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

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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, FMNH₂ and – after blue sepharose affinity purification of the enzyme – MVH₂ 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

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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solution containing 0.5 mM KNO₃ and $1 \mu \text{M}$ Na₂MoO₄ (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 CO₂ cooling.

After filtration the homogenate was centrifuged for 30 min at $30,000\times g$. Protein was precipitated by adding solid $(NH_4)_2SO_4$ to 50% saturation. After centrifugation (25 min at $30,000\times g$) 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, $10~\mu$ m 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 $0-150~\mu m$ 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 KNO₃. NAD(P)H-NR was assayed in the presence of 0.15 mm NAD(P)H. MVH₂-NR activity was assayed using 0.2 mm MV and 10 mm Na-dithionite in 10 mm NaHCO₃ 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 FMNH₂-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



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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 ($100 \text{ mm} \times 145 \text{ mm} \times 1.5 \text{ mm}$) 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 FMNH2 can be used for measuring NR activity at each step of purification, MVH₂-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%, FMNH₂ 58% and MVH₂ 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

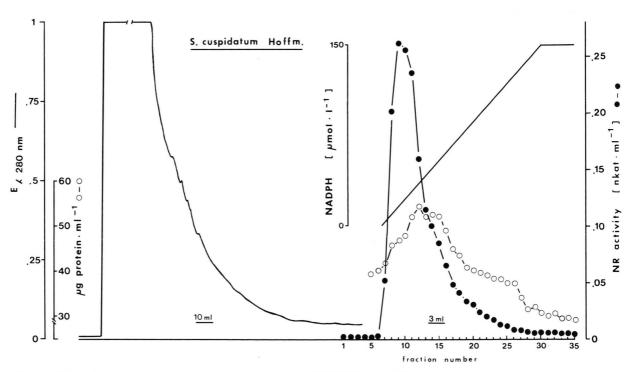


Fig. 1. Affinity chromatography of NR from *S. cuspidatum*. $(NH_4)_2SO_4$ (s=0.5) precipitated proteins were loaded onto the blue sepharose column, and the column washed with buffer C until the non-binding proteins were removed as indicated by the low level of the absorbance $E_{280\,\mathrm{nm}}$ (———).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

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Table I. NR activities from the different *Sphagnum* species. NR activities were determined after (NH₄)₂SO₄ fractionation of crude extracts.

Sections and species		kat mg protein NADH-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.

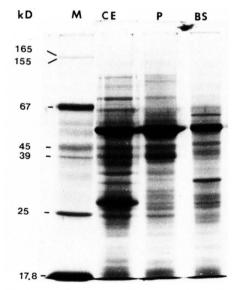


Fig. 2. Protein pattern of fractions obtained during purification of S. cuspidatum NR. M, molecular weight marker proteins (myoglobin – 17.8 kDa, chymotrypsinogen A – 25 kDa, ovalbumin – 45 kDa, BSA – 67 kDa and RNA polymerase – 39, 155, 165 kDa); CE, crude extract; P, (NH₄)₂SO₄ precipitate; BS, combined post blue sepharose fractions 8, 9 and 10 (Fig. 1). The gel was loaded with 50 μ g crude extract and (NH₄)₂SO₄ precipitated proteins and 40 μ g post blue sepharose fraction proteins. The gel was stained with Coomassie Brilliant Blue G-250.

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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).

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