Nitrate Reductase from *Sphagnum* Species: Isolation, *in vitro* Assays and Partial Purification

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Z. Naturforsch. **42c,** 653–656 (1987); received October 17, 1986

Nitrate Reductase, *Sphagnum* Species, Affinity Chromatography, *in vitro* Assays

Nitrate reductase (EC 1.6.6.2) has been isolated from 11 *Sphagnum* (Bryophyta) species and purified up to 500-fold by a procedure involving $(NH_4)_2SO_4$ precipitation and blue sepharose affinity chromatography.

The purest fraction showed a nitrate reductase activity of 7.7 nkat mg protein^{-1} with NADPH as the electron donor. Beside NADPH and NADH, $FMNH_2$ and – after blue sepharose affinity purification of the enzyme – MVH_2 were used as electron donors in different assays.

Introduction

While purified nitrate reductases from various sources (higher plants, algae, bacteria and fungi) are well characterized [1], little information on NR isolated from bryophytes is available. Recently Takio *et al.* [2], using an extraction buffer, containing polyvinylpyrrolidone, cysteine and EDTA, succeeded in extracting a NADPH dependent NR from a liverwort. While *in vivo* measurement of NR activity in musci does not present methodological difficulties [3–5], attempts to isolate this enzyme from *Sphagnum* species have failed [5].

In this paper, a method for the isolation and partial purification of *Sphagnum* NR is described for the first time.

Material and Methods

Plant material

Sphagnum species were collected from different mires in Schleswig-Holstein and cultivated according to Rudolph *et al.* [6]. NR was induced by applying a solution containing 0.5 mM KNO_3 and 1 μM Na_2MoO_4 (0.5 ml per plant) 6 h before enzyme extraction.

Enzyme extraction and purification

About 75 g plant material (capitula with green stem) was used for a typical purification. The plants were homogenized in 200 ml buffer A (80 mM K-phosphate buffer, pH 8.2, containing 5 mM cysteine, 2 mM DTT, 2 mM K-EDTA, 15 μM FAD, 4 mM PMSF and 20% methanol), using a bottle homogenizer (Braun Melsungen) with CO_2 cooling.

After filtration the homogenate was centrifuged for 30 min at 30,000 × *g*. Protein was precipitated by adding solid $(NH_4)_2SO_4$ to 50% saturation. After centrifugation (25 min at 30,000 × *g*) the pellet was redissolved in 25 ml buffer B (20 mM K-phosphate buffer, pH 8.2, containing 2.5 mM cysteine, 1 mM DTT, 1 mM K-EDTA, 10 μM FAD, 2 mM PMSF and 20% methanol) and applied at a flow rate of 40 ml h^{-1} to a blue sepharose column (K9/30, Pharmacia), equilibrated with buffer C (same as buffer B, but without PMSF and methanol). The column was washed with about 170 ml buffer C until the absorbance ($E_{280\,nm}$) of the eluate indicated that non-binding proteins had been removed.

NR was eluted with a linear gradient of 0–150 μM NADPH in 30 ml of buffer C. The gradient was applied at a flow rate of 14 ml h^{-1}. The collected fractions were analyzed for NR activity and protein content.

All operations were carried out at +4 °C.

Assay methods

NR activity was measured at 30 °C in a total volume of 0.8 ml 75 mM K-phosphate buffer, pH 7.5, containing 10 mM KNO_3. NAD(P)H-NR was assayed in the presence of 0.15 mM NAD(P)H. MVH_2-NR activity was assayed using 0.2 mM MV and 10 mM Na-dithionite in 10 mM $NaHCO_3$ as the reducing system. The reaction was stopped by adding 0.5 ml of a solution containing 10 mg sulfanilamide, 0.2 mg NED and 0.25 ml HCl (32%) per ml.

The $FMNH_2$-NR assay contained 10 mM K-EDTA, and 0.15 mM riboflavin-5-phosphate was employed as the reductant, which was reduced by white light (1000 μmol photons m^{-2} s^{-1}), provided by a Leitz slide projector (Osram Bellaphot lamp). The reaction was stopped by immersing the reagent tubes

Abbreviations: BSA, bovine serum albumin; DTT, DL-dithiothreitol; K-EDTA, ethylenediaminetetraacetic acid – potassium salt; MVH_2, methylviologen – reduced form; NED, naphthylethylenediammoniumdichloride; NR, nitrate reductase; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethyl sulfonylfluoride; SDS, sodium dodecyl sulfate.

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0341–0382/87/0500–0653 $ 01.30/0

in a boiling waterbath for 90 s. The diazo-coupling was carried out after all riboflavin was oxidized.

The colour was allowed to develop for 20 min. The absorbance was read at λ 540 nm.

Protein determination

Protein determination was based on the method of Bradford [7]. A commercial protein assay solution (Bio Rad) and γ-globulin as a standard were used.

SDS-PAGE

SDS-PAGE was based on the method described by Laemmli [8]. The stacking gel was 5%, the running gel 10% polyacrylamide. Protein samples (50 µg) contained 10% glycerol, 1% SDS, and 1% mercaptoethanol in 62.5 mM Tris-HCl buffer, pH 6.8. The samples were heated for 5 min at 90 °C.

The gels (100 mm × 145 mm × 1.5 mm) were run at a current of 15 mA until the marker dye (bromophenol blue) reached the running gel. Then the current was set to 25 mA.

Results and Discussion

The method described allows the successful extraction of NR from *Sphagnum* species. Mainly the use of PMSF as a protease inhibitor, DTT, L-cysteine and FAD are responsible for maintaining the isolated enzyme in an active state. As shown in Table I, NR extracted from 11 *Sphagnum* species was measured by three different *in vitro* assays. While NADPH, NADH and $FMNH_2$ can be used for measuring NR activity at each step of purification, MVH_2-NR activity can only be determined in samples purified by blue sepharose chromatography. For instance, post blue sepharose NR from *S. squarrosum* accepted all electron donors mentioned above: as compared with NADPH (100%) all other reductants brought about less enzyme activity: NADH 43%, $FMNH_2$ 58% and MVH_2 18%. Since Solomonson [9] introduced blue sepharose affinity chromatography as a tool in the purification of NR, this technique was been widely applied (*e.g.* [10–13]). Fig. 1 shows that NR from *S. cuspidatum* binds to blue sepharose, thus allowing a 348-fold

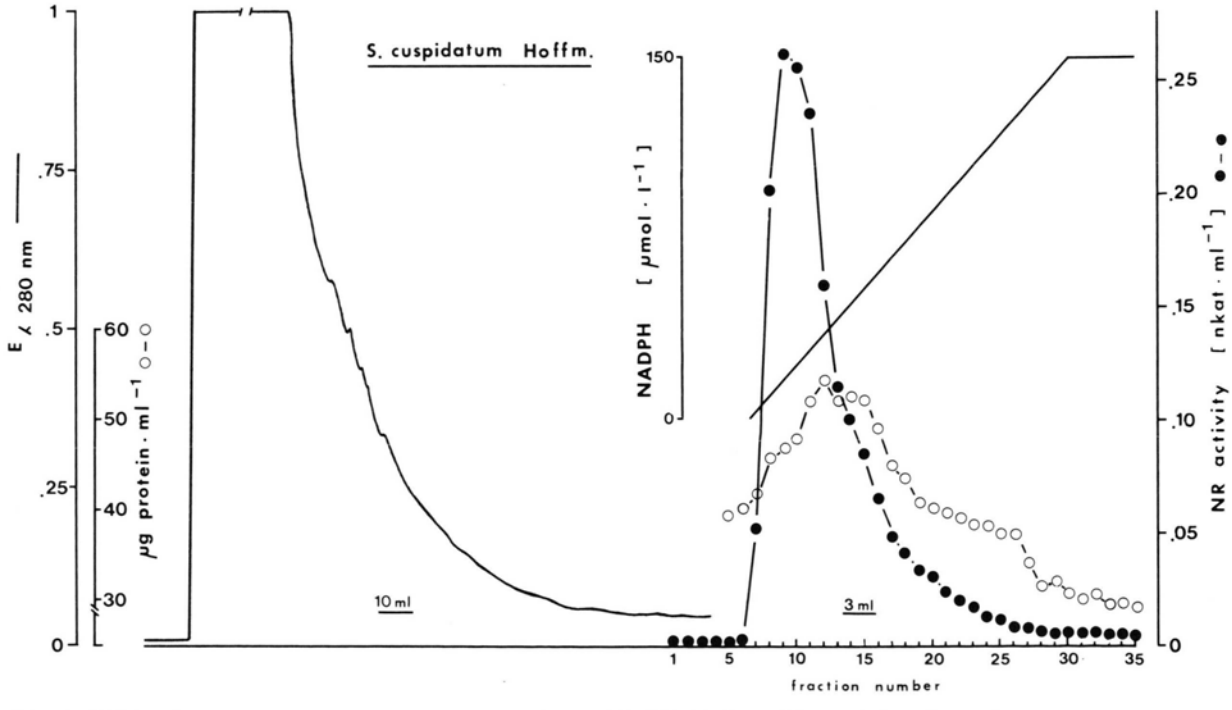

Fig. 1. Affinity chromatography of NR from *S. cuspidatum*. $(NH_4)_2SO_4$ (s = 0.5) precipitated proteins were loaded onto the blue sepharose column, and the column washed with buffer C until the non-binding proteins were removed as indicated by the low level of the absorbance $E_{280\,nm}$ (———). Then a linear gradient of 0–150 µM NADPH was applied and fractions of 1.23 ml were collected. NADPH-NR activity (●) and protein content (○) of each fraction were measured.

Table I. NR activities from the different *Sphagnum* species. NR activities were determined after $(NH_4)_2SO_4$ fractionation of crude extracts.

Sections and species	NR activity [pkat mg protein^{-1}] NADPH-NR	NADH-NR	$FMNH_2$-NR
Sphagnum			
S. magellanicum Brid.	34.1	13.1	21.2
S. palustre L.	11.9	4.2	8.5
S. papillosum Lindb.	6.1	1.9	1.0
Squarrosa			
S. squarrosum Crome	4.6	2.0	2.2
Subsecunda			
S. subsecundum Nees	8.0	3.5	4.5
Cuspidata			
S. cuspidatum Hoffm.	10.3	4.2	6.3
S. fallax (Klinggr.) Klinggr.	7.3	3.7	3.5
Acutifolia			
S. fimbriatum Wils.	12.9	4.6	7.3
S. molle Sull.	6.6	2.7	3.6
S. rubellum Wils.	9.1	3.4	5.0
S. subnitens Russ. & Warnst.	10.2	3.3	6.4

purification. Applying a linear NADPH gradient, the enzyme was eluted at very low NADPH concentrations. Similar results were obtained with NR from *S. fallax, S. fimbriatum, S. fuscum, S. magellanicum* and *S. squarrosum*.

The purification factors range from 322 *(S. magellanicum)* to 507 *(S. squarrosum)*. The overall recovery of at least 71% is higher than reported for NR from higher plants [13] or algae [9]. The highest specific activity, measured in the peak fraction of the elution profile of *S. magellanicum* NR, was 7.7 nkat mg protein^{-1}.

At each step of purification, samples were collected and subjected to SDS-PAGE (Fig. 2). During the purification certain protein bands are enriched (*e.g.* at 39, and 66 kDa), but even after blue sepharose affinity chromatography multiple bands are visible throughout the gel. This result is explained by the fact that all proteins possessing the dinucleotide fold bind to blue sepharose [14]. It should be emphasized that the described procedure, involving blue sepharose chromatography, results in only partially purified *Sphagnum* NR. These findings, however, are in agreement with those of Fido and Notton [12], who have shown that post blue sepharose fractions of spinach contain only partially purified NR.

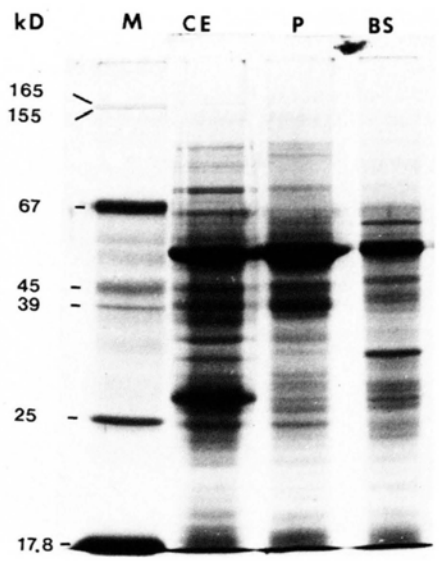

Fig. 2. Protein pattern of fractions obtained during purification of *S.* cuspidatum NR. M, molecular weight marker proteins (myoglobin – 17.8 kDa, chymotrypsinogen A – 25 kDa, ovalbumin – 45 kDa, BSA – 67 kDa and RNA polymerase – 39, 155, 165 kDa); CE, crude extract; P, $(NH_4)_2SO_4$ precipitate; BS, combined post blue sepharose fractions 8, 9 and 10 (Fig. 1). The gel was loaded with 50 μg crude extract and $(NH_4)_2SO_4$ precipitated proteins and 40 μg post blue sepharose fraction proteins. The gel was stained with Coomassie Brilliant Blue G-250.

Recent experiments, in which the Western blots technique and monospecific antibodies, raised against purified NR from corn were used, showed that the 66 kDa band is the subunit of *Sphagnum* NR (Deising and Rudolph, unpublished). The facts, that the enzyme from *Sphagnum* can be immunotitrated by *anti* corn NR serum and that only one distinct band appears on the nitrocellulose membrane after the Western blot procedure ensured, that NR from *Sphagnum* was specifically identified. As compared to NR from higher plants, the subunit of the bryophyte enzyme possesses a much smaller molecular weight [10, 15, 16].

In conclusion, this study has demonstrated a method for the extraction of NR from *Sphagnum* species. For the first time, a bryophyte NR was partially purified by affinity chromatography and recognized as an NAD(P)H dependent enzyme. In contrast to nitrate reductase from higher plants, which, in general, specifically use NADH, and from fungi, which are strictly NADPH dependent, NR from *Sphagnum* is an NAD(P)H dependent enzyme (EC 1.6.6.2).

Acknowledgement

I thank Prof. Dr. H. Rudolph for numerous discussions and critical reading of the manuscript, and Prof. Dr. A. Oaks for the generous gift of monospecific antibodies.

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