Enzymatic Nitrate Assay by a Kinetic Method Employing *Escherichia coli* Nitrate Reductase

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An enzymatic assay system for nitrate employing the membrane-bound nitrate reductase (EC 1.7.99.4) of *E. coli* is described. Contrary to previous enzymatic assay systems, the present method is a kinetic one, *i.e.* the substrate, nitrate, is assayed by measuring the reaction rate of the nitrate reductase-catalyzed reaction. Based on the observation that the nitrate reductase-catalyzed reaction obeys pseudo-first order kinetics, a test system is described allowing the assay of nitrate at a concentration as low as 1 ppm. The relatively high Michaelis-Menten constant for nitrate (0.3 mM) of the *E. coli* nitrate reductase favours nitrate assay by the kinetic method.

1. Introduction

In the last thirty years, the increasing use of nitrate in agricultural fertilization and preservation of meat products has caused a nitrate contamination problem both of drinking water and foodstuffs. Until now, nitrate was mainly assayed by chemical methods. Thus, for determination of nitrate in water, a colorimetric method employing the strychnin-derivative brucin is recommended as a standard procedure for water analysis in Germany [1]. Nitrate in meats and other foodstuffs is determined as nitrite after chemical reduction with metallic cadmium as reductant [2]. Both methods are highly unsatisfactory from a toxicological point of view, as well as with respect to reproducibility. Although a variety of alternative assay methods have been described (including electrochemical assays with nitrate specific electrodes), a simple enzymatic test system for nitrate would be highly desirable. Recently, an enzymatic assay for nitrate using purified *Escherichia coli* nitrate reductase has been described [3]. However, the purification procedure is rather complicated and the commercially available enzyme is very expensive. We therefore have investigated the possibility to use a crude membrane fraction of nitrate-grown *E. coli* as enzyme source for the nitrate assay. In contrast to a previous report on enzymatic nitrate assay using a bacterial nitrate reductase [4], our assay system is based on measurements of the nitrate reductase-catalyzed reaction rate and is, therefore, much less time consuming.

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3. Materials and Methods

Escherichia coli strain K12 (DSM 498) (kept in the culture collection of the institute) is maintained on agar slants (pH 7.2) containing 0.3% (w/v) meat extract, 0.5% (w/v) peptone and 1.5% (w/v) agar. Large cell batches were grown semianaerobically in 5 liter carboys filled to 4/5 of their volume with a culture medium containing 1% (w/v) nutrient broth, 0.2% (w/v) NaCl and 0.4% (w/v) KNO_3. The pH was adjusted to 7.2 with 1 N NaOH before autoclaving. The cultures were inoculated with 25 ml of a nutrient broth culture and grown overnight at 30 °C with gentle magnetic stirring until the turbidity of the culture (measured at 546 nm in 1 cm-cuvettes in the PM 2000 photometer of R. Riele KG, Berlin) had attained a value of 0.9 to 1.0. The cells were harvested by centrifugation, washed twice in 50 mM K-phosphate (pH 7) containing 0.9% (w/v) NaCl, and resuspended in 50 mM K-phosphate (pH 7) to a concentration of about 1 g wet weight per 20 ml buffer.

The cells were ruptured by ultrasonic oscillation using the ultrasonic desintegrator of Schoeller & Co, Frankfurt at maximal output for 30 s per ml. Unbroken cells were removed from the homogenate by centrifugation (4 °C; 10 min at 5000 rpm in the SS34 rotor of the Sorvall RC5 centrifuge). The membrane fraction was sedimented from the crude extract by 45 min of centrifugation (4 °C) at 15000 rpm in the rotor mentioned before. The sediment (= membrane fraction) was resuspended in 100 mM K-phosphate (pH 7) to a concentration of 1 mg protein per ml and was stored frozen at −18 °C without significant loss of nitrate reductase activity for several weeks.

Protein contents of membrane fragments were determined by the Lowry method [5] with bovine

serum albumine as standard. Nitrate and nitrite reductase activities were determined at 30 °C as described in [6] and [7], respectively. One unit of nitrate reductase is the activity catalyzing the formation of 1 μmol NO_2^- per min at 30 °C. Reaction mixtures for enzymatic nitrate assay (total volume: 1 ml) contained 80 μmol K-phosphate, pH 7, 0.1 μmol benzylviologen, 5 μmol $Na_2S_2O_4$, 10 μmol $NaHCO_3$, *E. coli* K12 membranes with 0.3 units of nitrate reductase (about 0.07 mg protein), and 200 μl of the sample to be assayed for nitrate containing up to 200 nmol NO_3^-. The enzymatic reduction of nitrate was performed at 30 °C. The reaction was started by the addition of a freshly prepared $Na_2S_2O_4$–$NaHCO_3$-mixture and stopped – if not otherwise stated – after 10 min by the addition of 2 ml ethanol (96%). For determination of initial nitrite concentrations, a second tube containing the same reaction mixture was treated with ethanol immediately after the addition of $Na_2S_2O_4$–$NaHCO_3$-mixture. If a precipitate was formed it was centrifuged off at 4000 rpm and nitrite was assayed colorimetrically at a wavelength of 546 nm [8] in a 200 μl-aliquot of the supernatant.

With the exception of benzylviologen and bovine serum albumin which were obtained from Serva, Heidelberg, all chemicals and culture medium constituents were purchased from E. Merck, Darmstadt.

4. Results and Discussion

By following the procedure outlined in the foregoing section, a membrane fraction of *E. coli* K12 was prepared which contained nitrate reductase (EC 1.7.99.4) at a high specific activity (4.5 units/mg protein). The use of such membrane fragments for nitrate analysis in our test system requires that the preparation is devoid of nitrite reductase activity. As a bacterium capable of nitrate ammonification, *E. coli* contains both nitrate and nitrite reductase [9]. In the *E. coli* strain used in this study (strain K12, DSM 498), soluble nitrite reductase activity was more or less completely separated from nitrate reductase when the membranes were sedimented by centrifugation.

Our nitrate assay with *E. coli* nitrate reductase makes use of the fact that metabolite concentrations can be determined by measuring the reaction rate as function of the metabolite concentration. This method requires that the enzyme catalyzed reaction is of first or pseudo-first order and that the Michaelis-Menten constant of the respective substrate is large in respect to the substrate concentrations to be assayed [10]. Although the nitrate reductase catalyzed reaction with its two reactants nitrate and electron donor is of second order, it can be treated as of pseudo-first order when the concentration of the reductant is considerably higher than that of nitrate. For first (or pseudo-first) order reactions, the decrease of substrate concentration [S] (here nitrate) during the course of the reaction can be described by equation (1):

$$[S] = [S]_0 \cdot e^{-kt}, \tag{1}$$

where $[S]_0$ is the initial substrate concentration, t the reaction time, and k the rate constant. If one considers a certain time interval $t_1 - t_2$ and the corresponding difference in substrate concentration [S], (1) can be rearranged to

$$[S]_0 = \frac{-\Delta[S]}{e^{-kt_1} - e^{-kt_2}}. \tag{2}$$

Since the concentration of the reaction product (here nitrite) emerging from the substrate considered (here nitrate) can be expressed as

$$[P] = [S]_0 - [S], \tag{3}$$

the term $\Delta[S]$ in Eq. (2) equals $-\Delta[P]$. Thus, Eq. (2) can also be written as

$$[S]_0 = \frac{\Delta[P]}{e^{-kt_1} - e^{-kt_2}}, \tag{4}$$

i.e. the change of product concentration $\Delta[P]$ (here nitrite) in a time interval Δt with experimentally fixed measurement times t_1 and t_2 is directly proportional to the initial substrate concentration $[S]_0$. Since product concentrations usually are assayed colorimetrically, the term $\Delta[P]$ is proportional to the corresponding ΔA-value (A = absorbancy).

Fig. 1 depicts the time course of nitrate reduction catalyzed by membrane-bound *E. coli* K12 nitrate reductase for initial nitrate concentrations ranging from 0.05 to 0.2 mM. In order to examine whether the data of Fig. 1 obey first order kinetics, the terms $-\ln([P]_\infty - [P]_t)$ (where $[P]_\infty$ denotes the product concentration at the end of the reaction and $[P]_t$ the product concentration at time t) were plotted against the reaction times t. For first order reactions, such

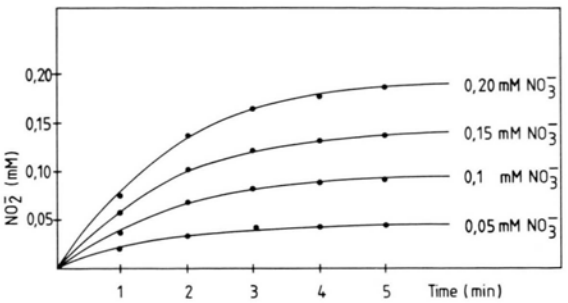

Fig. 1. Time course of nitrate reduction catalyzed by *E. coli* K12 nitrate reductase at 30 °C. The reaction mixtures (3 ml) contained $Na_2S_2O_4$ (5 mM), benzylviologen (0.1 mM), *E. coli* K12 membranes corresponding to 0.3 units of nitrate reductase per ml, and the KNO_3 concentrations as indicated. At various times, 200 µl samples were withdrawn from the reaction mixture, stopped by the addition of 400 µl ethanol (96%) and centrifuged to remove the precipitate. Nitrite was analyzed in 200 µl aliquots of the supernatants.

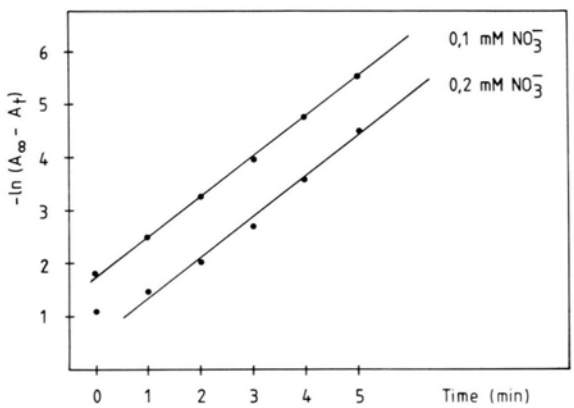

Fig. 2. Treatment of the reaction kinetics of Fig. 1 acoording to the equation $-\ln(A_\infty - A_t) = k\,t - \ln(A_\infty - A_0)$ valid for first or pseudo-first order reactions.

plots should yield straight lines [10]. Fig. 2 shows that this is the case for the nitrate reductase-catalyzed reaction. Since the Michaelis constant for nitrate of the *E. coli* K12 nitrate reductase is relatively high (0.3 mM in our test system), the enzyme fulfils the requirements necessary for nitrate assay by the kinetic method.

Since, in our test system, the reaction proceeds without lag-phase, the assessment of Δ[P]-values was simplified by measuring ΔA_{546}-values of samples after only one fixed time interval of 10 min. Fig. 3 shows that, by plotting such ΔA_{546}-values against the initial concentration of nitrate, a straight line was obtained for nitrate concentrations ranging from 10

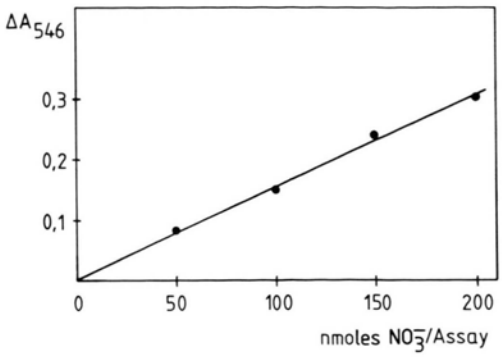

Fig. 3. Calibration curve for nitrate assay with the kinetic method employing *E. coli* K12 nitrate reductase. Assay mixtures (1 ml) with 0.3 units nitrate reductase, 5 mM $Na_2S_2O_4$, 0.1 mM benzylviologen and the indicated initial concentrations of KNO_3 (given as nmol per 200 µl of the water sample to be assayed) were incubated at 30 °C. After 10 min reactions were stopped by the addition of 2 ml ethanol (96%) and the mixtures were analyzed for nitrite. The ΔA_{546}-values represent absorbancy differences between reaction mixtures stopped after 10 min and zero time, respectively.

to 200 nmol per 200 µl-sample. The lower value corresponds to a detection limit of about 3 mg nitrate per liter. By increasing the volume of the nitrate containing sample from 200 to 600 µl in the enzymatic reaction system, the detection limit for nitrate can easily be lowered to 1 mg/liter (= 1 ppm). Thus, with nitrate contents of drinking water ranging from 1 to 100 ppm, the assay system described in this paper is potentially suitable for determination of nitrate in drinking water and other sources.

Nitrite ions present in the water samples to be assayed do not significantly interfere with the nitrate assay up to concentrations of about 5 ppm, corresponding to 0.1 mM NO_2^-. Note that none of the more frequent cations or anions which are normally present in drinking water at low concentrations (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}, HCO_3^-) tested at a concentration of 1 mM do interfere with our enzymatic test system. In view of the increasing use of autoanalyzers in routine diagnostics, it must also be remembered that the simplicity of our test system allows the development of an automated enzymatic nitrate assay with anyone of the commercially available autoanalyzer systems.

Acknowledgement

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