many animal tissues (20, 28). The enzyme appears to link to the electron transport system through the flavin moiety, and such a linkage is similar to those of succinate dehydrogenase and α -glycerol-P oxidoreductase (10).

Activity of Proline Oxidase in Relation to Rate of Proline Accumulation. After taking into consideration the per cent recovery of the isolated mitochondria from the leaves (11), we calculate the total *in vitro* proline oxidase activity to be 144 μ mol/day·g

fresh weight at 25 C using the DCPIP assay. The value is 17 μ mol/day·g fresh weight at 29 C employing the O₂ uptake assay. The lower O₂ uptake activity of the enzyme preparation is presumably due to the instability of the complete intact electron transport system. When the enzyme preparation was stored at 4 C, the DCPIP reduction activity remained unchanged for several days whereas the O₂ uptake activity decreased to about 50% after 24 h. With either value, the *in vitro* proline oxidase activity under optimal assay conditions is high enough to prevent proline accumulation (11 μ mol/day·g fresh weight; see Fig. 1) under water stress conditions. We suppose that the activity of the enzyme *in vivo* is somehow restrained under water stress.

DISCUSSION

We have shown that O₂-dependent proline oxidase exists in higher plants. This supports the indirect evidence of the existence

of such an enzyme in plant mitochondria (3). We found the enzyme in the particulate fraction of the endosperm of castor bean seedlings as well as in spinach leaves. Because a decrease in proline oxidation has been shown to contribute to proline accumulation during water stress (23, 24), proline oxidase should be a focal point for the elucidation of the mechanism of proline accumulation. In animal tissues, the enzyme was best studied in insect flight muscle in relation to energy production during the initial flight period. The enzyme has never been studied in relation to water stress-induced proline accumulation in eucaryotes.

Our finding of proline accumulation in spinach leaf discs under water stress is in agreement with this phenomenon in many other plant species (2, 5, 26). Spinach leaf discs accumulate proline only after a certain threshold of water stress. This finding reinforces our belief (5) that most if not all plant tissues have the ability to accumulate proline provided that the imposed water stress is severe enough.

The physiological significance of proline accumulation in plant tissues is unclear. It may well be different in different species. Hanson's data (9) indicate that the accumulation is a pathological consequence in barley leaves. Other plant species may accumulate proline for a beneficial reason of enduring water and temperature stresses (5, 6, 26). The role of proline as a cellular osmoticum is well documented in the algae, *Cyclotella cryptica* (13), and in certain fish and marine vertebrates living in fluctuating saline environment (7, 19). No convincing evidence exists to show directly the role of proline in regulating cellular osmosis in higher plants.

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