Environ Chem Lett (2006) 4: 69–73
DOI 10.1007/s10311-006-0035-4

ORIGINAL PAPER

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Nitrate reductase for nitrate analysis in water

Received: 18 January 2006 / Accepted: 27 February 2006 / Published online: 28 March 2006
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Abstract Nitrate analysis in water is one of the most frequently applied methods in environmental chemistry. Current methods for nitrate are generally based on toxic substances. Here, we show that a viable alternative method is to use the enzyme nitrate reductase. The key to applying this Green Chemistry solution for nitrate analysis is plentiful, inexpensive, analytical grade enzyme. We demonstrate that recombinant *Arabidopsis* nitrate reductase, expressed in the methylotrophic yeast *Pichia pastoris*, is a highly effective catalyst for nitrate analysis at 37°C. Recombinant production of enzyme ensures consistent quality and provides means to meet the needs of environmental chemistry.

Keywords Nitrate reductase · Recombinant expression · Nitrate · Green chemistry · Water · Enzymes

Introduction

Enzymes are biological catalysts and one of the components responsible for the existence of life on earth. Biochemistry of enzymes is well understood and it is now possible to produce essentially any enzyme using recombinant DNA technology. As a consequence, specifically designed enzymes are being used in industry to replace older methods. The introduction of enzymes into environmental chemistry is one of the next logical steps to enhance the sustainability of analytical methods being employed for analysis of chemical components in water in this discipline.

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Nitrate content of drinking water is regulated by government agencies at 10 mg nitrate as nitrogen per liter (ppm nitrate-N), which may also be expressed as 44 mg nitrate per liter. This limit is utilized to prevent the occurrence of methemoglobinemia in infants (Clesceri et al. 1998). Nitrate is also suspected of other negative effects on human health and is toxic to livestock. The more frequent occurrence of drought today, resulting in increased nitrate content in animal feeds, places livestock at greater risk. In addition, the general increase in the availability of nitrogen in the environment has resulted in persistent problems with nitrate pollution in ground and surface waters. For all these reasons, nitrate analysis of water samples is one of the most common methods used in environmental chemistry.

Of the many analytical methods used in environmental chemistry, nitrate analysis is one that can be done with an enzyme rather than with inorganic or organic reagents (Campbell 1999). Nitrate analysis is accomplished by reduction of nitrate to nitrite, and reaction of the resulting nitrite with the Griess reagents to yield a highly colored compound which can be quantified by using a colorimeter or spectrophotometer (Patton et al. 2002). The reduction step in the existing methods is carried out using copper–cadmium granules or hydrazine (Clesceri et al. 1998). Both these reagents are toxic.

An alternative approach is to use nitrate reductase (NaR) to catalyze the reaction and the natural reducing agent of this enzyme, namely, reduced nicotinamide dinucleotide (NADH), to drive the conversion of nitrate to nitrite. Nitrate analysis using NaR and NADH has a long history, which was reviewed by Campbell (1999). The enzyme method for nitrate analysis in natural water samples has been described for an automated system where commercial corn leaf NaR was employed (Patton et al. 2002). The challenge in making the enzyme method a viable alternative to existing methods is to produce NaR of analytical grade in sufficient quantities and at a competitive price.

We describe production of NaR, derived from the plant *Arabidopsis thaliana* via recombinant DNA technology, using the methylotrophic yeast *Pichia pastoris* as host for expression. This expression system is widely used for

production of enzymes (Higgins and Cregg 1998) and NaR has been successfully expressed in this system (Su et al. 1997; Skipper et al. 2001). To simplify purification of the enzyme, a series of six histidine residues (His-tag) was engineered near the amino-terminus of the *Arabidopsis* NaR2. Thus, immobilized metal ion affinity chromatography (IMAC), which takes advantage of the His-tag engineered into the *Arabidopsis* NaR2 polypeptide chain, was employed as a single purification step to produce analytical grade enzyme. Employing this recombinantly engineered NaR with NADH as reducing agent, was shown to yield an effective method for nitrate analysis. The enzymatic reduction nitrate analysis method was applied to water samples to demonstrate its utility for environmental chemistry.

Experimental

Nitrate analysis was carried out by reconstituting freeze-dried *Arabidopsis* NaR2 (EC 1.7.1.1) in 25 mM potassium phosphate, 0.1 mM Na_2EDTA, pH 7.5, at 22–25°C, to a final concentration of 1 unit/ml. The unit of enzyme activity is defined as the amount of NaR catalyzing conversion of 1 μmol nitrate to nitrite per minute at pH 7.5 and 30°C. The nitrate analysis reagent was prepared by bringing the reconstituted NaR2 solution (3 units) to 54 ml by mixing with additional phosphate buffer, and the addition of 1.0 ml of 2.82 mM NADH solution, prepared in deionized water. The enzymatic reduction step in the nitrate analysis method was carried out by adding 50 μl of aqueous sample or nitrate standard to 1.0 ml of nitrate analysis reagent and incubating the mixture at 37°C for 15 min in a water bath. The color development step was carried out by addition of 100 μl of sulphanilamide reagent (10 g l^{-1} of ~ 1.2 N HCl), followed by addition of 100 μL *N*-(1-naphthyl)ethylenediamine dihydrochloride reagent (1.0 g l^{-1} of deionized water) and reacting mixture at 22–25°C or 37°C, for at least 5 min. Finally, the absorbance at 540 nm was determined for each sample and nitrate standard using a Hewlett-Packard spectrophotometer. A standard curve was generated from the absorbance of nitrate standards prepared in the range of 0.2–10 ppm nitrate-N using a certified 1000 ppm nitrate standard (Fisher Scientific Co.). The nitrate content of the samples was determined from their absorbance using the nitrate standard curve. Nitrate-free deionized water was used, and all containers and reaction vessels were also free of nitrate.

Arabidopsis NaR2 was engineered using the polymerase chain reaction carried out with synthetic oligonucleotide primers designed to introduce restriction enzyme sites at the termini of the enzyme's gene with the previously cloned complementary DNA for the enzyme's messenger RNA transcript as the template (Crawford et al. 1988). The primers also introduced nucleotides encoding a hexahistidine sequence near the amino-terminus of the protein and a proteinase site for removing the His-tag after expression. The engineered *Arabidopsis* NaR2 gene (GenBank Accession no. AY575950) was purified and cloned into the *P. pastoris* vector pPICZa (Invitrogen, Carlsbad, CA) using the restriction enzyme sites introduced in the gene. The construct was linearized by restriction enzyme digestion with *Pme*I, which is a unique site in the vector, and transformed into *P. pastoris* KM71H competent cells (Barbier et al. 2004). Transformed cells were selected by resistance to the antibiotic Zeocin™, which is a feature of the pPICZa vector, and grown in cultures induced with methanol for expression of NaR2 from the alcohol oxidase promoter which is also a feature of the vector. Cell lines expressing the highest NaR activity per gram wet cell weight were selected. The methanol utilization phenotype of the cell line chosen for production, which is called AtNaR2-45, was determined to be of the slow growth type with methanol as the sole carbon source, known as Mut^S, which was expected based on the *P. pastoris* KM71H strain used as host (Higgins and Cregg 1998). The AtNaR2-45 cell line was grown in 10 l BioFlo3000 fermenter (New Brunswick Scientific, Edison, NJ) using a protocol similar to those previously described for methanol-induced expression of NaR in *P. pastoris* (Barbier et al. 2004; Mertens et al. 2000; Skipper et al. 2001).

Recombinant NaR2 was purified by IMAC using His-Select™ resin (Sigma-Aldrich, St. Louis, MO), loaded with Ni^{2+}, which was carried out using a batch protocol (Barbier et al. 2004). Briefly, the AtNaR2-45 cell extract was gently mixed on a stir plate at 4°C with ~300 ml Ni-His-Select™ resin for 45 min and recovered by vacuum filtration. Enzyme-loaded resin was washed six times with 2 l of 50 mM Na-phosphate, 0.3 M NaCl, 10 mM imidazole, 10% glycerol, pH 7.0, and recovered by filtration. Washed, enzyme-loaded resin was transferred to a 1 l glass column and eluted with "washing buffer" with the imidazole concentration increased to 300 mM. Eluted fractions with highest NADH NaR activity were pooled and buffered exchanged in 25 mM MOPS, pH 7.0, using an Amicon stirred ultrafiltration cell equipped with YM30 membrane (Millipore, Bedford, MA). Concentrated enzyme was freeze-dried in aliquots of 1–3 units and stabilized using a sugar. The concentration of *Arabidopsis* NaR2 protein was determined by absorbance at 413 nm using an extinction coefficient of 120 mM^{-1} cm^{-1} (Skipper et al. 2001). Quality of the *Arabidopsis* NaR2 preparation was determined using denaturing gel electrophoresis (Barbier et al. 2004).

Results and discussion

Nitrate determination with *Arabidopsis* NaR2

Development of a reliable and accurate nitrate determination method using NaR at 37°C has been difficult due to the instability of the enzyme at this analysis temperature where it must remain active for at least 15 min to catalyze full reduction of nitrate to nitrite. In Fig. 1, we demonstrate that *Arabidopsis* NaR2 provides a highly accurate and reproducible nitrate determination standard curve at 37°C, when employed in the method described here.

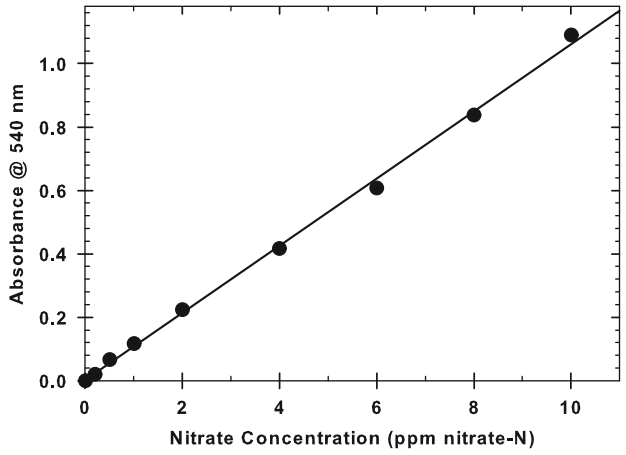

Fig. 1 Enzymatic reduction nitrate analysis standard curve generated using *Arabidopsis* NaR2 at 37°C, as described in the "Experimental" Section. Each point represents mean of 10 determinations with a standard error of less than 3%. The least-squared line fit has a correlation coefficient = 0.998. The inverted slope of the line = 9.42 ppm nitrate-N per $A_{540\ nm}$

Production of *Arabidopsis* NaR2

The production of NaR in *P. pastoris* has been worked out over the past 10 years, beginning with the work of Su et al. (1997). The eukaryotic nature of pyridine nucleotide dependent NaR forms obtained from plants, fungi, yeasts, and algae (EC 1.7.1.1–3) appears to explain why these enzymes are not expressed in active form in *Escherichia coli* (Campbell 1999). In addition to complete enzyme, various catalytically active fragments or modules of NaR have also been produced in *P. pastoris*, including the nitrate reducing module containing the molybdenum–molybdopterin cofactor involved in catalysis of the conversion of nitrate to nitrite (Barbier et al. 2004; Mertens et al. 2000). A structure model for the NaR active site has been derived from X-ray diffraction analysis of crystalline forms of the nitrate-reducing module of *Pichia angusta* NaR (Fisher et al. 2005). The structural results indicate that the yeast NaR expressed in *P. pastoris*, which is called YNaR1, is a dimer of two identical subunits and fully loaded with molybdenum cofactor. On the other hand, biochemical analysis of *Arabidopsis* NaR2 showed that it assembled as a tetrameric structure of four identical subunits and, on average, only one of the subunits contained a molybdenum (Skipper et al. 2001). Thus, recombinant plant and yeast NaR, which are 57% homologous in amino acid sequence, have quite different structural forms. To confirm this conclusion and gain greater insight into the regulation of plant NaR activity, the crystallization of *Arabidopsis* NaR2 has been initiated (Schwarz 2006, personal communication).

The differences in the structures and composition of the yeast NaR1 and *Arabidopsis* NaR2 suggest that there will also be differences in biochemical properties. To take advantage of these expected differences, we engineered *Arabidopsis* NaR2 by recombinant DNA methods and expressed it in *P. pastoris*. Engineered NaR2 was designed to have a metal binding site near the amino terminus (His-tag), which provides a facile handle for cost-effective purification of the recombinant enzyme in downstream processing. Furthermore, we generated *P. pastoris* cell lines expressing *Arabidopsis* NaR2 with a slow methanol growth phenotype, since this has been found to be advantageous for production of NaR (Barbier et al. 2004; Skipper et al. 2001). In addition, selection of production cell lines was directed toward those with multiple copies of the NaR2 expression cassette, since there seems to be a linear correlation between gene copy number and the level of NaR activity expressed (Barbier et al. 2004). The *P. pastoris* cell line selected for production of *Arabidopsis* NaR2 (called AtNaR2-45) had the highest NADH NaR activity per wet cell weight among 45 cell lines selected with a high resistance to the antibiotic Zeocin^{TM}, which indicates a high copy number of the resistance factor and therefore, the gene of interest. The AtNaR2-45 *P. pastoris* cell line was shown to have the Mut^{S} phenotype by growth on methanol plate cultures side-by-side with wild type and alcohol oxidase mutant *P. pastoris* cell lines. When the AtNaR2-45 cell line was grown in the fermenter using a standardized protocol for production of Mut^{S} strains of *P. pastoris*, which begins with glycerol batch and fed-batch stages to bulk up cell mass prior to methanol feeding (Mertens et al. 2000), the cell growth during the 21 h methanol feeding stage was not significant, as expected, with no net increase in the gram wet cell weight per liter of culture. However, during the methanol phase, the NaR2 activity in the fermenter increased to ~5 units/g wet cell weight. Overall, ~2.5 kg wet cell weight and ~13,000 units of NADH NaR activity were produced in a typical fermenter run, which requires ~6 person-days of labor and a low cost in materials when carried out in an established production facility.

Purification of *Arabidopsis* NaR2, which requires ~2 person-days of labor, was done with immobilized metal ion affinity chromatography (Table 1). Overall, ~25% of the total enzyme produced in the cells is recovered as purified NaR2. Purified *Arabidopsis* NaR2 had specific activity equivalent to the value previously reported for this recombinant enzyme, when the protein content is based on the heme-Fe absorption (Skipper et al. 2001). The purified enzyme is essentially homogenous when analyzed by denaturing gel electrophoresis and the size of the polypeptide chain is ~100,000 Da, as expected (Fig. 2). The UV-Vis spectra of oxidized and NADH-reduced enzyme were virtually identical to previously reported spectra (Skipper et al. 2001).

Nitrate analysis of real world water samples

Determination of nitrate in natural water samples was done in the same way as the nitrate standards plotted in Fig. 1, as described in the "Experimental" Section. Five water samples of known nitrate content (Patton et al., 2005, personal communication), were determined and found to have a nitrate content within 5% of the value determined by the cadmium reduction method. Local pond and stream water were determined to have 0.2 and 0.1 ppm nitrate-N, respectively,

Table 1 Summary of the purification of *Arabidopsis* NaR2

Purification step	Total activity (units)[a]	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Extract[b]	2690	9100	0.32	100
IMAC[c]	1410	50	28	52

[a]Activity unit: μmol nitrite produced per min

[b]Extract made from ∼1.2 kg wet cell weight

[c]IMAC: immobilized metal ion affinity chromatography

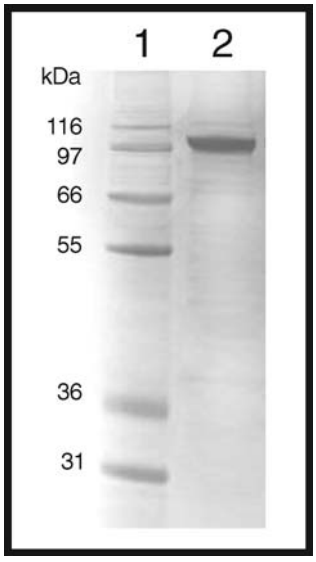

Fig. 2 Purity and molecular size of *Arabidopsis* NaR2. A 10% Bis-Tris Nu-PAGE™ gel (Invitrogen) was used. *Lane 1*, marker proteins (Sigma); *lane 2*, purified *Arabidopsis* NaR2 after IMAC and buffer exchange. Approximately 1 μg protein was loaded on the gel and run for 45 min at 200 V

which corresponds well with the natural watershed of these bodies of water. Local ground water obtained from a drinking water well had no detectable nitrate, which reflects the lack of agriculture in the area and the local geology. Lake Linden, Michigan, tap water had 0.3 ppm nitrate-N, which corresponds well with the value for nitrate content reported by the village water manager, based on certified nitrate analysis done with the cadmium reduction method. Thus, it is clear that the enzymatic reduction nitrate analysis method using *Arabidopsis* NaR2 at 37°C works well for determining nitrate in real world samples. Since the volume of sample required for analysis is quite small, colored samples such as the pond water analyzed here, do not interfere with the determination. In general, the only inhibitors of the enzyme found in natural water samples are heavy metals and this is overcome by inclusion of EDTA in the system buffer to chelate these ions. In addition, salt water and estuary water samples, containing chloride, can also be handled by this method without interference (data not shown).

It should be noted that all nitrate methods report the sum of nitrate and nitrite in water samples (Clesceri et al. 1998). The nitrate method reported here can be easily adapted for nitrite analysis in the same water samples. This is accomplished by omitting the enzyme and NADH from the analysis reagent and carrying out the protocol. This was done on the water samples described above and all were found to contain no nitrite. This is expected for natural water samples and agrees with the certified analysis for Lake Linden, Michigan, tap water.

Natural water samples from undisturbed regions generally have low nitrate content. This is illustrated by the natural water samples analyzed here, in which the nitrate content was found to be near the limit of detection of the enzymatic reduction nitrate method. However, the method described here can be extended into a low range by simply increasing the volume of sample analyzed. For example, a low range enzymatic reduction nitrate method could be formulated to use 0.5 ml of sample and 0.5 ml of analysis reagent containing NaR2 and NADH with an increased concentration of buffer. The low range nitrate analysis would extend the detection limit to 0.05 ppm nitrate-N (data not shown). Thus, the enzymatic reduction nitrate analysis method is effective for analysis of water with very low nitrate content, including samples from the open ocean.

Conclusion

We show here that recombinant *Arabidopsis* NaR2 is effective in nitrate determinations carried out at 37°C using enzymatic reduction method. Recombinant enzyme was engineered to facilitate downstream processing and produced in large quantities using commercial yeast expression system. *Arabidopsis* NaR2 was easily purified to analytical grade enzyme, as shown by gel electrophoresis and spectrophotometry. Highly efficient production of recombinant enzyme results in a low cost per assay, requiring only 0.055 units of *Arabidopsis* NaR2. Furthermore, the method can be easily adapted to existing automated instruments and is highly suited to the new robotic autoanalyzers now being introduced for environmental analysis.

Acknowledgements We thank Prof. N. M. Crawford for supplying the *Arabidopsis* NaR2 containing plasmid. We also thank Troy Kinnunen-Skidmore and Michael J. B. Campbell for assistance with the experiments. This work was supported, in part, by the Michigan Tech Research Fund.

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