Phytochemistry, Vol. 26, No. 9, pp. 2467–2470, 1987.
Printed in Great Britain.

0031–9422/87 $3.00 + 0.00

AN ENZYME LINKED IMMUNOSORBENT ASSAY FOR NITRATE REDUCTASE USING MONOCLONAL ANTIBODIES

PETER N. WHITFORD, ROGER J. FIDO and BRIAN A. NOTTON

Department of Agricultural Sciences, University of Bristol, Long Ashton Research Station, Long Ashton, Bristol BS18 9AF, U.K.

(*Received* 1 *December* 1986)

Key Word Index—*Spinacea oleracea*; Chenopodiaceae; spinach; immunoassay; nitrate reductase; monoclonal antibodies.

Abstract—An indirect sandwich enzyme linked immunosorbent assay (ELISA), which used both rabbit polyclonal and rat monoclonal antibodies to nitrate reductase (NR) (E.C. 1.6.6.1), was adapted to measure NR protein in crude extracts of spinach (*Spinacea oleracea*) plants. Conditions were optimised for maximum dose response with respect to coating times and dilutions of antibodies and antigen. These were polyclonal, 1 hr and 1/3000; antigen, 1 hr; monoclonal, 1 hr and 50 μg protein/ml for MAC 74; and antispecies, 40 min at 27° and 1/2000 sheep anti-rat. The ELISA signal was a linear function of the amount of NR up to *ca* 1.5 ng and of the log of the amount of NR over the range 20–1200 ng. All our monoclonals (AFRC MAC 74–79) gave positive dose responses and the method is illustrated with MAC 74. Changes in antigenic NR in leaves of spinach plants on nutrient-nitrate removal followed by resupply and the distribution of NR in roots, petioles and leaves of spinach plants, were measured by ELISA and shown to be related to changes in NADH–NR activity.

INTRODUCTION

Nitrate reductase (NR) (E.C. 1.6.6.1) is the rate limiting enzyme for the assimilation of inorganic nitrogen in eukaryotic species. Its properties have been the subject of a number of reviews [1–4]. In most higher plants, NR preferentially utilizes NADH to reduce nitrate and also displays two partial activities; (a) the reduction of nitrate by either reduced FMN or methyl viologen (MV), a reaction which is independent of the NADH site and (b) the reduction by NADH of cytochrome *c*, a reaction which is independent of the nitrate binding site. The M_r is 200 000–250 000 and is a homodimer. Structural subunits contain FAD and cytochrome b_{557}. The nitrate binds at a molybdopterin complex which has a structure common to most molybdoenzymes [5]. NR is unstable both *in vivo* and *in vitro*, requiring the addition of protective agents such as leupeptin [6], FAD, EDTA and a sulphydryl compound in extracting media in order to retain *in vitro* activity (see [1–4] for references) which, from some species, is up to 1.67 μkat nitrite produced/mg protein [7–9].

An alternative to NADH–NR activity determination is some form of immunoassay to measure the amount of NR protein, such as that recently reported [10] for corn (*Zea mays*). NR is a good immunogen and comparatively small amounts of pure enzyme are required to raise antisera. Polyclonal antibodies raised to the enzyme from one species have been shown to inhibit the enzyme activity of a number of other related species [11], indicating that some degree of homology exists between NR protein structures. Monoclonal antibodies, designated AFRC MAC 74–79, have been obtained to spinach (*Spinacea oleracea*) NR [12] which differentially inhibit its activities (NADH–NR and either MV–NR or NADH–cytochrome *c* reductase), and bind at different sites on the enzyme.

Using these monoclonals, together with polyclonal antiserum raised in a different animal species, we have adapted an enzyme-linked immunosorbent assay (ELISA) and determined optimum conditions to measure the amount of antigenic NR present in crude extracts of spinach plants. Although all the monoclonals produced a dose response, we have concentrated on MAC 74 because of its ability to bind to, and inhibit the NR activity of, roots and leaves of all plant species so far tested and which may therefore have more general application.

RESULTS AND DISCUSSION

The purity of the NR [9] used to raise the monoclonals and their ability to inhibit NADH–NR activity [12] decreased the possibility that they were raised to a highly antigenic contaminating protein present in small amounts in the NR preparations used as the immunogen. It was, however, necessary to determine optimum conditions for the ELISA and to establish a response to changes in the NR content of crude plant extracts. Conditions finally selected reflect economy of both time and materials with minimum loss of sensitivity of the signal.

Coating of the ELISA plates with a five-fold dilution series of polyclonal antiserum gave a maximum signal with 1/3000 dilution using 25 ng of NR, MAC 74 and peroxidase-labelled rabbit anti-rat (Fig. 1A). Increasing the concentration of antiserum decreased the signal, possibly because of multilayering of the polyclonal in the wells and formation of antibody/antigen complex which was not attached to the plastic. Extending the polyclonal coating time beyond 1 hr only slightly increased the signal after 3 hr and overnight coating produced no further increase, therefore 1 hr was chosen. The ELISA signal increased up to the limiting concentrations of each of the

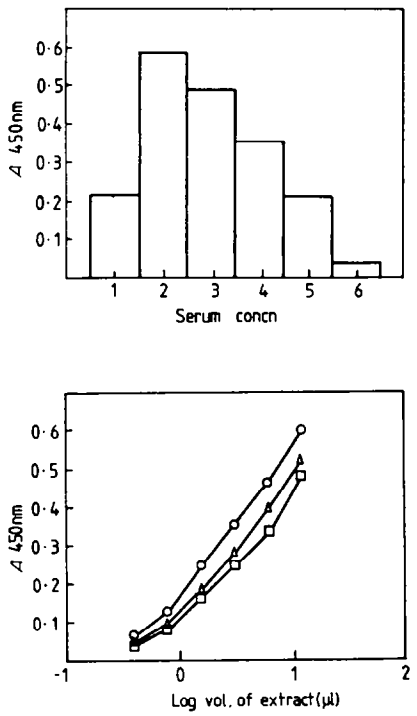

Fig. 1. Optimum parameters for ELISA. (top) Effect of increasing concentration of polyclonal anti-NR on ELISA signal, with 25 ng NR and 50 μg/ml MAC 74. Dilution of anti-NR in 0.1 M $NaHCO_3$ pH 8.3. (1) 1/15 625; (2) 1/3125; (3) 1/625; (4) 1/125; (5) 1/25; (6) 1/5. (bottom) Effect of increasing concentration of MAC 74 on ELISA signal plotted vs log vol (μl) of spinach leaf extract. Polyclonal anti-NR at 1/3000 dilution. Coating time 1 hr. Conc of MAC 74 100 μg/ml (O); 10 μg/ml (Δ); 5 μg/ml (□).

monoclonal antibodies tested, depending on the avidity of the monoclonal for the NR and the concentration of the monoclonal in the ascites fluid. The results obtained for MAC 74 are shown in Fig. 1B. The signal increased with increasing concentrations of MAC 74 from 5 to 100 μg/ml: 50 μg/ml was chosen as adequate. Similarly the concentration of peroxidase-labelled anti-rat was optimised at 1/2000, because increased concentrations tended to raise all values by the same extent, including that of the controls.

Mixing two monoclonals (MAC 74 and 78), which bind at different sites on the NR and which are non-competitive [12], at their pre-determined optimum concentrations, increased the ELISA signal (result not shown). However, MAC 78 appears to be spinach NR-specific [12] and this effect would not be of general application.

An ELISA standard curve using pure NR with MAC 74 resulted in a signal which was a linear function of the amount of NR up to 1.5 ng and a log function of the amount of NR from 20 to 1200 ng (Fig. 2). A linear response to corn NR up to 10 ng has been shown using a polyclonal/polyclonal sandwich ELISA [10]. In other work, using a polyclonal/mixed monoclonals sandwich ELISA, a linear response to phytochrome from oat (*Avena sativa*) up to 1.2 ng/well has been shown [13]. Over a larger range of up to *ca* 500 ng phytochrome, using a polyclonal/polyclonal sandwhich ELISA, the signal was

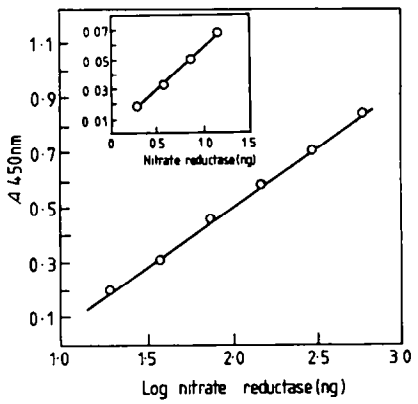

Fig. 2. Standard calibration curve for NR. ELISA signal plotted vs log amount of NR (20–1200 ng). Insert shows ELISA signal plotted vs amount of NR (up to 1.5 ng).

found to be an approximate linear function of the log of the phytochrome concentration [14].

Using crude extracts of spinach leaves, an NADH–NR activity dilution curve was a linear function of volume and/or time (results not shown), while the ELISA signal was a linear function of the log of the volume from 12.5 μl down to 0.1 μl/well (Fig. 3).

Addition of varying amounts of pure NR, from 37.5 to 300 ng, to a crude spinach leaf extract, produced ELISA signals which were linear to the log of the extract (plus NR) volume from 25 μl down to 0.1 μl/well (Fig. 4). Increasing amounts of NR increased both the slopes and intercept values and a plot of the change in signal against the log of added NR produced a straight line.

The nitrate-inducible nature of NADH–NR was used to test the ELISA response. Young spinach plants growing in complete nitrate nutrient were deprived of nitrate for two days and then resupplied with nitrate for a further two days. Samples were taken each day and extracts assayed for total protein, NADH–NR activity and antigenic-NR by ELISA. The ELISA responses showed a linear relationship to the log of volume of extracts as in Fig. 3 and the NR protein values calculated from the standard curve were commenserate with the NADH–NR activities rather than the total protein (Table 1).

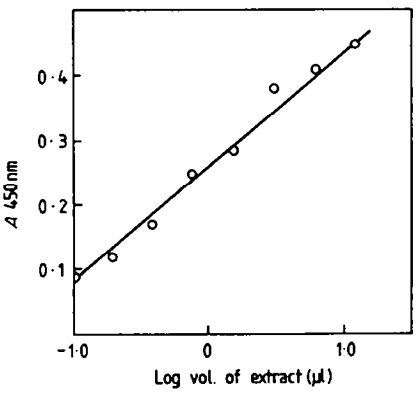

Fig. 3. Relationship between ELISA signal and log vol (μl) of crude spinach leaf extract.

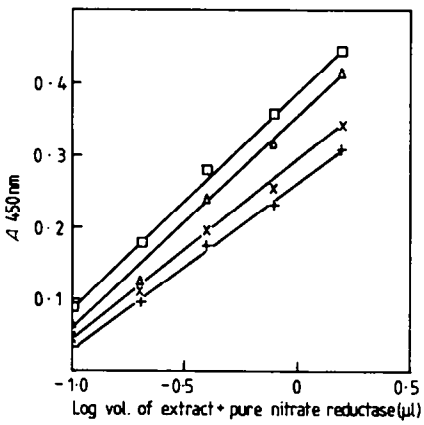

Fig. 4. Effect on ELISA signal of addition of pure NR to crude spinach leaf extracts plotted vs log vol (μl) of extract plus NR. 300 ng NR (□); 150 ng NR (Δ); 75 ng NR (×); 37.5 ng NR (+).

The distribution of antigenic-NR in the roots, petioles and individual leaves of a young spinach plant was similarly independent of the total protein differences and related to NADH–NR activity (Table 2). It would seem probable that, provided a polyclonal antiserum raised to NR of a plant species binds to NR of the species under examination, the method described using MAC 74 could be used to estimate the antigenic-NR content.

EXPERIMENTAL

Plant material. Spinach plants were grown using a nutrient-flow technique (NFT) with complete nitrate nutrient or nitrate-free nutrient [15].

Enzyme purification. Spinach NR was purified from leaves using a multistage procedure [9] up to 3400-fold to a sp. act. of *ca* 1.67 μKat NO_2^- produced/mg protein (using BSA as ref. protein) and was substantially free of 'nicked' sub-units as judged by SDS-PAGE [9].

Enzyme assay. NADH–NR activity of cell-free extract was determined in test tubes [16] or adapted to microtitre plates. Protein was determined in microtitre plates [17].

Antibodies. (1) Monoclonal antibodies (AFRC MAC 74–79) were obtained from rats as previously described [12]. Ascites fluid was purified by pptn with $(NH_4)_2SO_4$ at 50% satn, dil. to 10 mg/ml and stored at −20°. (2) Polyclonal antibodies were raised in rabbits by injection with 1 mg pure NR in Freund's complete adjuvant and boosted with 1 mg pure NR after four and eight weeks. Antigenicity was determined by the ability of the antiserum to inhibit NADH–NR activity. (3) Rabbit anti-rat IgG peroxidase labelled was obtained commercially.

Table 1. Effect of nitrate starvation and reassimilation on total protein, NADH–NR activity and NR-protein measured by ELISA of extracts of leaves of young spinach plants

Nutrient status of plants	Protein (μg/mg leaf)	NADH–NR activity (nKat NO_2^- produced/mg leaf)	NR-protein (ELISA) 10 μl extract (3.3 μg leaf)	
			A_{450}	ng NR-protein*
+ Nitrate nutrient	37.6	3.85	0.71	315
− Nitrate (24 hr)	36.6	2.70	0.63	203
− Nitrate (48 hr)	35.4	1.75	0.54	124
+ Nitrate (24 hr)	37.8	2.80	0.65	227
+ Nitrate (48 hr)	40.8	3.50	0.76	414

*Antigenic NR-protein calculated from standard ELISA curve see Fig. 2.

Table 2. Total protein, NADH–NR activity and NR-protein measured by ELISA of extracts of roots, petioles and individual leaves of a healthy spinach plant

Material	Protein (μg/mg material)	NADH–NR activity (nKat NO_2^- produced /mg material)	NR-protein (ELISA) 10 μl extract (3.3 μg material)	
			A_{450}	ng NR-protein*
Roots	3.6	0.05	0.107	12
Petioles	2.9	1.53	0.452	77
Leaf 1	36.0	6.08	0.661	241
Leaf 2	26.4	6.69	0.673	258
Leaf 3	15.0	2.29	0.469	84
Leaf 4	20.1	5.28	0.675	261
Leaf 5	18.0	2.89	0.582	156
Leaf 6	19.5	4.86	0.641	217

*Antigenic NR-protein calculated from standard ELISA curve see Fig. 2.

Standard ELISA protocol. The composition of coating, blocking and substrate solns were those used for screening supernatant solns of hybridomas [18]. Optimum parameters for the ELISA were determined (see Results) and the standard protocol was as follows: wells of polystyrene flat-bottomed ELISA plates (Nunc Gibco) were coated with 50 μl of polyclonal anti-NR rabbit serum diluted 1/3000 in 0.1 M $NaHCO_3$ pH 8.3. After incubating for 1 hr at room temp., wells were emptied and blocked with 3% BSA in PBS (8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g K_2HPO_4 in 1 l of H_2O) and incubated for 1 hr. Test soln (50 μl serially dil. eight-fold with blocking soln) was added to each well and incubated for 1 hr. The wells were emptied, washed twice with blocking soln and 50 μl of monoclonal antibody, appropriately dil. with blocking soln, added. After 1 hr incubation, the wells were emptied, washed twice with blocking soln and 50 μl of anti-rat antibody (dil 1/2000 in blocking soln) added. After 1 hr incubation, the plates were emptied, washed × 10 with cold H_2O and 150 μl of substrate (13.5 ml H_2O, 1.5 ml 1M NaOAc buffer, pH 6, 150 μl of 10 mg/ml tetramethylbenzidine in DMSO and 15 μl of 6% H_2O_2 added. The plate was incubated for up to 30 min at 27° and the reaction stopped with 25 μl of 2 M H_2SO_4. A_{450} was determined using an ELISA plate reader. Data were captured on disc and slopes, intercepts, regression coefficients and standard errors calculated using 'in-house' software on a microcomputer (D. P. Hucklesby, personal communication).

Acknowledgement—Long Ashton Research Station is funded through the Agricultural and Food Research Council.

REFERENCES

1. Notton, B. A. and Hewitt, E. J. (1979) in *Nitrogen Assimilation of Plants* (Hewitt, E. J. and Cutting, C. V., eds) pp. 227–244. Academic Press, London.
2. Hewitt, E. J. and Notton, B. A. (1980) in *Molybenum and Molybdenum Containing Enzymes* (Coughlan, M. ed.) pp. 273–325. Pergamon Press, Oxford.
3. Guerrero, M. G., Vega, J. M. and Losada, M. (1981) *Annu. Rev. Plant Physiol.* **32**, 169.
4. Campbell, W. H. (1985) in *Nitrogen Fixation and CO_2 Metabolism* (Ludden, P. W. and Burris, J. E. eds) pp. 143–151. Elsevier, Amsterdam.
5. Johnson, J. L. (1980) in *Molybdenum and Molybdenum Containing Enzymes* (Coughlan, M. ed.) pp. 347–383. Pergamon Press, Oxford.
6. Wray, J. L. and Kirk, D. W. (1981) *Plant Sci. Letters* **23**, 207.
7. Kuo, T., Somers, D. A., Kleinhofs, A. and Warner, R. L. (1982) *Biochem. Biophys. Acta* **708**, 75.
8. Redinbaugh, M. G. and Campbell, W. H. (1983) *Plant Physiol.* **71**, 205.
9. Fido, R. J. and Notton, B. A. (1984) *Plant Sci. Letters* **37**, 87.
10. Campbell, W. H. and Remmler, J. L. (1986) *Plant Physiol.* **80**, 435.
11. Smarreli, J. and Campbell, W. H. (1981) *Plant Physiol.* **68**, 1226.
12. Notton, B. A., Fido, R. J. and Galfre, G. (1985) *Planta* **165**, 114.
13. Shimazak, Y., Cordonnier, M.-M. and Pratt, L. H. (1983) *Planta* **159**, 534.
14. Thomas, B., Crook, N. E. and Penn, S. E. (1984) *Physiol. Plant.* **60**, 409.
15. Watson, E. F. and Hewitt, E. J. (1980) *Acta Hort.* **98**, 179.
16. Wray, J. L. and Filner, P. (1970) *Biochem. J.* **119**, 715.
17. Redinbaugh, M. G. and Campbell, W. H. (1985) *Anal. Biochem.* **147**, 144.
18. Galfre, G. and Milstein, C. (1981) *Methods Enzymol.* **73**, 3.