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A High-Throughput Assay Format for Determination of Nitrate Reductase and Nitrite Reductase Enzyme Activities

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ABSTRACT

The authors describe a microplate-based high-throughput procedure for rapid assay of the enzyme activities of nitrate reductase and nitrite reductase, using extremely small volumes of reagents. The new procedure offers the advantages of rapidity, small sample size-nanoliter volumes, low cost, and a dramatic increase in the throughput sample number that can be analyzed simultaneously. Additional advantages can be accessed by using microplate reader application software packages that permit assigning a group type to the wells, recording of the data on exportable data files and exercising the option of using the kinetic or endpoint reading modes. The assay can also be used independently for detecting nitrite residues/contamination in environmental/food samples.

Index Entries: *Candida utilis*; enzyme assay; environment; food yeast, microplate; nitrate; assimilation; nitric oxide synthase; nitrite; water.

INTRODUCTION

The assimilation of inorganic nitrogen by plants and microbes is catalyzed by activities of the enzymes, NAD(P)H: nitrate oxidoreductase

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(EC 1.6.6.2) and NAD(P)H: nitrite oxidoreductase (EC 1.6.6.4) *(1)*. These are among the most well-studied enzymes both at biochemical and molecular genetic levels, because of their important physiological roles and their structural intricacies at the molecular level, which have been coming to light only recently. Conventionally, these two enzymes are assayed by a standard colorimetric method that measures the color intensity of the azo dye formed by the coupling of diazotized sulfanilamide with *N*(1-naphthyl) ethylenediamine (NED) and the nitrite in the reaction mixture, using a spectrophotometer or a simple colorimeter such as the Klett Summerson Colorimeter or a Spectronic 20. Nitrite is the reaction product of nitrate reductase, the first enzyme of the pathway, and is substrate for nitrite reductase, the second enzyme. This methodology thus requires a few to several milliliters of reagents and can read only one sample at a time, costing considerable amounts of time and reagents to analyze multiple samples. Further, to study the properties of these two enzymes, especially fast kinetics, or to monitor enzyme activity of multiple fractions collected during purification procedures where one has to rapidly assay multiple samples and document and analyze the data, the speed of the assay becomes even more critical. The need for a faster assay is further accentuated when one attempts to use an *in situ* procedure as we do, to assay one of these enzymes in the whole cell milieu, where a number of cellular reactions are active in a dynamic equilibrium, impacting the reaction rate of one another. To fulfill these and analogous needs we examined the feasibility of adapting for our enzyme assays the microplate format, which requires only microliters of reagents and completes reading 96 samples within a fraction of a minute, and the results are reported in this paper.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical grade. Dithiothreitol (DTT), Fe_2SO4, NED, sodium pyrophosphate, sodium tungstate, sulfanilamide, and zinc acetate were obtained from Sigma Biosciences, St. Louis, MO, and dextrose, EDTA, sodium nitrite ($NaNO_2$), sodium nitrate ($NaNO_3$), sodium and potassium phosphate salts, and trichloroacetic acid (TCA) from Fisher Scientific, Pittsburgh, PA.

Organism

The food yeast, *Candida utilis* (ATCC 9950), was obtained from the American Type Culture Collection, Rockville, MD. The composition of the medium and maintenance of the yeast were as described *(1)*.

Yeast Culture

One hundred mL of a rich medium (YPD, yeast extract-peptone-dextrose, Difco, Detroit, MI) was inoculated with *C. utilis* and allowed to grow overnight at 28°C on a gyratory shaker shaking at 120 g force. The yeast culture was centrifuged at 3000 g force for 10 min, the cell pellet resuspended in 50 mL fresh synthetic medium containing 10X Yeast Nitrogen Base (Difco, Detroit, MI), 1% (w/v) dextrose, and 0.5% $NaNO_3$, and further growth was allowed for 3.5 h in order to induce nitrate reductase activity. Nitrite reductase was induced by continuing the growth of YPD-grown yeast overnight (ca. 10 h) in the induction medium, consisting of 10X Yeast Nitrogen Base, supplemented with 1% (w/v) dextrose, 0.5% (w/v) 1 m*M* ferrous sulfate, 1m*M* sodium tungstate, and 50 m*M* $NaNO_3$. After this time, the yeast cells were washed (three times) and resuspended in 25 mL of 50 m*M* potassium phosphate buffer, pH 7.0, 0.1 m*M* DTT, and 1.0 m*M* EDTA.

Permeabilization of Yeast

Yeast cells, resuspended in 50 m*M* potassium phosphate buffer, pH 7.0, containing 1 m*M* EDTA, and 0.1 m*M* DTT, were distributed in 1.0 mL aliquots in chilled 1.5 mL microfuge tubes. One hundred microliters of chilled toluene: ethanol (1:4, v/v) was added to each tube and the tubes were vortexed at maximal speed for 90 s. Ten microliters aliquots of this preparation were used to assay nitrate- and/or nitrite-reductase activities *in situ* using the microplate method as described below or using the standard macro-version (spectrophotometric) of the colorimetric method.

Enzyme Assays

Nitrate Reductase (EC 1.6.6.2)

Nitrate reductase activity was assayed as a function of the product (nitrite) formed at the end of the reaction. In the microplate assay procedure, to each of the wells in a round-bottomed microplate (Dynatech Labs Inc., Chantilly, VA) were added 10 µL of the permeabilized yeast cell preparation, 40 m*M* sodium pyrophosphate buffer, pH 7.0, and 20 m*M* sodium nitrate, in a total reaction volume of 20 µL. The plates were then gently shaken briefly to ensure mixing of the assay reagents and were incubated in an air incubator at 37°C for 1, 5, 10, 15, 20, 30, 60, or 90 min, respectively. The reactions were terminated by the addition of 20 µL of 1% sulfanilamide in 10% TCA and cooling the plate on ice for 2 min. The plates were centrifuged for 30 s at 6000*g* (optional step), and 10 µL of the supernatant were transferred to a clean microplate using a multi-channel pipettor, and color development was carried out by the addition of 10 µL of NED. After 30 min at room temperature, the optical density (OD) of all the samples was read at 560 nm in a microplate reader (UVmax Kinetic

Microplate Reader equipped with the application software package—'Softmax'; Molecular Devices Corp., Menlo Park, CA). Recording the O.D. of 96 samples took 14 s.

The relative concentrations of nitrite corresponding to the observed A_{560} values were read directly from a nitrite calibration curve, generated by plotting nitrite reference dilutions in the range of 1 μmole to 1 mmole against the corresponding A_{560} values. All assays were done in triplicate.

Nitrite Reductase (EC 1.6.6.4)

Nitrite reductase activity was assayed as a function of the depletion of the substrate (nitrite). Aliquots of permeabilized yeast cells of 10 μL each, with 50 m*M* phosphate, pH 7.0 and 0.1 m*M* $NaNO_2$, in a total reaction volume of 20 μL per well, were incubated in 96-well microplates for various time periods, 1, 5, 10, 15, 20, 30, 60, or 90 min, respectively, at 30°C. The enzymatic reaction was initiated by the addition of permeabilized yeast cells and was terminated by the addition of 10 μL of 1.0*M* zinc acetate and 10 μL of 1% sulfanilamide in 50% TCA at the end of the desired time period and placing the assay-plate on ice. Procedures for color development and reading and for nitrite estimation were similar to those described for the nitrate reductase assay.

Assays of nitrate reductase and nitrite reductase activities using the standard spectrophotometric procedures were performed as described *(2)*, using a 50-fold greater reaction volume and reagents. The A_{560} values of the reaction products were read in a Beckman DU-6 spectrophotometer using 1-mL cuvets.

Protein Estimation

The concentration of the total extractable protein-content of permeabilized cells determined by the Lowry procedure as modified by Hartree *(3)*, using bovine serum albumin as standard, was found to be 55 mg/g wet weight of packed yeast cells.

RESULTS AND DISCUSSION

Figures 1 and 2 compare the microplate method with the standard spectroscopic method in determining the kinetics of nitrate reductase and nitrite reductase activities, respectively. The data show the microplate assay, with all its advantages, to be on par with the standard assay both in sensitivity and reproducibility (Fig. 1). Nitrate reductase activity by both methods followed linear course between 5 and 60 min. Similarly, nitrite reductase activity, in both formats of assay, peaked at 20 min and maintained a near steady state level for the rest of the assay duration (Fig. 2). We have been using the microplate assay routinely and reproducibly for the assay of nitrate reductase and nitrite reductase activities during various purification steps of these two enzymes.

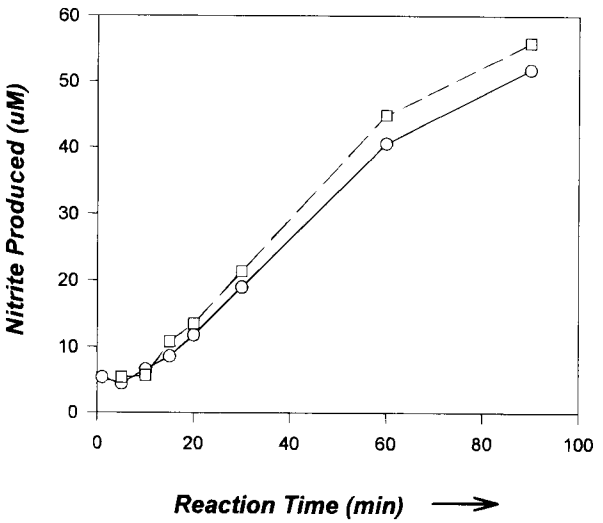

Fig. 1. Time-course of yeast nitrate reductase activity. Nitrate reductase activity was assayed as a function of the production of nitrite. In the microplate assay (-○-), 10 µL of permeabilized preparation of *C. utilis* was incubated with nitrate reductase *in situ* assay mixture in a total reaction volume of 20 µL at 37°C, and the reaction was terminated at the end of the time periods shown in the figure, by the addition of acidified sulfanilamide. A 50-fold higher reaction volume and contents were used in the standard spectrophotometric assay (-□-) of nitrate reductase activity, as described earlier (*2*). The reaction end-product processing and detection were accomplished as described in the text, under the Methods section.

The microplate assay, in addition to facilitating rapid assay of nitrate reductase and nitrite reductase activities and estimation of nitrite, offers additional advantages. The microplate reader allows analysis of 96 samples simultaneously within a short time period of 14 s, in a striking contrast to the extremely time-consuming conventional spectrophotometric procedure that requires reading of individual samples separately one at a time, each requiring about 50-fold more reagents. Since the microplate procedure can analyze as little as 10 µL, the volumes of the reagents required and the cost and time spent are all decreased considerably. Relative to other assays available for nitrite estimation, this microplate format is simple to perform and facilitates detection of nitrite with much greater rapidity, without compromising its sensitivity. Further advantages include automated data processing features offered by the application software packages. Microplates can be mapped with the location and values of blanks, controls, unknowns, and empty wells. Group types can be assigned to any well (i.e., unknown, control, and so on). Plate blanks can be subtracted from all wells on the plate as can group blanks. Both kinetic and endpoint reading modes are available, the kinetic mode offering an added advantage to enzyme assays. Additionally, raw data collected from

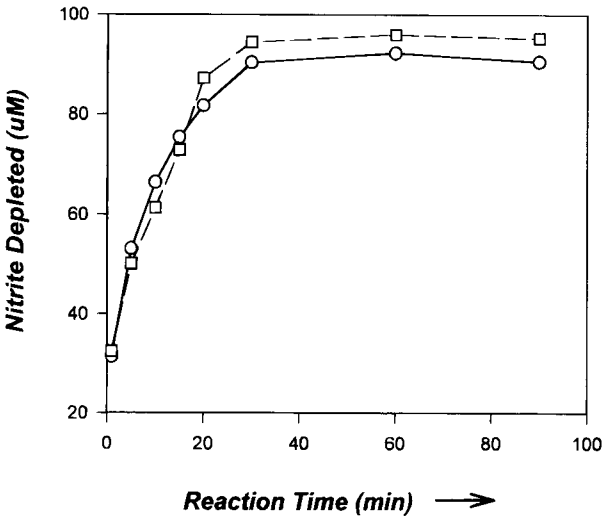

Fig. 2. Time-course of nitrite reductase activity. Nitrite reductase activity was assayed by following the depletion of nitrite in the reaction mixture. The assay mixture contained 50 m*M* potassium phosphate buffer, pH 7.5, 0.1 m*M* sodium nitrite and 10 µL permeabilized yeast cell preparation, in a total reaction volume of 20 µL, in the microplate assay format (-○-). A 50-fold higher reaction volume and contents were used in the standard spectrophotometric (-□-) assay of nitrite reductase activity, essentially as described for nitrate reductase. The reaction initiation and termination conditions and substrate (depletion)-monitoring protocol were as described in the text, under the Methods section.

microplates can be stored as data files and displayed in several different formats, or can be exported for analysis in a separate context of analysis or in other programs, such as Lotus 1-2-3.

The application of the microplate format is envisioned beyond the assay of nitrate reductase and nitrite reductase enzyme activities, e.g., in the studies of the nitric oxide synthase-mediated biosynthesis of nitric oxide, a cellular mediator of growing importance. A simple, sensitive, and precise assay requiring almost no sample preparation to measure physiological changes in the concentrations of both nitrite and nitrate, commonly used as indices of the generation of nitric oxide, which is difficult to quantify because of its extremely short half life *(4)*, would be a highly valuable asset.

Further, nitrites are ubiquitous in their distribution and are formed naturally as process intermediates or end-products in some contexts, or are added deliberately to the source, in others. Ground water, fertilizers, fresh agricultural produce, preserved meats, drugs, and mammalian cells contain significant levels of nitrites. The levels of nitrite present in the environment as a result of biological denitrification, industrial waste and fertilizer runoff are assessed by monitoring nitrite concentrations in envi-

ronmental samples such as lake or river water, rain, and underground water. Presence of nitrite in concentrations exceeding those specified by public health authorities is reported to culminate in health hazards such as induction of methaemoglobinaemea, and formation of carcinogenic nitrosamines as a consequence of the reaction of nitrites with secondary and tertiary amines *(5)*, and has thus created a need for monitoring of diets, saliva, serum samples *(6)*, processed foods, and fresh produce, in order to facilitate accurate assessment of potential risk posed by these toxic chemicals to human health. The proposed procedure has the potential to be extremely valuable to analyses of environmental contamination that requires on-site screening of multiple samples, out in the field and away from the laboratory where instrumentation is located.

A number of sensitive assays are available for nitrite estimation, but none matches the cost-effectiveness and simplicity of the technique proposed here. The ion exchange chromatographic methods developed by Small et al. *(7)*, Gjerde et al. *(8)*, and Jackson et al. *(9)* are highly sensitive but suffer from interference because of the presence of common anions such as chloride, nitrate, and phosphate. The gas chromatographic method developed for the determination of nitrite using ethyl 3-oxobutoanate as a derivatization reagent and applied successfully to the determination of nitrite in river water and human saliva, with the detection limit of 2 ng/mL *(10)* is useful, but is again dependent on heavy instrumentation.

In conclusion, the proposed procedure offers an unique combination of speed, simplicity, sensitivity, cost-effectiveness, and field-applicability to multiple sample-analysis. It removes the tedium from the analysis of multiple samples, by reducing the analysis time from several hours to a few minutes.

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