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SEROLOGICAL ESTIMATION OF SPINACH NITRATE REDUCTASE

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Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach; nitrate reductase; antibodies; biochemistry; enzyme inactivation.

Abstract—An antiserum raised in guinea pigs to a preparation of spinach nitrate reductase inactivated the enzyme. The amount of antiserum required to produce 50% inactivation of a standardized nitrate reductase preparation was increased by addition of enzymically inactive nitrate reductase. A quantitative relationship exists between the original activity of the added enzyme and the required increase in antiserum.

INTRODUCTION

NADH nitrate reductase (NR) (E.C. 1.6.6.1) from higher plants is unstable both *in vivo* [1–3] and *in vitro* [4] and the rate of loss of NR activity in a crude plant extract varies with the species [5]. Furthermore, the amount of extractable active NR may decline because of inactivation *in vivo* or during extraction; for example by cyanide [6,7], phenolics [8], amino acids [9,10], dark [1,3] or by ammonium compounds or other agents [11–13], or because of protein turnover *in vivo* [14,15]. The enzyme has yet to be isolated in a homogeneous state from higher plants, and the supposed reasons for the losses in activity in different circumstances must therefore be based only on indirect evidence. As loss of activity occurs for many reasons, of which some are of potential physiological importance, it is of interest to determine the extent to which the enzyme protein is present, irrespective of its catalytic activity.

Antibodies have been raised against NR extracted from *Aspergillus nidulans* [16] and *Escherichia coli* [17], but not previously from a higher plant. We report here the use of antibodies raised against a semipurified NR preparation from spinach leaves to detect and estimate the total amount of soluble cross-reacting NR protein measured in terms of enzyme activity units but not necessarily having NR activity.

RESULTS AND DISCUSSION

Preliminary tests established that immune serum completely inactivated and non-immune serum slightly stimulated NR activity. An enzyme preparation having an initial activity of 2000 units/ml and an activity of 1000 units/ml when used 4 days after preparation, was completely inactivated with immune serum diluted up to 40-fold; non-immune serum stimulated activity up to 20% of a no-serum control. When the experiment was repeated with a freshly-prepared enzyme with an activity of 6600 units/ml, the immune serum at 40-fold dilution produced only 61% inactivation. Since the concentration of NR obtained from different preparations varied, different amounts of serum were required to produce the same degree of inactivation. A "standard" enzyme was therefore used, NR prepared as described in Experimental was diluted with 0·1 M potassium phosphate, pH 7·5 containing 10^{-3} M EDTA, to an activity of 2800 units/ml

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at the end of the 2-day purification. This activity was selected as being in the most sensitive assay range using a 0·1 ml aliquot of the enzyme-serum mixture. It was postulated that equivalence between NR antibodies and enzyme would be a function of the total amount of NR protein present in spite of loss of catalytic activity. This was tested by adding to a catalytically-active enzyme an NR preparation which had lost its enzyme activity but retained its immunochemical activity. This addition should cause an increase in the concentration of serum required to inactivate the catalytically-active enzyme, due to competition between the active and inactive enzymes for antibodies. An NR preparation having an activity of 12900 units/ml when fresh was diluted 4·6-fold to an activity of 2800 units/ml with the following: (a) potassium phosphate buffer, pH 7·5 containing 10^{-3} M EDTA; (b) a sterile inactive 8-month-old NR preparation which had been stored at 4°, whose original activity was 5800 units/ml; and (c) the same inactive NR preparation previously diluted 1:5 with buffer. Each of these solutions were mixed with equal volumes of serially diluted immune serum, incubated for 1 hr, and NR activity determined in the supernatant. Additionally, the buffer-diluted enzyme was mixed with equal volumes of serially diluted, non-immune serum. Results were plotted as amounts of serum protein against residual enzyme activity calculated as a percentage of a non-serum control using instead 0·15 ml buffer. Calculations based on 50% NR inactivation are shown in Table 1, columns A.

In an attempt to quantify the shift in the NR inactivation curve by addition of inactive enzyme, a fresh NR preparation having an activity of 8400 units/ml was diluted three-fold to a "standard" activity of 2800 units/ml either with buffer or with various pre-diluted solutions of a different inactive sterile enzyme preparation 10·5 months old whose original activity was 16400 units/ml and final activity after storage at 4° was 4 units/ml. The total dilutions of inactive enzyme in the final active enzyme solution were 1·5, 5, 10, 20 and 100-fold. Each of the active enzyme solutions were mixed with successive dilutions of immune serum and residual activities determined as before. Inactivation curves were progressively shifted to higher serum concentrations and calculations based on 50% inactivation points are shown in Table 1, columns B. When the log of the original activities of the added inactive enzyme (x axis) were plotted against the log of the resultant increases in serum required to produce 50% inactivation of the NR (y axis), a linear relationship was obtained with the following characteristics: $x = 1{\cdot}35$ (s.e. $\pm$ 0·10)y − 1·04. Results of both experiments in Table 1 were included in the plot and indicate the reproducibility of the relationship based on separate proportions of different initial activity. Enzymically-inactive NR may therefore be detected and quantified in terms of active NR by using the inactivation of a standardized enzyme solution with antibodies raised against a semi-purified preparation. By using the methods described, it seems possible to quantify enzymically-inactive protein, and this technique may have wider applications, especially where several alternative causes of loss of catalytic activity are suspected.

Table 1. Effect of additions of enzymically inactive NR to a "standardized" NR preparation on the amount of immune serum required to cause 50% enzyme inactivation

Inactive enzyme added (units × 10^{-1}/ml)		Serum concn at 50% inactiv. (μg × 10^{-1}/ml)		Increase in serum concn at 50% inactiv. (μg × 10^{-1}/ml)	
A	B	A	B	A	B
0		122		0	
91		207		85	
457		454		332	
	0		141		0
	16		172		31
	82		225		83
	164		245		104
	329		368		227
	1095		748		607

Guinea pig immune serum protein content 73·6 mg/ml.

Inactive NR A. Purified preparation, aged 8 months, original activity 5800, final activity zero. B. Purified preparation, aged 10·5 months, original activity 16400, final activity 4.

"Standard" NR preparation A. Purified to activity of 12900, diluted 4·6-fold to 2800. B. Purified to activity of 8400, diluted 3-fold to 2800.

EXPERIMENTAL

Spinach plants (*Spinacia oleracea* L.) were grown in sand using a Long Ashton complete nutrient [18]. NR was extracted and purified as previously described [19] using $Ca_3(PO_4)_2$ gel absorption, $(NH_4)_2SO_4$ pptn and Biogel A 0·5 m chromatography, with one additional passage through the agarose column. The final eluate was concentrated by dialysis under pressure to *ca* 5 mg/ml gross protein and sterilised by filtration. Specific activities (defined below) ranged from 1313 to 4700 NR

activity units/mg protein, a purification factor of 20- to 58-fold.

Antibodies to NR were raised in guinea pigs (strain Dunkin Hartley, smooth haired, aged 6 weeks) by serial injections initially subcutaneously with 1 ml (1·8 mg gross protein) of a 30-fold purified NR preparation emulsified in Freund's complete adjuvant and 3× at 2- or 4-week intervals intravenously without adjuvant with 0·2 ml (0·8–1·5 mg gross protein) of enzyme purified 20- to 58-fold. Serum was obtained from immunized and control animals after 16 weeks, sterilized by filtration and stored frozen. Antibodies to the enzyme were assayed by mixing equal vols (0·15 ml) of serum serially diluted in 0·1 M K phosphate, pH 7·5 and enzyme of activity 2800 units/ml, standing at 4° for 1 hr, centrifuging (14000 *g* for 5 min) and determining residual activity in the supernatant. Control experiments used non-immune serum. NR activity was measured by the NADH method [20], and 1 unit of enzyme is defined as 1 nmol of NO_2^- produced at 27°/15 min either per mg protein or per ml of soln as appropriate. Serum protein was determined by A at 280 nm with reference to serum albumin, and plant protein by the Folin method [21].

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