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IMMUNOLOGICAL COMPARISON OF SPINACH AND *CHLORELLA* NITRATE REDUCTASE

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

Abstract—Polyclonal antibodies raised against spinach (*Spinacea oleracea*) and *Chlorella vulgaris* nitrate reductases, cross-react with the enzymes with limited recognition. Monoclonal antibodies, previously raised against spinach NR, which bound to *Chlorella* NR were detected by direct enzyme linked immunosorbent assay (ELISA) and immunoblotting but did not inhibit *Chlorella* NR enzyme activities. Two new monoclonal antibodies raised against spinach NR (designated AFRC MAC 231 and 232) have been obtained, which recognised both native and denatured spinach NR and *Chlorella NR* with high avidity without inhibiting their enzyme activities.

INTRODUCTION

Nitrate reductase (NR) (E.C. 1.6.6.1), catalyses the initial and rate limiting step in nitrate assimilation. The enzyme has been isolated from a variety of eukaryotic sources including algal (e.g. *Chlorella vulgaris*), fungal (*Neurospora crassa*), yeast (*Candida nitratophila*) and higher plants and its properties have been the subject of a number of reviews [1–5]. In addition to the full activity, the transfer of reducing equivalents from NADH to nitrate, the enzyme, a multimeric molybdohaemoflavoprotein, exhibits a number of partial activities such as NADH–ferricyanide reductase (NADH-Fed Rase), NADH–cytochrome *c* reductase (NADH-cyt *c* Rase) and reduced methyl viologen–nitrate reductase (MV–NR). These reactions require the involvement of one or more of the prosthetic groups of the enzyme and have been used in the study of functional domains [1–5].

Immunological methods, using polyclonal antibodies, have been widely used to compare structural similarities, in terms of conservation of antigenic recognition sites, between NRs isolated from different sources. These include spinach (*Spinacea oleracea*) [6], barley (*Hordeum vulgare*) [7], squash (*Cucurbita maxima*) [8], *N. crassa* [9] and *Chlorella* [10, 11]. In several cases, immunodetection methods, such as Ouchterlony double diffusion and rocket immunoelectrophoresis and inhibition of enzyme activities, have been used to compare cross reactivity of the NRs. The extent of cross reaction, i.e. whether immunoprecipitation occurred as well as enzyme inhibition, appeared to depend on the phylogenetic distance between the various organisms but all NRs had some common antigenic determinants [8, 9, 11].

Monoclonal antibodies have also been used for comparative purposes. Since individual monoclonals recognise a single antigenic determinant, this presents an assay for the conservation of specific antigenic sites. Monoclonal antibodies have been obtained against NR from spinach [12], squash [13] maize (*Zea mays*) [13, 14] and barley (Kleinhofs, A., personal communication). While cross-reactivity has been demonstrated between some of these monoclonals and NRs from other plant species, no cross reactivity, as judged only by inhibition of enzyme activity, was found between the monoclonals raised against maize NR and the enzyme from an algal source (*Chlorella pyrenoides*) [15].

As part of a study on the comparative structure and function of NR from spinach and *Chlorella* [16], we report here the relative ability of seven monoclonal antibodies raised against spinach NR to antigenically recognise the *Chlorella* enzyme.

RESULTS AND DISCUSSION

Polyclonal antibodies raised against spinach NR and *Chlorella* NR inhibited the NADH–NR activity of both enzymes (Fig. 1). However, the cross reaction required a higher concentration of antibodies to produce equivalent inhibition, confirming the previously reported effects of antibodies raised against squash NR on *Chlorella* NR [8], and against *Chlorella* NR on spinach NR [11]. In neither case was there sufficient homology to produce an immunoprecipitate. The ability of the antibodies from one source to inhibit the enzyme activities of the enzyme from another source, suggests that there is sufficient commonality of antigenic sites to ensure that some of the monoclonal antibodies raised against spinach NR would recognise the *Chlorella* NR. Similar inhibition curves were obtained for the various partial activities of NR (not shown).

Monoclonal antibodies raised against spinach NR had been selected for their ability to inhibit the NADH–NR activity and differentially inhibit the partial activities of the enzyme [12]. When these were tested against the pure *Chlorella* enzyme, no inhibition of any of the activities was found. An apparent three to four-fold stimulation of the NADH–NR activity by these monoclonals was

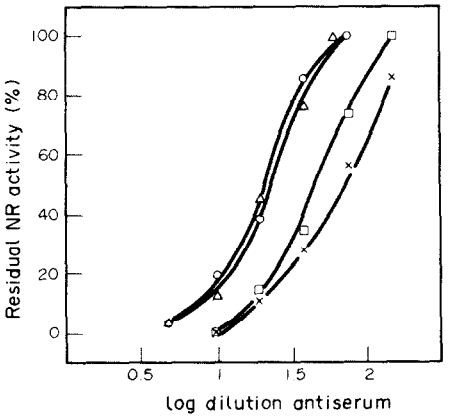

Fig. 1. Inhibition of NADH-NR activity of spinach NR and *Chlorella* NR by polyclonal antisera raised against the two enzymes. *Chlorella* NR v. anti*Chlorella* NR (X); spinach NR v. antispinach NR (□); *Chlorella* NR v. antispinach NR (△) and spinach NR v. anti*Chlorella* NR (○). 100% activity of spinach NR was 3 nKat NO_2 produced/ml and *Chlorella* was 4.3 nKat NO_2 produced/ml.

shown, by using monoclonals which were unrelated to NR but of the same subclass (see Experimental) and at the same protein concentration, to be due to stabilisation of the enzyme. In the absence of any monoclonal, the enzyme was inactivated at the large dilution used in the test solution (Table 1).

Exudates from previously uncloned cell lines raised against spinach NR [12] were tested by direct enzyme linked immunosorbent assay (ELISA) against *Chlorella* NR. This produced six positive reactions. The two most positive cultures were cloned [17] and rescreened against *Chlorella* NR. A positive cloned cell line from each culture was selected for production of supernatant and ascites fluid [17]. Both monoclonals were characterized as IgM (Butcher, G. M., personal communication). They were designated AFRC MAC 231 and 232. Neither of these monoclonals was able to inhibit the activities of spinach NR or *Chlorella* NR while producing very strong ELISA responses with both enzymes.

The ability of the monoclonals to recognise *Chlorella* NR was tested by direct ELISA and the responses compared with those obtained from spinach NR, at approximately the same enzyme concentration as judged by NADH–NR activity (Fig. 2). The ELISA response for MACs 232 and 231 was such that the reaction was stopped after five and 11 min respectively, whereas the reaction with the other monoclonals was allowed to proceed for 45 min. Monoclonals unrelated to NR served as controls. Apart from MACs 231 and 232, chosen for their ability to recognise both NRs, the order of response was: spinach: MAC 75, >77, >79, >74 and 78; Chlorella: MAC 77, >79, >75, >78 and 74. MAC 77, which has been shown to inhibit NADH–NR and MV–NR activities of a number of different plant species [12], bound relatively strongly to the *Chlorella* NR without inhibition of activity. This would suggest a conformational difference between the two NRs, without a change in the particular antigenic site. MAC 74, which has similar inhibitory properties to MAC 77 [12], and which has been used to immunopurify the spinach NR [18] and to measure antigenic spinach NR by indirect ELISA [19], failed to recognise *Chlorella* NR, suggesting a considerable difference at the antigenic site of MAC 74 between algal and higher plant NRs. This is an effect similar to that found for monoclonal 96(9)25 raised against maize NR [15], which bound only to higher plant NRs and not to NRs of gymnosperms, *C. pyrenoides* or *N. crassa*.

The relative affinities of MACs 77, 231 and 232 for *Chlorella* NR is shown in Fig. 3. A direct ELISA was done using a constant concentration of *Chlorella* NR (25μg/ml) with a two-fold dilution series of the monoclonals down from 200 μg ammonium sulphate precipitated protein/ml. MAC 77 showed a linear response only down to 25 μg/ml whereas MAC 231 and 232 responded over the whole of the range and saturated at 50 μg/ml. Optimum concentrations for maximum NR-specific responses were determined as 100 μg/ml for MAC 77 and 12.5 μg/ml for 231 and 232. Spinach NR gave similar responses.

A direct ELISA, using the optimum concentration of MAC 232, over the nominal range 50 to 600 ng/well of spinach NR and *Chlorella* NR, resulted in dose response curves (Fig. 4), plotted as the absorbance (A_{450}) against the log of the amount of enzyme [19]. The ELISA response obtained with the *Chlorella* NR being larger than that for the spinach NR, at the same nominal concentration, confirming the result obtained with the screening shown in Fig. 2.

MACs 232, 231, 77, 75 and 79 recognized native spinach NR and *Chlorella* NR after dot blotting the enzyme on to nitrocelluose with the same relative intensities found with direct ELISA. MAC 231 and 232 also recognized the denatured subunits of both enzymes, after treatment of the enzymes with SDS, followed by SDS-PAGE and 'western blotting' on to nitrocellulose. These properties resembled those of class 4 and 5 monoclonals raised against maize NR [14]. MAC 77 failed to recognize the denatured enzymes and therefore binds at a conformation-dependent site.

The ELISA response of *Chlorella* NR to MAC 77 showed similar intensity to the response of spinach NR to MAC 74. This suggests that MAC 77 may be useful for the immunopurification of *Chlorella* NR using a method similar to that employed for spinach NR using MAC 74 [18]. On the other hand, the very avid MAC 232 would be useful for quantification of NR-protein by indirect sandwich ELISA [19] and for the identification of spinach and *Chlorella* NR in mRNA translation products, after separation by SDS-PAGE.

EXPERIMENTAL

Enzyme purification. Spinach NR was purified by immunoaffinity chromatography [18] and *Chlorella* NR by affinity purification [20].

Enzyme assays. NADH–NR and NADH–cyt *c* Rase activities were determined by the method of ref. [21]; NADH–Fed Rase as described in ref. [22] and MV–NR as described in ref. [23].

Antibodies. Polyclonal antibodies were raised in rabbits, the IgG fraction was isolated by chromatography on Protein A Sepharose–CL 4B (Pharmacia) according to manufacturers instructions, pptd with 50% satd $(NH_4)_2SO_4$ and redissolved in Pi buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 1.15g Na_2HPO_4, 0.2 g K_2HPO_4 in 1 l of H_2O).

Table 1. Effect of monoclonal antibodies on the NADH-NR activity of spinach and *Chlorella* nitrate reductase

Monoclonal* antibody	Class (subclass)	Inhibition of NADH-NR activity† (expressed as % of appropriate monoclonal control)‡	
		Spinach NR§	*Chlorella* NR§
MAC 74	IgG (2a)	95.5	2.0
MAC 75	IgG (1)	62.5	0
MAC 77	IgG (2b)	92.0	2.0
MAC 78	IgG (2a)	74.0	5.0
MAC 79	IgG (2a)	63.5	7.5
MAC 231	IgM	0	0
MAC 232	IgM	0	0
PBS		52.4	73.5
Monoclonal control‡		0	0

*Monoclonals as 50% saturated $(NH_4)_2SO_4$ precipitate redissolved in PBS to a concentration of 10 mg/ml.

†Equal volumes of monoclonal (or PBS) and enzyme mixed and allowed to stand for 1 hr at 4° before determination of NADH-NR activity.

‡Monoclonal controls were MAC 80 (IgG 2a), MAC 83 (IgM), MAC 218 (IgG 2b), and MAC 221 (IgG1) (see Experimental).

§Purified spinach and *Chlorella* NR respectively diluted 50-fold and 200-fold with 5 mM Pi buffer, 1 mM EDTA, 10 μM FAD pH 7.5 to activities of 14.0 nKat and 18.4 nKat NO_2^- produced/ml.

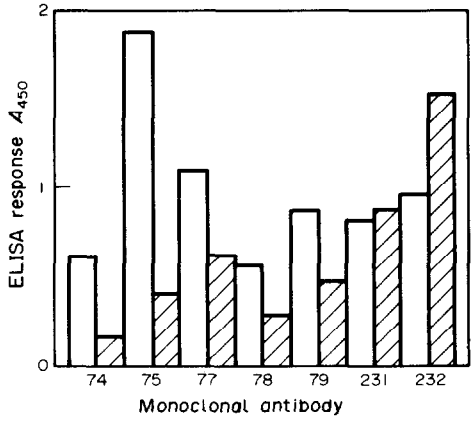

Fig. 2. Direct ELISA response (A_{450}) of spinach NR (plain) and *Chlorella* NR (hatched) to monoclonal antibodies. 1.25 μg of enzyme and 2.5 μg of monoclonal/assay. Reaction stopped after 5 min (232); 11 min (231) and 45 min (remainder).

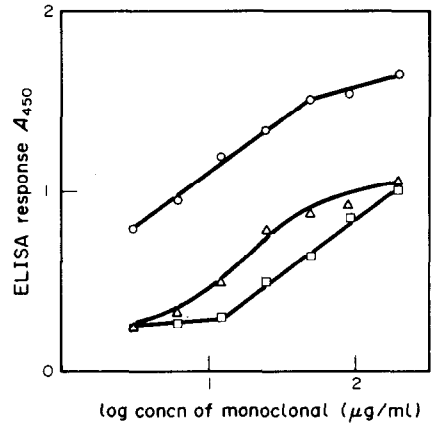

Fig. 3. Effect of increasing concentrations (3–250 μg/ml) of monoclonal antibodies 232 (○); 231 (△) and 77 (□), on direct ELISA response (A_{450}) of *Chlorella* NR. (1.25 μg enzyme/assay). Reaction stopped after 5 min (232); 11 min (231) and 45 min (77).

Monoclonal antibodies MACs 74, 75, 77, 78, and 79, were obtained previously [12]. MACs 231 and 232 were obtained after screening 18 previously uncloned cell lines for cross reactivity against *Chlorella* NR. The two most positive cell lines were selected, cloned in soft agar and individual colonies picked [17]. Ascites fluid was partially purified by pptn. with $(NH_4)_2SO_4$ at 50% satn, the ppt. redissolved in PBS and excess salt removed by dialysis against PBS. Solns were diluted to 10 mg/ml PBS and stored at −20°.

Control monoclonal antibodies, raised against animal proteins, were a gift from Dr G. M. Butcher (AFRC Monoclonal Antibody Centre, Babraham, Cambridge, U.K.). These were MAC 80 (IgG 2a), anti-pig T lymphocyte sheep erythrocyte receptor; MAC 83 (IgM), anti-pig CD2 molecule (lymphocyte); MAC 218 (IgG 2b), anti-ovine placental lactogen; MAC 221 (IgG1), anti-ovine placental lactogen.

Enzyme inhibition. Equal vols of the enzyme and antibody, diluted with 5 mM Pi buffer, 1 mM EDTA, 10 μM FAD pH 7.5, were mixed at 4°, allowed to stand in ice for 1 hr and the mixture assayed for the various activities.

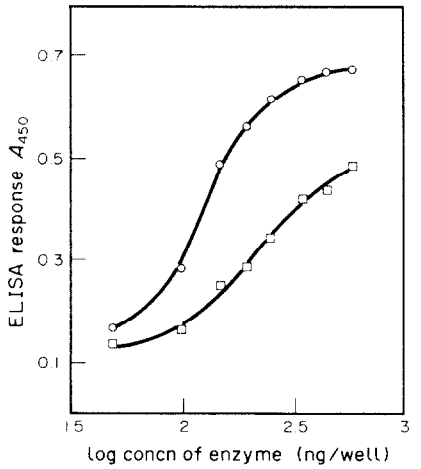

Fig. 4. Direct ELISA response (A_{450}) using 232 (0.25 μg/assay) of increasing concentrations (50–600 ng/well) of spinach NR (□) and *Chlorella* NR (○). Reaction stopped after 5 min.

Direct ELISA. Reaction between enzyme and monoclonals used the ELISA described for screening of hybrids [17], with dilutions of enzymes and monoclonals as stated in the Results and Discussion.

Electrophoresis. Denaturing electrophoresis used the method of Laemmli [24] with 7.5% separating gel and 3.75% stacking gel. The enzymes were heated to 90° for 5 min in the presence of SDS and 2-mercaptoethanol before running.

Western blotting. After electrophoresis the denatured enzymes were transferred electrophoretically to nitrocellulose [25]. After blocking with BSA, immunoreaction was identified with phosphatase-labelled rabbit antirat antibodies [26].

Dot blots. Native enzyme was absorbed onto nitrocellulose sheets as 5 μl dots. Immunoreactivity was identified as above using serial dilutions of both enzyme and antibody to eliminate non-specific binding at higher concentrations.

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