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Ferredoxin-nitrite reductase from a cyanobacterium *Spirulina platensis*

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Ferredoxin-dependent nitrite reductase (Fd-NiR) (EC 1. 7. 7. 1) was purified about 4,100-fold, with a yield of 14%, from a cyanobacterium, *Spirulina platensis* by a procedure involving acetone fractionation, DEAE-cellulose chromatography, Butyl-Toyopearl chromatography, Sephadex G-75 gel filtration and ferredoxin-Sepharose affinity chromatography. The purified enzyme was apparently homogeneous, as judged on polyacrylamide disc gel electrophoresis, with a specific activity of 222 units/mg of protein. The molecular weight of the enzyme was estimated to be 57 kilodaltons by gel filtration. On subunit analysis by SDS-PAGE, a single band corresponding to molecular weight of 58 kilodaltons appeared. The purified enzyme (Fd-NiR) showed 1.4 times higher methyl viologen-linked activity than ferredoxin-dependent activity. In the oxidized form, the enzyme exhibited absorption maxima at 277, 390 (Soret band), 573 (α band) and 695 (CT band) nm, indicating that siroheme is involved in the catalysis of nitrite reduction. The absorbance ratios, A_{390}: A_{277} and A_{573}: A_{390} were 0.58 and 0.26, respectively. The Km values calculated from Lineweaver-Burk plot of the data were 4.8×10^{-4}M (nitrite) and 2.0×10^{-6}M (reduced ferredoxin).

Key Index Words: cyanobacterium—ferredoxin linked enzyme—nitrite reductase—Spirulina platensis.

The nitrite reductases (NiRs) from plants catalyze the reduction of nitrite to ammonia in the presence of reduced ferredoxin, which is the physiological donor of electrons, or in the presence of reduced methyl viologen, which is an artificial electron donor. This enzyme has been purified from several plants and characterized (Ho and Tamura 1973, Shimizu and Tamura 1974, Hirasawa and Tamura 1980, Hirasawa-Soga and Tamura 1981, Hirasawa-Soga *et al.* 1982, Hirasawa-Soga *et al.* 1983, Nagaoka *et al.* 1984, Hirasawa *et al.* 1984, Ishiyama and Tamura 1985, Ishiyama *et al.* 1985, Ide and Tamura 1987). However, nitrite reductases from cyanobacteria have not received the same attention as those from higher plants. NiRs from *Anabaena cylindrica* (Hattori and Uesugi 1968), *Anacystis nidulans* (Manzano 1977), *Anabaena* sp. 7119 (Mendez and Vega 1981, Mendez *et al.* 1981), *Spirulina platensis* (Yabuki *et al.* 1985) and *Phormidium laminosum* (Arizmendi and Serra 1990) have been partially characterized, but only the enzymes from *S. platensis* and *P. laminosum* have been purified to a high degree.

In the present report, we highly purified NiR from *S. platensis* and described several properties of the NiR.

Materials and Methods

Materials

The following chemicals were purchased from commercial sources: DEAE-cellulose DE-52 (Whatman, Maidstone, U. K.); Butyl Toyopearl 650S (Toso, Tokyo, Japan); Sephadex G-75, CNBr-activated Sepharose 4B, Electrophoresis Calibration Kit, Blue Dextran 2000 (Pharmacia, Uppsala, Sweden); Coomassie brilliant blue G-250, R-250 (Fluka, Buchs, Switzerland); sodium dithionite (Koso Chemical, Tokyo, Japan); methyl viologen (Tokyo Kasei, Tokyo, Japan); albumin fraction V, Calibration Proteins II (Boehringer Mannheim, Mannheim, F. R. G.); finely granulated sugar (local market). Other chemicals were reagents of analytical grade from Wako Pure Chemical (Tokyo, Japan).

Cell culture

Spirulina platensis strain OU-1, kindly supplied by professor K. Wada of Kanazawa University, was grown photoautotrophically at 35°C in inorganic artificial medium (SOT medium; Ogawa and Terui 1970) in a 100 liter plastic container (AL-LL Reactor, supplied by Mitsubishi kakoki, Tokyo, Japan). The culture medium was agitated by a stream of air from a pump and continuous illumination was provided by four white fluorescent lamps (FLR40SW/M40W; Mitsubishi) at 10,000 lux. About 300 g (wet wt.) of cells were harvested by suction filtration at the stationary phase, which was reached after one week. The harvested cells were immediately placed in a freezer at −30°C and stored until use.

Assay of enzymatic activity

The method for the assay of nitrite reductase (NiR) activity was essentially the same as that described in our previous paper (Yabuki and Tamura 1985). The reaction mixture contained, in a total volume of 1 ml, 20 μmol of Tris-HCl buffer, pH 7.5, 2 μmol of $NaNO_2$, 3 μmol of methyl viologen or 2 mg of ferredoxin from *S. platensis*, 3.75 mg of sodium dithionite (freshly dissolved in 0.3 M $NaHCO_3$) and an aliquot of enzyme preparation. In the enzymatic reaction, reduced methyl viologen was used as electron donor unless otherwise stated.

The reaction was started by the addition of sodium dithionite after a 2 min of preincubation at 35°C and was incubated for 4 min at the same temperature. The reaction was terminated by vigorous shaking in a cyclomixer. The decrease in the level of nitrite was measured by the method of Snell and Snell with sulfanilamide and N-1-naphthylethylenediamine dihydrochloride.

In the inhibitory experiments, the effect of inhibitors on the reduced form of NiR was measured by addition of sodium nitrite after the preincubation of the enzyme in a solution containing Tris-HCl, methyl viologen, sodium dithionite and each inhibitor for 7 min at 35°C. The inhibitory effects on the oxidized form was measured by addition of sodium dithionite.

One unit of NiR activity was defined as the amount of enzyme that reduced 1 μmol of nitrite per min under the conditions of the assay.

Purification of ferredoxin

Ferredoxin from *S. platensis* were purified by acetone fractionation, chromatography twice on DEAE-cellulose and once on Sephadex G-75. In this study, an absorbance ratio for ferredoxin (A_{423}/A_{277}) of more than 0.45 was used. Amounts of ferredoxin were estimated using a molar absorption coefficient of 9.2 $mM^{-1}cm^{-1}$ at 423 nm (Hall *et al.* 1972).

Preparation of ferredoxin-Sepharose

Ferredoxin-Sepharose 4B was prepared by the method of Shin and Oshino (1978).

Purification of NiR

All the purification procedures were performed in a cold room at 4°C. Dialysis was performed overnight against the indicated buffer, and centrifugation was carried out at 10,000 × g for 15 min.

(1) *Extraction of the Enzyme*

Frozen cells of *S. platensis* (300 g) were thawed and mixed in the four volumes (1,200 ml) of extraction buffer (20 mM Tris-HCl buffer, pH 7.5, containing 200 mM NaCl). The suspension was sonicated (Sonicator; Otake seisakusho, Tokyo, Japan) at 15 kHz for 4 min, and a crude extract was obtained after removal of cell debris by centrifugation.

(2) *Acetone fractionation*

The precipitate formed from the crude extract (1340 ml) after the addition of cold acetone (−30°C) to a final concentration of 35% (v/v) was removed by centrifugation and discarded. Cold acetone (−30°C) was further added to the supernatant to give a final concentration of 75%, then the resultant precipitate was collected by centrifugation, resuspended in 200 ml of 20 mM Tris-HCl

buffer, pH 7.5, and dialyzed overnight against the same buffer. The dialyzed sample was centrifuged to remove the precipitate.

(3) *Column chromatography on DEAE-cellulose*

The dialyzed solution (300 ml) was loaded on an anion-exchange column of a DEAE-cellulose DE-52 (5 cm × 22 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.5. After the column had been washed with same buffer, the elution was performed as follows. A linear concentration gradient of NaCl was established with 600 ml of 20 mM Tris-HCl buffer, pH 7.5, in the mixing vessel and 600 ml of 20 mM Tris-HCl buffer, pH 7.5, containing 300 mM NaCl in the reservoir. The flow rate of elution was 30 ml/hr, and fractions of about 10 ml each were collected. The fractions containing NiR activity were pooled.

(4) *Column chromatography on Butyl-Toyopearl*

The pooled fractions (70 ml) were supplemented with ammonium sulfate to bring them to 40% saturation. The precipitate formed was removed by centrifugation. The resultant supernatant was loaded on a hydrophobic column of Butyl-Toyopearl 650S (2.2 cm × 21 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.5, that was 40% saturated with ammonium sulfate. After the column was washed with the same buffer, a linear concentration gradient of ammonium sulfate was established with 250 ml of equilibration buffer in the mixing vessel and the same volume of 20 mM Tris-HCl buffer, pH 7.5, in the reservoir. The flow rate of elution was 20 ml/hr and fractions of about 4 ml were collected. The fractions containing NiR activity were combined.

(5) *Column chromatography on Sephadex G-75*

The pooled fractions (35 ml) were concentrated in a dialysis bag immersed in finely granulated sugar overnight and then dialyzed against 20 mM Tris-HCl buffer, pH 7.5, containing 200 mM NaCl. The resultant dialyzed solution of the enzyme was filtered through a gel filtration column of Sephadex G-75 (3 cm × 90 cm) equilibrated with the dialysis buffer. The flow rate of elution was 10 ml/hr and fractions of about 3 ml were collected. The fractions active for NiR were pooled.

(6) *Column chromatography on ferredoxin-Sepharose 4B*

After the pooled (20 ml) fractions has been dialyzed against 20 mM Tris-HCl buffer, pH 7.5, they were loaded on an affinity column of ferredoxin-Sepharose 4B (1 cm × 2 cm) equilibrated with the same buffer. After washing with same buffer, elution of the enzyme was performed with 20 mM Tris-HCl buffer, pH 7.5, containing 400 mM NaCl. The flow rate of elution was 15 ml/hr and fractions of about 1 ml were collected. The fractions containing NiR activity were pooled.

Storage of the enzyme

After the enzyme solution purified by the procedure described above was dialyzed against 20 mM Tris-HCl buffer, pH 7.5, containing 200 mM NaCl and 10% glycerol (v/v, final concentration), the preparation was stored in a freezer (−80°C) and aliquots were used for subsequent analysis.

Absorption spectra

The absorption spectra of oxidized form of the enzyme were recorded at room temperature against a buffer blank in a spectrophotometer Hitachi U-3200 in cuvettes of 1 cm path length.

Other analytical methods

Protein was determined by the methods of Bradford (1976) with albumin fraction V as a standard. Analytical gel electrophoresis was carried out by the method of Davis (1964) for PAGE and by that of Laemmli (1970) for SDS-PAGE. Protein bands on the gel slab were stained with Coomassie brilliant blue R-250. Activity staining of methyl viologen-dependent NiR activity was performed by the modified version of the method of Hucklesby and Hageman (Vega and Kamin 1977).

Table 1. Summary of purification of nitrite reductase from *Spirulina platensis*

Step	Activity (units)	Protein (mg)	Specific Activity (units/mg protein)	Purification	Yield (%)
Crude extract	411	7560	0.0544	1.0	100
Acetone 35–75%	528	1130	0.467	8.6	129
DEAE-Cellulose	230	224	1.03	19	56
Butyl Toyopearl	163	14.7	11.1	200	40
Sephadex G-75	83.5	0.980	85.2	1600	20
Ferredoxin Sepharose	55.6	0.250	222.4	4100	14

These data were obtained during the processing of 300 g (wet wt.) of the cells.

Results

Purification of enzyme

Table 1 shows the summary of a typical purification that started with 300 g (wet wt.) of *Spirulina platensis* cell. Nitrite reductase (NiR) was purified more than 4,100-fold, with a yield of 14%, to a specific activity of 222 units/mg protein at 35°C. The ratio of the activity with ferredoxin to that with methyl viologen was 0.72.

After the last step (Fig. 1.) of the purification, PAGE in 7% gels and SDS-PAGE in 10% gels gave a single band of protein upon staining with Coomassie brilliant blue R-250 (Fig. 2.). Furthermore, the band visualized by activity staining corresponded to the band of purified enzyme after non-denaturing PAGE (Fig. 2a).

Absorption spectrum

The solution of oxidized form of NiR gave the absorption spectrum shown in Fig. 3. In addition to the protein peak at 277 nm, the purified NiR exhibited absorption maximum at 390 nm (Soret band), 573 nm (α band) and 695 nm (CT band), respectively. The ratio of A_{390}/A_{277} was 0.58, and the ratio of α band to Soret peak was 0.26.

Molecular weight

The molecular weight of the purified NiR

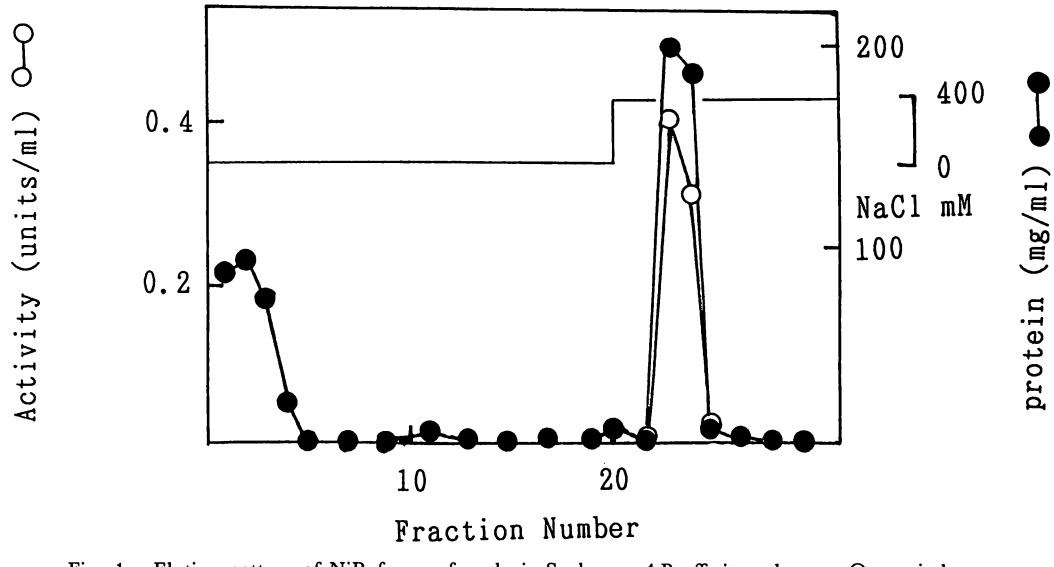

Fig. 1. Elution pattern of NiR from a ferredoxin-Sepharose 4 B affinity column. Open circle, enzyme activity in units per ml; closed circle, amount of protein in mg per ml. Experimental conditions are described in the text. Nos. 23–24 of the fractions were pooled.

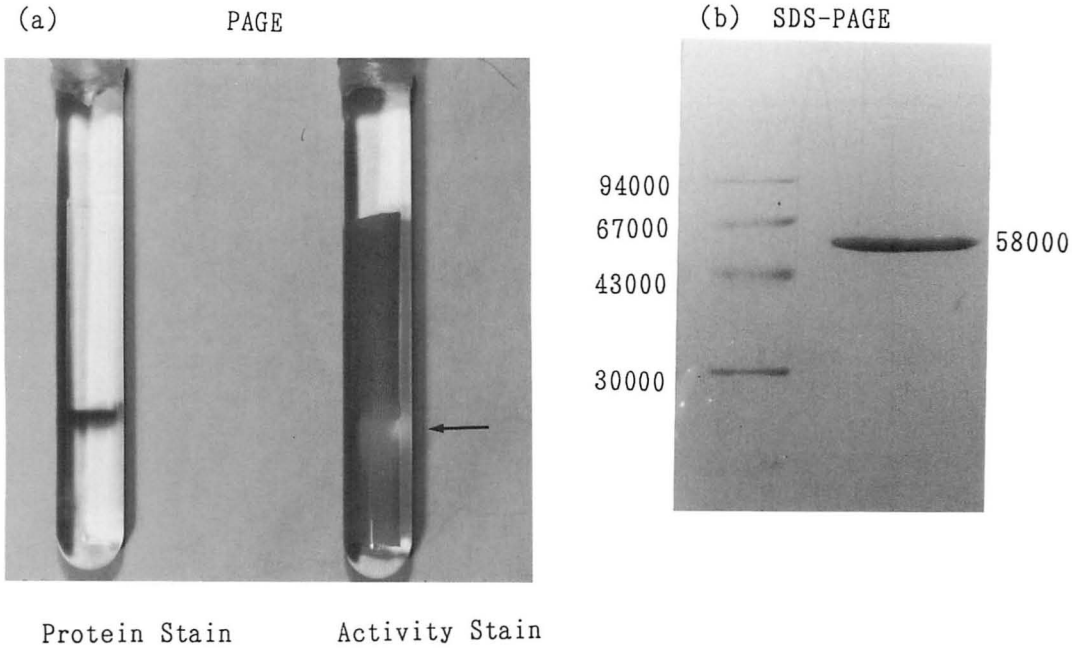

Fig. 2. Electrophoretograms of purified NiR on PAGE and SDS-PAG. (a): about 50 μg of the enzyme was subjected to electrophoresis on a 7% polyacrylamide gel disc. A constant electric current (5 mA per tubes) was applied for 5 hr. The NiR was detected by protein staining and activity staining. Activity is shown by an arrow. (b): about 50 μg of the enzyme was incubated at 100°C in a water bath for 5 min with 0.1% SDS and 1% β-mercaptoethanol. The treated enzyme was subjected to electrophoresis on a 10% SDS-polyacrylamide gel. A constant electric current (5 mA) was applied for 6 hr. The NiR was detected by protein staining.

was estimated by gel filtration on Sephadex G-75 (Fig. 4a) and SDS-PAGE (Fig. 2b). In Figure 4, the elution volumes for the marker proteins are plotted against their molecular weights. The molecular weight of NiR was estimated to be 57 kDa by gel filtration and 58 kDa by SDS-PAGE.

Effect of substrate concentration on enzyme activity

The effects of sodium nitrite and ferredoxin on NiR were determined by varing their concentration. From the double-reciprocal (Lineweaver-Burk) plot (Fig. 5), Km values for sodium nitrite and ferredoxin were determined to be 4.8×10^{-4} M and 2.0×10^{-6} M, respectively.

Optimum pH of NiR

The effects of pH on the NiR activity were studied under the same conditions as those described in Materials and Methods, except that the pH of the reaction mixture was varied. The pH-activity curve obtained were rather flat with an optimum pH around pH 7.5 (data not shown).

Effects of inhibitors

The effects of various compounds on the reaction catalyzed by NiR were examined (Table 2). Both the oxidized and the reduced form of the enzyme were strongly inhibited by cyanide. p-Chloromercuribenzoic acid (p-CMB) had an inhibitory effect only at high concentration as 2 mM, and this reagent inhibited more than two times oxidized form of NiR than reduced one. An inhibitor of hemoproteins, NaN_3 and a metal-chelating reagent, EDTA, had slightly inhibitory effect at 2 mM.

Heat stability

Methyl viologen-supported and ferredoxin-supported activities were both stable to heating at 40°C for 5 min, but more than 90% of

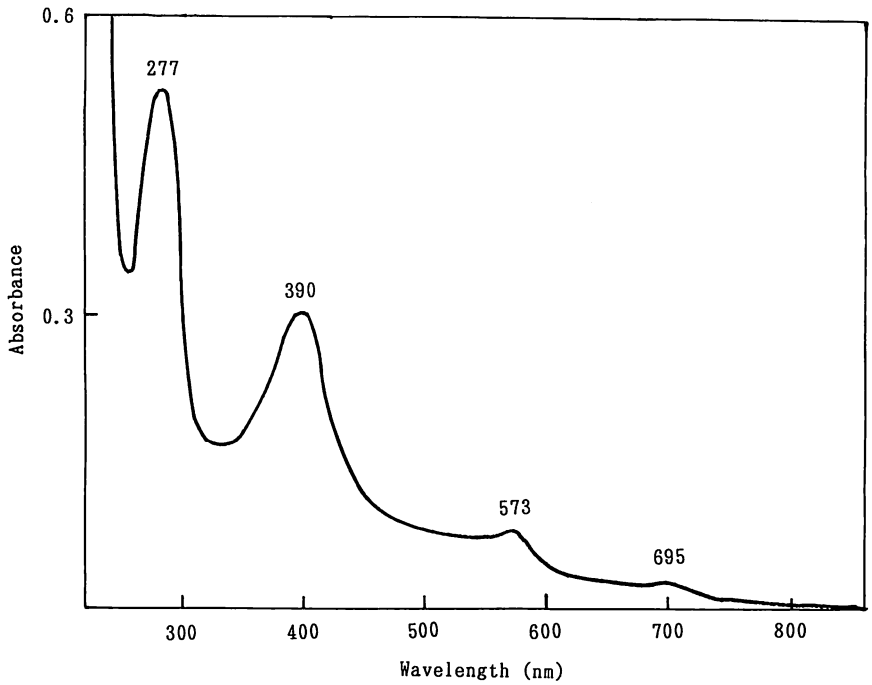

Fig. 3. Absorption spectrum of NiR. The purified enzyme preparation (400 μg/ml; oxidized form) was used. The protein was dissolved in 20 mM Tris-HCl buffer, pH 7.5 containing 200 mM NaCl and 10% glycerol (v/v). The absorption spectra were measured at room temperature.

both activities was lost after heating at 60°C for 5 min. Heating at 70°C for 5 min completely eliminated both activities. Ferredoxin-supported activity was somewhat less stable than methyl viologen-supported activity (Fig. 6).

Discussion

The present paper described a new methods for purifying ferredoxin dependent-nitrite reductase (Fd-NiR) from cells of a cyanobacterium *Spirulina platensis*. This enzyme was purified 4,100-fold (Table 1), and behaved as an almost homogeneous protein on polyacrylamide gel electrophoresis (Fig. 2). This purified enzyme showed a specific activity of 222 units/mg protein at 35°C. Compared with the specific activity reported in other works on NiRs from cyanobacteria, this value is very high, and it was almost same value as that reported previously from our laboratory (194 units/mg protein, Yabuki *et al.* 1985) and lower than the value for NiR from *Phormidium laminosum* (625 units/mg protein at 50°C, Arizmendi and Serra 1990). The ratio of the activities with ferredoxin and methyl viologen was 0.72. This ratio is close as that for the enzyme from *Anabaena cylindrica* (0.88, Hattori and Uesugi 1968) and *Anabaena* sp. 7119 (0.93, Mendez *et al.* 1981). However, NiRs from spinach leaves (Hirasawa and Tamura 1980) and green shoots of bean (Ishiyama *et al.* 1985) have the ratio of 1.74 and 1.43, respectivery. This difference may be due to the nature of the individual enzymes.

The absorption spectrum of this enzyme was similar to that of NiRs from other cyanobacteria and eukaryotic organisms, suggesting the presence of a siroheme as the prosthetic group. Yabuki *et al.* (1985) purified the NiR from *S. platensis*, a same strain in our study, 4,200-fold. However their purified enzyme showed many minor peaks such as at 350, 360, 462, 534, 588 and 658 nm in addition to the three peaks characteristic of siroheme. These small peaks might be due to

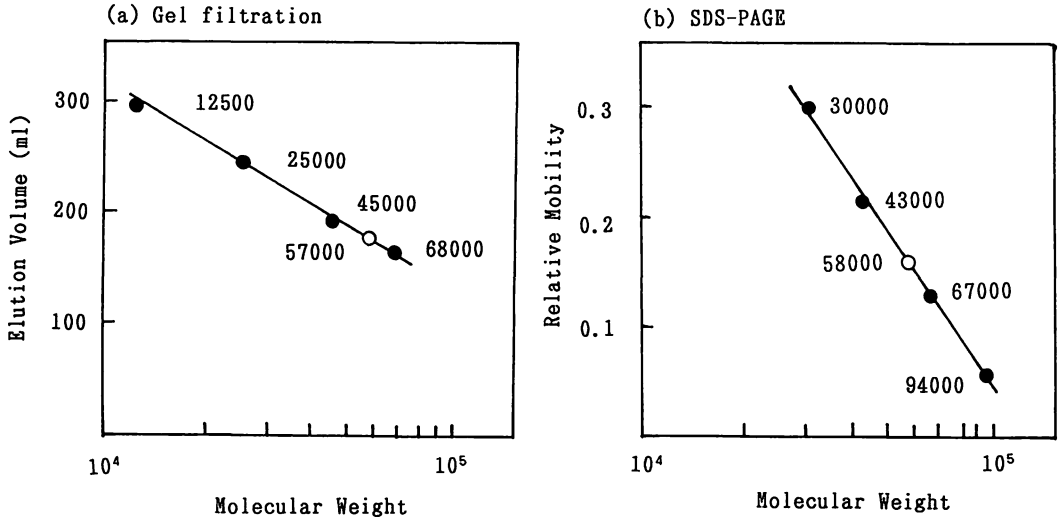

Fig. 4. Determination of the molecular weight of NiR. (a): NiR (500 μg/ml) and each marker protein (10 mg/ml) were separately filtered through a column of Sephadex G-75 (2 × 90 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 200 mM NaCl and 10% glycerol (v/v). The marker proteins used were cytochrome c (12500), chymotrypsinogen A (25000), ovalbumin (45000), bovine serum albumin (68000). ○, NiR. (b): Electrophoretic mobilities of marker proteins and NiR are shown in the figure. The condition of SDS-PAGE was shown in the legend of Fig. 2(b). The marker proteins have following molecular weights: carbonic anhydrase (30000), ovalbumin (43000), bovine serum albumin (67000), phosphorylase b (94000).

some contaminants in their preparation.

The molecular weight of this enzyme (57-58 kDa) is slightly larger than that of other cyanobacterial NiRs (52-54 kDa, Manzano 1977, Mendez and Vega 1981, Arizmendi and Serra 1990) with the exception of NiR from *A. cylindrica* (68 kDa, Hattori and Uesugi 1968).

The Km values for sodium nitrite reported from other cyanobacterial NiR are 5×10^{-5} M (*A. cylindrica*, Hattori and Uesugi 1968) and 4×10^{-5} M (*P. laminosum*, Arizmendi and Serra 1990). The Km value for sodium nitrite of NiR from *S. platensis* was calculated to be 4.8×10^{-4} M. However, this value is a little larger than those of other cyanobacterial

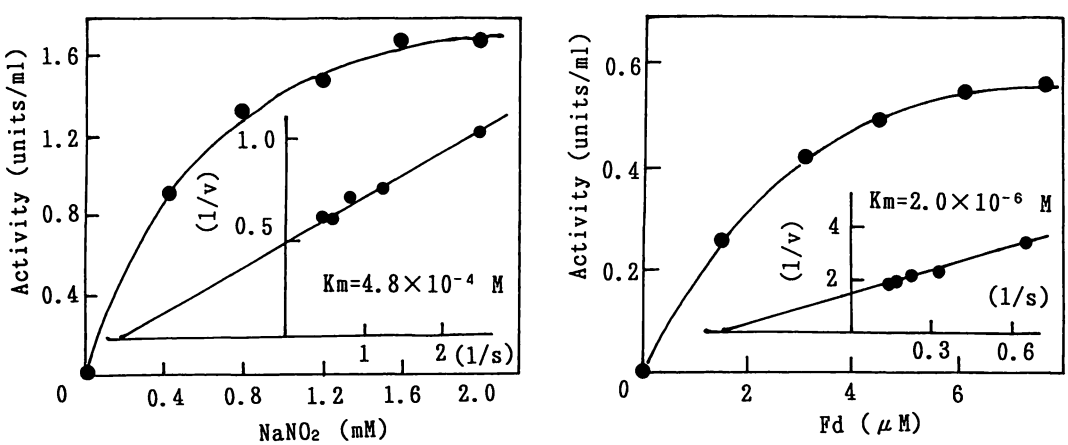

Fig. 5. Effects of nitrite and ferredoxin concentrations on NiR. The reaction mixture was varied in nitrite concentration (a) and ferredoxin concentration (b). For detailed see Materials and Methods. The amount of protein used for assay was 10 μg. Km values were calculated from Lineweaver-Burk plots.

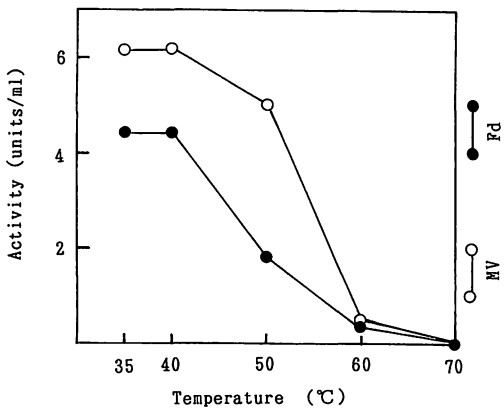

Fig. 6. Heat stability of NiR. The remaining activities were measured by the methods as described in Materials and Methods after a heat treatment of the enzyme for 5 min. The amount of protein used in each assay was 10 μg. Fd, ferredoxin-supported activity; MV, methyl viologen-supported activity.

NiRs. The Km value for ferredoxin of NiR from *Spirulina platensis* was 2.0×10^{-6} M. This value was almost same as that of NiR from *A. cylindrica* (5×10^{-6} M, Hattori and Uesugi 1968).

The optimum pH (7.5) for activity of this enzyme is almost same as that of NiR from *A. cylindrica* (pH 7.6, Hattori and Uesugi 1968).

The effect of inhibitors on the NiR from *S. platensis* were similar to those on NiRs from other cyanobacteria (Mendez *et al.* 1981, Arizmendi and Serra 1990).

The NiR from *S. platensis* is a little more stable to heating than the NiR from spinach leaves (Ho and Tamura 1973).

From our present study on NiR from *S. platensis*, we conclude that this enzyme is similar to NiRs from other plants, with the exception of its molecular weight.

Table 2. Effects of inhibitors on NiR.

Inhibitor	Concentration (mM)	Inhibition (%)	
		Oxidized form	Reduced form
KCN	0.02	55.9	57.8
	0.2	100	100
NaN_3	0.2	0	0
	2	6.4	13.7
p-CMB	0.2	6.1	6.1
	2	87.7	39.0
EDTA	0.2	0	0
	2	7.9	6.1

Purified enzyme (10 μg of protein) was employed in each reaction. In the control experiment, inhibitors were omitted. Other experimental conditions were as described in Materials and Methods.

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宮地竜郎・政田正弘・田村五郎：藍藻 *Spirulina platensis* のフェレドキシン依存性亜硝酸還元酵素

窒素固定を行わない藍藻である *Spirulina platensis* の同化型フェレドキシン依存性亜硝酸還元酵素を簡便な方法で4100倍に精製した。精製酵素は比活性が 222 units/mg protein であり，PAGE 及び SDS-PAGE 的に均一な標品であった。精製酵素の吸収スペクトルは 277, 390 (Soret band), 573 (α band), 695 (CT band) nm に吸収極大が見られた。分子量はゲル濾過法により 57 kDa, SDS-PAGE により 58 kDa と推定された。亜硝酸とフェレドキシンに対する Km 値はそれぞれ 4.8×10^{-4} M, 2.0×10^{-6} M と算出された。(271 千葉県松戸市松戸648　千葉大学園芸学部生物化学研究室)