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IN VITRO STABILITY OF NITRATE REDUCTASE FROM BARLEY LEAVES*

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Abstract—Barley seedling nitrate reductase was stabilized *in vitro* without the use of extraneous protein by optimizing the buffer components. The extraction buffer (NRT 8.5) consists of 0.25 M Tris–HCl, pH 8.5, 3 mM DTT, 5 μM FAD, 1 μM sodium molybdate and 1 mM EDTA. This buffer stabilizes the extracted nitrate reductase at 0° and 30°, whereas the addition of extraneous protein to standard extraction buffers stabilizes the enzyme only at 0°.

INTRODUCTION

Nitrate reductase (NR) from many plant species is unstable *in vitro* [1]. *In vitro* stability can be improved by addition of bovine serum albumin or casein [2], or phenylmethylsulfonyl fluoride [3] to the standard extraction medium, indicating that *in vitro* instability of NR may be due to proteolytic degradation. The *in vitro* stability of barley nitrate reductase is a function of seedling age, growth temperature and extraction buffer [4]. The enzyme is most stable when extracted from young seedlings grown at 16°. Casein improves the *in vitro* stability of NR, but is undesirable in some situations [4, 5]. We report here a study concerning the development of an extraction buffer for stabilization of barley seedling NR without the use of external proteins and a comparison of NR stability in the presence and absence of casein.

RESULTS AND DISCUSSION

Tris(hydroxymethyl)aminomethane (Tris) was chosen as the basic buffer because NR was more stable when extracted with Tris than with phosphate, particularly in the absence of casein (unpublished). The *in vitro* stability of NR was significantly improved by increasing the Tris concentrations from 0.01 to 0.50 M (Fig. 1A). Maximal *in vitro* NR stability was obtained at Tris concentrations of 0.25 M or higher. Some of the protection afforded by the high Tris buffer concentrations may be a simple salt effect. Addition of NaCl to the low Tris buffer concentrations increased NR stability but not to the same extent as high concentrations of Tris. Evaluation of buffer pH indicated that pH 8.5 resulted in the most stable NR (Fig. 1B). Tris extraction buffer above pH 8.7 and below pH 8.2 resulted in a more rapid inactivation of NR. The optimal catalytic activity of barley NR is at pH 7.5 [6], thus the stabilizing effect of high pH is not the same as the optimal pH for enzyme catalysis. Purified NR from wheat leaves, however, was most stable at the NR activity optimal pH [7]. Optimum NR stability was achieved with 2 mM cysteine or 3 mM dithiothreitol (DTT). DTT, however, provided much greater NR stability than cysteine (Fig. 1C). Barley NR requires functional sulfhydryl groups for catalytic activities [8] and requires exogenous reducing agents to prevent *in vitro* enzyme oxidation. Nevertheless, concentrations of DTT at 5 mM reduced *in vitro* NR stability (Fig. 1C). Similar inhibitory effects of high concentrations of sulfhydryls have been observed in *A. nidulans* by Dunn–Coleman and Pateman [9] and in spinach by Palacian *et al.* [10]. Flavin adenine dinucleotide (FAD) concentrations greater than 10^{-6} M provided maximum stabilizing effect on NR (Fig. 1D). Sodium molybdate provided only small levels of NR protection compared with FAD. NR has been shown to be a molybdoflavoprotein involving Mo and FAD in the electron transport chain [11]. The effect of exogenous FAD and Mo may be attributed to maintaining prosthetic group integrity. EDTA (ethylenediamine tetraacetic acid) concentrations of 0.3 mM or greater provided maximum *in vitro* NR stability (Fig. 1F).

By optimizing the extraction buffer components for maximum *in vitro* NR stability, a new extraction buffer, NRT 8.5 (0.25 M Tris–HCl, pH 8.5, 3 mM DTT, 5 μM FAD, 1 μM sodium molybdate, and 1 mM EDTA) was developed. The NR extracted from 6-day-old barley with NRT 8.5 gave a decay rate of less than 5% per hr at 30° for at least 90 min. In contrast, in the buffer (0.1 M Tris–HCl, 1 mM cysteine, pH 7.5) used by Wray and Filner [8], 80% of the NR was lost within 30 min at 30°. Other buffers, including Bicine [*N,N* - bis(2 - hydroxyethyl)glycine], Tricine [*N* - tris(hydroxymethyl)methylglycine and glycylglycine at pH 8.5 each stabilized NR as well as Tris buffer.

The presence of casein in a phosphate buffer [1] improves the NR stability at 0°, but fails to do so at 30° (Table 1). However, the stability of barley NR in NRT 8.5 is only slightly affected by an elevated incubation temperature. This result indicates that the major stabilizing effect of casein might be different from that of NRT 8.5 which inhibits acidic proteases predominant in higher plants [12]. Perhaps casein

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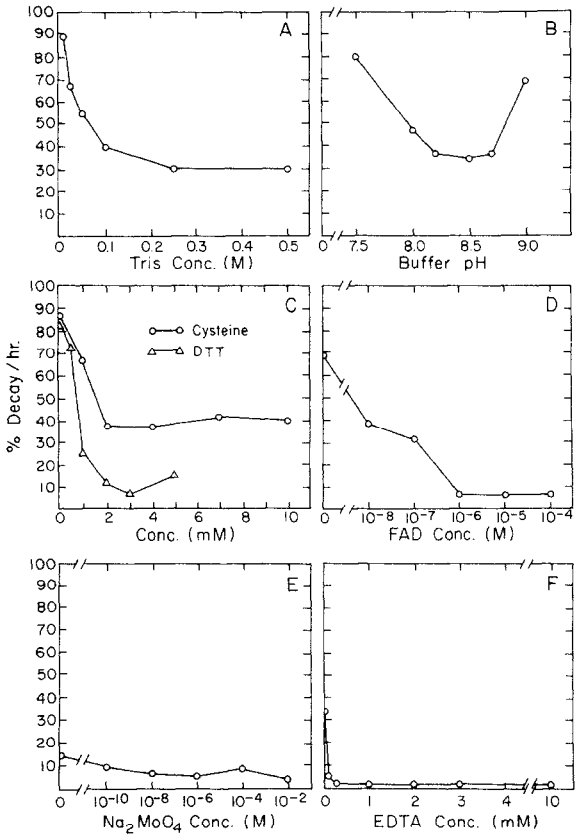

Fig. 1. (A) Effect of Tris concentration in the extraction buffer on *in vitro* stability of NR. Seedlings grown at 16° for 6 days were ground in different concentrations of Tris–HCl (pH 8.2), each containing 10 mM cysteine, 1 μM FAD, 1 μM Na_2MoO_4, and 1 mM EDTA. The crude extracts were maintained at 30° and the activities were determined as described in the Experimental. (B) Effect of pH on the *in vitro* stability of NR. The extraction buffer contained 0.25 M Tris–HCl at pHs indicated and other components as in (A). (C) Effect of sulfhydryl concentration on *in vitro* stability of NR. The leaf extracts were made in 0.25 M Tris–HCl (pH 8.5) and variable concentrations of cysteine or DTT as indicated. Other conditions as in (A). (D) Effect of FAD on *in vitro* stability of NR. The leaf extracts were made in 0.25 M Tris–HCl (pH 8.5) containing 3 mM DTT, 1 mM EDTA, and 1 μM Na_2MoO_4 in the presence of varying concentrations of FAD as indicated. Other conditions as in (A). (E) Effect of Mo on *in vitro* stability of NR. The leaf extracts were made in 0.25 M Tris–HCl (pH 8.5) containing 3 mM DTT, 1 mM EDTA, and 5 μM FAD in the presence of varying concentrations of Mo as indicated. Other conditions as in (A). (F) Effect of EDTA concentration on *in vitro* stability of NR. The leaf extracts were made in 0.25 M Tris–HCl (pH 8.5) containing 3 mM DTT, 5 μM FAD, and 1 μM Na_2MoO_4 in the presence of varying concentrations of EDTA as indicated. Other conditions as in (A).

serves as an effective adsorbent for polyphenols[13] in protecting NR from inactivation in addition to serving as a probable substrate for proteases[2]. Thus, addition of casein to NRT 8.5 would be beneficial in providing stable NR when the tissue–buffer ratio is high or when older tissue is used for study.

Table 1. Stability of barley (7-day-old) NR in various extraction media and at various incubation temperatures.

Extraction buffer	% decay per hr at 0°	% decay per hr at 30°
NRT 8.5	1	5
NRT 8.5 + 3% casein	0	6
Phosphate	10	89
Phosphate + 3% casein	6	85
Phosphate + 6% casein*	4	88

Phosphate buffer contained 25 mM KPi, pH 8.8, 10 mM cysteine, 1 mM EDTA[1]. In the presence of casein, the extraction medium was adjusted to its final pH with KOH.
*This is part of a single experiment; other figures represent an average of at least two additional experiments.

In this study 'stability' is used as a general term to represent detectable NR activity present in crude extracts. An unchanged NR activity may indicate that the NR is stable or that an activation of inactive NR occurs concurrently with inactivation of active enzyme. *In vitro* stability of barley NR is affected by a number of parameters including seedling age, growth temperature, concentration of buffer (salt), pH, FAD, sulfhydryl reagents, Mo, EDTA, and addition of external proteins. This indicates that many inactivating mechanisms may simultaneously affect NR.

EXPERIMENTAL

Plant culture and enzyme extraction. *Ca* 150–200 barley seeds (*Hordeum vulgare* L. cv Steptoe) were planted and watered daily by sub-irrigation with a modified Hoagland soln containing 15 mM nitrate as described in refs.[14, 15]. Leaf material above the coleoptile was harvested and immediately macerated with 6 ml buffer per g leaf material in a chilled mortar and pestle in the presence of acid-washed sand. The macerate was immediately squeezed through 2 layers of cheesecloth and centrifuged for 15 min at 30 000 *g*. All procedures were conducted at 0 to 2°. The starting extraction buffer was Tris–HCl, pH 8.2, containing 10 mM cysteine, 1 μM FAD, 1 μM Na molybdate, and 1 mM EDTA[6, 14]. For subsequent studies, the extraction buffer was sequentially modified to provide maximal NR stability.

Enzyme assay. NADH–NR was assayed as described in refs. [14, 15]. The *in vitro* decay rates of NR were determined by the ratio of NR activity at 30° after different incubation periods and plotted on a semilogarithm scale against incubation time[16, 17]. The decay of NR activity/unit time was calc. from the slope of the semilog plot and expressed as % loss/hr.

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