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Characterization and daily variation of nitrate reductase in *Gracilaria tenuistipitata* (Rhodophyta)

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Abstract

A daily rhythm in the activity of nitrate reductase (NR: EC 1.6.6.1) isolated from the marine red algae *Gracilaria tenuistipitata* is shown to be attributable to changes in amounts of the protein. The enzyme was purified in four steps: ion exchange Q-Sepharose separation, ammonium sulfate precipitation, gel filtration on Sephacryl S-300, and affinity chromatography on Affigel-blue resin. This purification procedure yielded an active purified NR of about 500-fold with a recovery of 85%. The SDS–PAGE silver staining of purified NR revealed a 110 kDa single band. Non-denaturated protein showed a molecular mass of 440 kDa on gel filtration comparing with SDS–PAGE, the enzyme is apparently composed of four identical subunits. In extracts of algae grown under either constant dim light or a light–dark cycle, the activity of NR exhibited a daily rhythm, peaking at midday phase as does photosynthesis. Staining with monoclonal antibodies, raised against NR from *Porphyra yezoensis*, showed that the amount of protein changes by a factor of about 12, with a maximum occurring in the midday phase. © 2002 Elsevier Science (USA). All rights reserved.

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The major source of nitrogen in the marine ecosystem is in the form of nitrate, which must be reduced to ammonia or amine to be assimilated into the biosynthesis of nitrogen-containing compounds such as proteins and nucleic acids [1–3]. The nitrogen assimilation process occurs in a two-step reaction catalyzed by two enzymes working sequentially, nitrate and nitrite reductases. The reduction of nitrate to nitrite is the first step in the reduction to ammonia; it is catalyzed by an NADH-dependent nitrate reductase (NR; EC 1.6.6.1), and may be rate-limiting in the nitrate assimilation process [4]. Nitrate reductase is a high molecular complex with three prosthetic groups, FAD-heme (cytochrome b_{557}) and Mo-pterin, that uses NADH or NADPH as the electron donor [2,3,5,6], and is subject to tight controls at the levels of enzyme activity, synthesis, and degradation. NR has been purified from several organisms and it occurs in a wide variety of molecular weights as well as number of subunits. NR size ranges from 200 kDa in spinach, with two subunits [7], to 500 kDa in the green alga *Ankistrodesmus braunii* with eight subunits of 58 kDa [8].

The marine red alga *Gracilaria tenuistipitata* var. *liui* Zhang and Xia is economically important, being one of the main sources for the production of phycocolloids [9]. This strain is extensively cultivated in ponds in southern China and Taiwan and exhibits a wide tolerance to environmental factors [9–11]. We describe in this paper the purification and characterization of NR and its daily variation of the enzyme in *G. tenuistipitata*. The present studies have shown that the amount of the NR protein changes in the cell, being abundant during the day phase but absent in the night extracts.

Materials and methods

Algae culture. Cultures of the marine red macroalga *G. tenuistipitata* were grown in von Stosch medium [12] under alternating periods of 12 h light (cool white fluorescent; 120 $\mu E\,m^{-2}\,s^{-1}$) and 12 h dark (LD

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12:12). The cultures were maintained at 20 °C under constant air bubbling. Algae were harvested in the middle of the light period. The beginning of the light period is defined as time zero (LD 0).

Purification of NR. The algae (400 g) were collected at the middle of the day when the NR activity is highest [4]. The algae were ground in liquid nitrogen using a mortar and pestle until a fine powder was obtained. The powder was suspended in 400 mL of extraction buffer (0.2 M phosphate buffer pH 8 containing 14 mM β-mercaptoethanol, 150 μg/mL phenylmethyl sulfonil fluoride, and 2 mM benzamidine). Cell debris was removed by centrifugation at 15,000*g* for 15 min. The supernatant was then submitted to ultracentrifugation at 105,000*g* for 1 h. The crude extract was maintained in a supplier Q-Sepharose FF batch, for 1 h under constant agitation, to promote the adsorption of the proteins to the resin. The resin was washed extensively with extraction buffer and centrifuged at 1000 rpm to wash off unbound proteins. The levels of protein in the buffer were monitored at 280 nm until levels lower than 0.05 absorbance units. The Q-Sepharose resin was packed in a XK16/30 (Pharmacia) column and the proteins were eluted with a saline linear gradient (75–700 mM NaCl in extraction buffer). The fractions containing NR activity were pooled and precipitated with 45% $(NH_4)_2SO_4$. The mixture was centrifuged at 16,000*g* for 30 min, and the precipitated protein was suspended in extraction buffer and desalted by dialysis overnight.

The product from dialysis was loaded on a XK16/70 (65 cm) Sephacryl S-300 column (Pharmacia) equilibrated with extract buffer and calibrated with molecular weight standards ferritin (430 kDa) and cytochrome *c* (12.3 kDa) (Boehringer Mannhein), dextran blue (2000 kDa), and flavin mononucleotide (478 Da) (Sigma). The fractions containing activity were pooled and loaded onto a 1.5 × 10 cm Affigel-blue column (Bio Rad) equilibrated with extraction buffer and washed extensively with the same buffer. The washings were discarded and NR was eluted with a linear NaCl gradient (0–1 M). Protein content was estimated in the eluted fractions by spectrophotometric absorbance at 280 nm, and determined quantitatively by reaction with the Coomassie blue dye-binding assay [13]. Bovine serum albumin (Sigma) was used as the standard protein for calibration curves.

NR activity assay. Crude extracts were preincubated in the reaction mixture (0.4 M phosphate buffer, pH 8.0, 10 mM KNO_3, 2 mM $MgSO_4$) at 20 °C for 10 min. NADH (0.4 mM) was added at zero time to start the reaction (controls lack NADH). The reaction was stopped by addition of 50 mM $ZnSO_4$ and 0.7% v/v cold-ethanol. Nitrite concentration was determined by absorption at 543 nm after addition of 0.75 mM sulfanilamide and 7.7 μM *N*-(1-naphthyl) ethylenediamine dihydrochloride. Thus the red color (measured at 543 nm) developed is proportional to nitrite concentration. One unit of NR is defined as the amount of enzyme required to produce 1 μmol of nitrite per minute at 20 °C. All assays were performed in triplicate.

Electrophoresis and Western blotting. SDS–PAGE was performed on 10% gels according to Laemmli [14]. The gels were stained with silver nitrate [15] or were transferred to a PVDF membrane for Western blots [16], using the ECL Detection System (Amersham Pharmacia) and hyperfilm for autoradiographic detection of bound antibody. The pre-stained molecular mass standards were purchased from Sigma Chemical (phosphorilase *b*, 94 kDa; serum bovine albumin, 67 kDa; egg albumin, 43 kDa; carbonic anhydrase, 30 kDa).

Antibody analyses. To analyze the NR protein levels over a circadian period two monoclonal antibodies raised against NR purified from *Porphyra yezoensis* (NR10 and NR20 [17]) were used. The antibodies showed to cross-react specifically with NR from *G. tenuistipitata* in both crude extract and purified enzyme. Both antibodies recognized the Mo-pterin domain, the most conserved motif in all NRs characterized [5]. For the antibody titration, samples of crude extracts containing 0.3 U of NR were incubated at 4 °C with different amounts of NR10 and NR20. The activity remaining after 1 h, when the reaction was completed, was measured as described above.

Results and discussion

Purification of NR

Although the overall enzymatic functions of NR from various different eukaryotes are similar, and they are typically oligomeric enzymes, the number and types of subunits can vary considerably [2,7,18,19]. Since blue-Sepharose binds NR from higher plants and algae [20], we purified *Gracilaria* NR utilizing the same concept, with a few modifications. The purification of NR started with the use of Q-Sepharose because of its high anionic charge. The active fractions from the Q-Sepharose column (Fig. 1A) were precipitated with 45% $(NH_4)_2SO_4$, desalted, and loaded on a Sephacryl S-300 column (Fig. 2). The eluted fractions containing NR activity were pooled and loaded on the Affigel-blue column (Fig. 1B). The enzyme was then eluted with a linear saline gradient

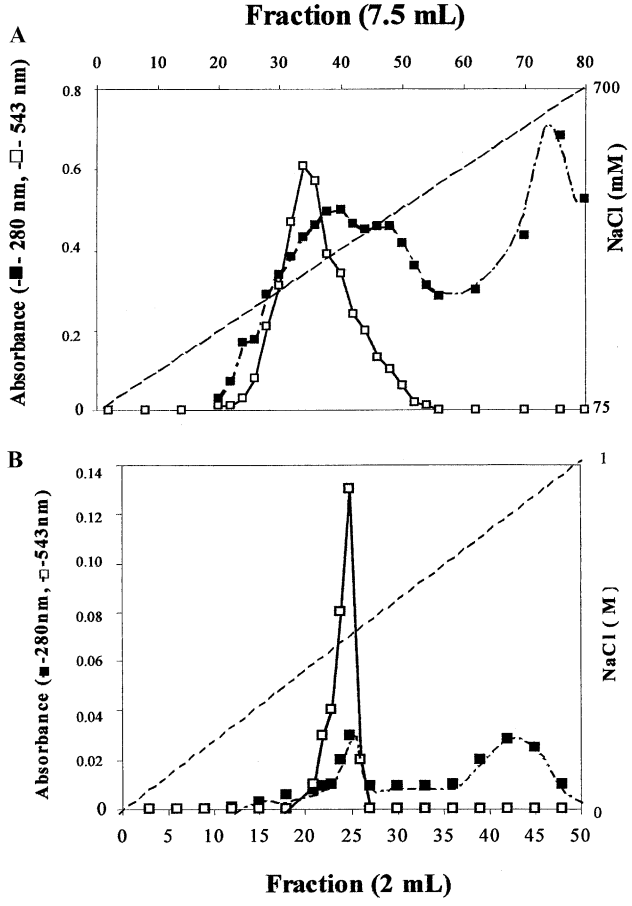

Fig. 1. NR purification. (A) Ion exchange chromatography. The crude extract was loaded on the Q-Sepharose and washed with 1 volume of extraction buffer. NR was eluted with a gradient of NaCl (75–700 mM dashed line). (B) Affigel blue chromatography. Fractions containing activity from a Sephacryl S-300 column were pooled and loaded onto an Affigel-blue column. After the colunm was washed with extraction buffer, NR was eluted by a gradient of NaCl (0–1 M, dashed line).

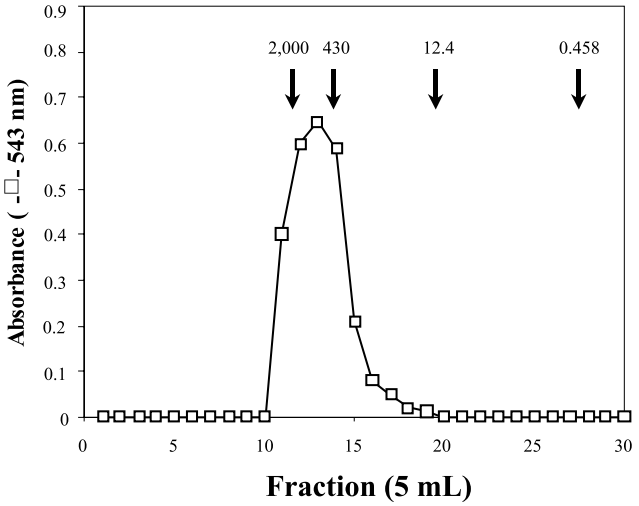

Fig. 2. Gel filtration of NR. Fractions eluted from Q-Sepharose containing NR activity were pooled and loaded on a Sephacryl S-300 column. The NR activity was measured for each fraction (ordinate). Activity peaked at about 440 kDa; the molecular size standards are noted by arrows.

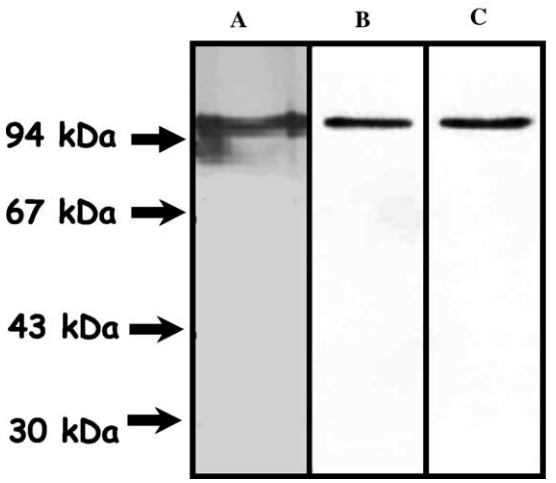

Fig. 3. SDS–PAGE of *Gracilaria* NR. (A) Fraction 26 from an Affigel-blue column containing NR activity was submitted to SDS–PAGE and silver stained. Immunoblotting of purified NR (B) and crude extract (C). Proteins were subjected to SDS–PAGE and either silver stained or transferred electrophoretically to a nitrocellulose membrane and revealed by the chemiluminescence assay. The positions of molecular mass markers are indicated to the left of the figure.

of NaCl. This resin utilizes the same principle for binding proteins as does blue-Sepharose resin. The procedure resulted in a protein purified almost 500-fold, with a recovery of about 85% (Table 1).

Based on SDS–PAGE (Fig. 3A), the protein at the final stages of purification was estimated to be about 95% pure and to have a subunit molecular mass of about 110 kDa. The apparent molecular mass of the undenatured NR was determined by gel filtration chromatography on a calibrated Sephacryl S-300 column to be about 440 kDa (Fig. 2), meaning that the native protein is a tetramer of approximately 110 kDa, corresponding to the SDS–PAGE value. The NR from the dinoflagellate *Lingulodinium polyedrum* is composed of six subunits of 52 kDa while *Chlorella vulgaris* NR has two identical subunits of molecular weight 100 kDa [21,22]. The *A. braunii* enzyme is composed of eight identical subunits with a subunit molecular weight of about 60 kDa [8]. Those isolated from *Neurospora crassa* and *Rhodotorula glutinis* are homodimers consisting of two subunits of 115 [23] and 118 kDa [24], respectively.

Antibody inactivation of enzyme

The NR family enzymes have three distinct motifs, Mo-pterin, heme, and flavin domains, well conserved among the enzymes characterized [5,6]. The Mo-pterin domain has about 80% amino acid homology when considering all NRs sequenced. The tests performed by Nakamura and Ikawa [17] using a set of 22 monoclonal antibodies raised against NR purified from *P. yezoensis* showed a high degree of conservation with the three domains among algae and higher plants. *Porphyra* and *Gracilaria* being from the same division, Rhodophyta, NR was expected to have antibody cross-reactivity. The monoclonal antibodies from *Porphyra* inhibited *Gracilaria* NR activity very effectively, as shown in Fig. 4. About 3 μL of both NR10 and NR20 inhibit approximately 50% of total enzyme activity after 1 h of incubation at 4 °C. On Western blots the antiserum reacts with a single band of approximately 110 kDa in crude extracts and purified enzyme (Fig. 3B and C). When primary monoclonal antibodies were omitted from the

Table 1
Purification of NR from *G. tenuistipitata*

Step	Protein (mg)[a]	Total activity (mU)[b]	Specific activity (mU/mg)	Recovery (%)
Supernatant 17,000*g*	547	3.74	0.0068	100
Supernatant 105,000*g*	537	2.92	0.0054	78
Q-Sepharose	67.3	16.34	0.2429	437
$(NH_4)_2SO_4$ precipitation	43.8	0.27	0.0069	7
S-300 HR	17.5	5.94	0.339	159
Affigel-blue	0.095	3.21	3.37	86

[a] Pooled peak fractions.
[b] Protein was measured according to [13]. 1 U = μmol of nitrite reduced per minute at 20 °C.

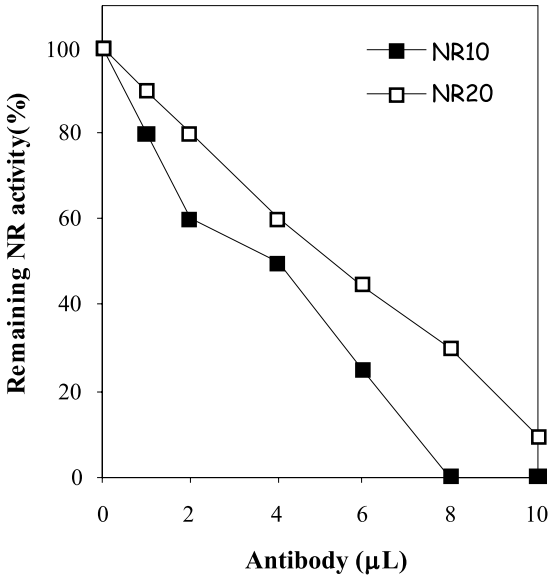

Fig. 4. Titration with monoclonal anti-NR. Crude extracts containing 0.3 U of NR were incubated with different amounts of either monoclonal NR10 (■) or NR20 (□) for 1 h at 4 °C and the remaining activity was determined (ordinate).

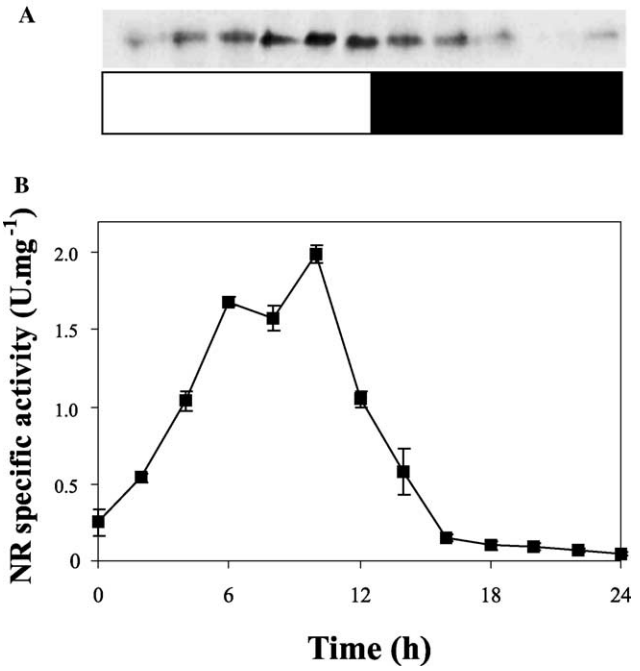

Fig. 5. Daily oscillation in the activity and amount of the NR protein. Cells maintained under a 12 h LD cycle were harvested every 2 h over a 24 h period. Equal amounts of soluble proteins were loaded onto gels, separated by SDS–PAGE and transferred electrophoretically onto a PVDF membrane, which was treated with NR10 monoclonal antibody and assayed by developing procedures using the ECL Detection System. The panel shows that the circadian rhythm in immunologically reactive NR protein (A) corresponds to an oscillation in NR activity (B).

assays no inhibitory properties and no bands were recognized on the Western blots (data not shown).

Daily changes in activities and amounts of extractable NR

The regulation of NR has been studied in several different species, both with respect to transcriptional expression and enzymatic activity. NR expression is a complex process, regulated by different factors such as levels of nitrate, CO_2, light, carbon skeletons, and nitrogen metabolites [25]. In addition, many authors have suggested that the biological clock controls the levels of NR in algae and higher plants, with maximum rates occurring during the light period and minimal activities in the dark period [26–33]. With *G. tenuistipitata* grown under a light–dark (LD) cycle, the NR activity exhibits a circadian rhythm [4], peaking at mid-day phase when activity is 30-fold higher than at night. The NR activity is clearly enhanced by light; when algae are kept under continuous light, they have a higher level of activity than *G. tenuistipitata* grown under a light:dark regime, and when kept in continuous darkness, only a basal activity is observed [4,28]. Using Western blot techniques we found that the extractable levels of NR protein change in parallel with its activity (Fig. 5), with amplitude equal to or even greater than the activity rhythm. Crude extracts (20 µg protein per lane), prepared every 3 h from cells grown under LD condition, were submitted to 10% SDS–PAGE, and transferred to a PVDF membrane, and probed with NR10 monoclonal antibody (Fig. 5A). The stained bands show that the amount of NR protein present in crude extracts is about 40-fold higher in cells extracted during the day phase. This indicates that the protein is probably synthesized and destroyed each day, as opposed to what might seem a more economical alternative to the same end, such as inhibition and activation by phosphorylation, for example. These studies further characterize NR and its expression in *G. tenuistipitata*. Understanding the nitrogen assimilation process can be helpful for improving cultivation and increasing the agar content in *G. tenuistipitata*.

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