Direct Determination of Nitrate Using Nitrate Reductase in a Flow System

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Nitrate was determined using nitrate reductase (NR) in a flow system. A merging zone method was applied in the system in which a zone of NR and that of nitrate in separate streams were merged to react. The NADPH decreased by the enzymatic reaction was detected at 340 nm. The length of the reaction coil used for the enzymatic reaction was 250 cm. Of the concentrations from 0 to 0.6 mM NADPH in a carrier, 0.02 mM gave the maximum peak area due to decreased NADPH, suggesting that NR may be inhibited by a coenzyme, NADPH. The buffer of pH 7.5 was found to be optimum in the pH range from 6.5 to 8.0 of the buffer used as a carrier medium. Of the various buffer types (pH 7.5) used as the medium of carriers, piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) buffer afforded the maximum peak area. The elevated temperatures of the water bath for enzymatic reaction gave reduced peak areas and the maximum peak area was observed at 32°C. Under the optimum conditions, a linear calibration curve (r = 0.996) was obtained in the nitrate concentration range from 5 to 100 μM and detection limit (S/N = 3) was 1.8 μM. The relative standard deviation of the peak area at 20 μM nitrate was 4.2% (n = 7). The method was applied to the determination of nitrate in samples of natural water. Nitrate content obtained by the present method agreed well with that determined by the JIS method.

Key words —— nitrate, nitrate reductase, flow injection analysis, natural water

INTRODUCTION

The nitrate anion is an important analyte for the environmental problem and for human health and its detection and quantification is essential. Nitrate is usually determined as nitrite after the reduction of nitrate by copperized-cadmium. Nitrite is spectrophotometrically determined by converting it to azo dye *via* azotization with nitrite and an azocoupling reaction. With respect to a sample containing both nitrite and nitrate, the amount of nitrate is obtained by subtracting the amount of nitrite from the total amount of nitrite after reduction of nitrate. Thus, this is an indirect method. Flow injection analysis has been applied for the determination of nitrite and/or nitrate using sulphanilamide and *N*-(1-naphthyl)ethylenediamine,[1–7] or *p*-aminoacetophenone and *m*-phenylenediamine,[8,9] as reagents for nitrite detection.

Determination of nitrate using nitrate reductase (NR) has also been investigated.[10–13] The monitoring of the decrease of NAD(P)H in the reaction below would permit a direct determination of nitrate. Nitrate determination in the flow system using NR has not yet been reported.

$$NO_3^- + H^+ + NAD(P)H \xrightarrow{NR} NO_2^- + NAD(P)^+ + H_2O$$

Thus, immobilization of NR was first studied to utilize immobilized enzyme in a flow system. However, the immobilized enzyme could not be used for analysis due to its instability in air. It was thus decided that nitrate reductase was utilized in the form of a soluble enzyme, not as an immobilized enzyme. The flow system used contained a zone merging method, in which the zone of enzyme in one stream and that of nitrate in another stream were merged to react. The decreased NADPH by the enzymatic reaction was monitored at 340 nm. The nitrate determination by the present method was applied to the analysis of nitrate in natural water samples.

MATERIALS AND METHODS

Materials —— NR from *Aspergillus species* was purchased from Boehringer Mannheim, and NADPH from Oriental Yeast Co. Ltd. NR in solid state (20 units) was dissolved in 2 ml of water and the solution was fractionated in 0.1 ml portions, which were stored at – 20°C until used. At the time of usage, 0.9 ml of 1/9 M buffer was added to make an enzyme solution containing 1 unit of enzyme in 1 ml of 0.1 M buffer, and this solution was injected into a flow system for analysis.

Apparatus —— A schematic diagram of the apparatus used in the study is shown in Fig. 1. Two car-

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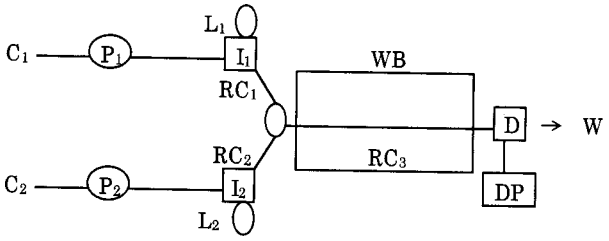

Fig. 1. Apparatus for Determination of Nitrate

C_1, C_2, carrier reservoir; P_1, P_2, pump; I_1, I_2, sample injector; L_1, L_2, sample loop; RC_1, RC_2, RC_3, reaction coil; D, spectrophotometer; DP, data processor; WB, water bath; W, waste.

riers were pumped with Shimadzu LC-10AD pumps (P_1 and P_2) at flow rates of 0.2 ml/min, respectively. NADPH in the merged carrier was monitored at 340 nm by a Hitachi L-4200H. The area of the peak observed was calculated with a Hitachi D-2500 data processor. The flow lines were made from polytetrafluoroethylene (PTFE) tubing (0.5 mm i.d.). The lengths of reaction coils, RC_1, RC_2, and RC_3 were 35, 40, and 250 cm, respectively, and RC_3 was immersed in a water bath (WB) thermoregulated at 32°C. The volumes of sample loops, L_1 and L_2 were 50 and 30 μl, respectively. The enzyme solution was injected to the flow system from I_1, and the nitrate sample from I_2. The carrier, C_1 was 0.1 M piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7.5) and C_2, 0.1 M PIPES buffer (pH 7.5) containing 0.02 mM NADPH.

Nitrate standards for a calibration curve were prepared by dissolving potassium nitrate in C_2 and diluting the solution with C_2. The natural water samples for analysis were prepared as solutions of 0.1 M PIPES buffer (pH 7.5) containing 0.02 mM NADPH by diluting the original water sample with appropriate PIPES buffers (pH 7.5) containing NADPH.

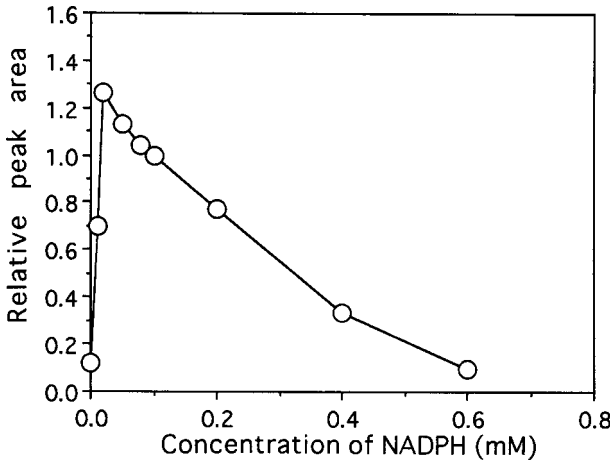

Fig. 2. Dependence of Peak Area on the NADPH Concentration in C_2

Peak area is shown as the relative area to that obtained when C_2 containing 0.1 mM NADPH was used. Carrier medium used was 0.1 M phosphate buffer (pH 7.5). The temperature of the water bath in which RC_3 was immersed was 30°C. Injected sample was a 100 μM nitrate solution.

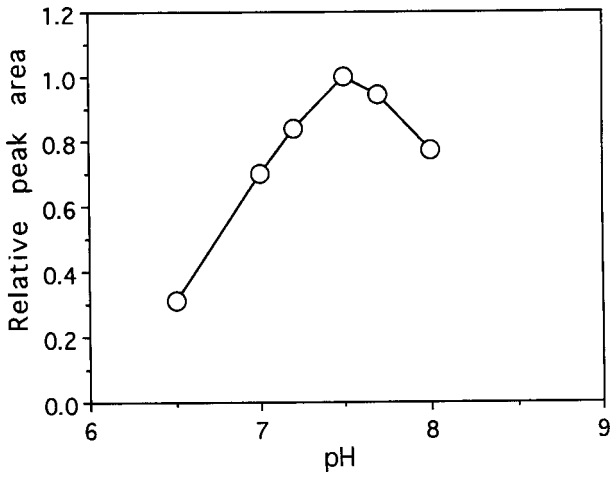

Fig. 3. Dependence of Peak Area on the pH of Carriers

Peak area is shown as the relative area to that obtained when the carrier of pH 7.5 was used. Carrier medium used was 0.1 M phosphate buffer and C_2 contained 0.02 mM NADPH. Other conditions were the same as described in the legend of Fig. 2.

RESULTS AND DISCUSSION

When a solution of NR and one of nitrate were injected simultaneously from I_1 and I_2 to the flow system, two zones of NR and nitrate were merged, the nitrate zone being completely included in the NR zone. The decrease of NADPH by the enzymatic reaction was observed as a negative peak at 2.1 min, which was inverted to a positive peak and its area obtained by a data processor. The dependence of peak area upon the length of RC_3 was studied in the length range from 100 cm to 400 cm. The reaction coil length of 250 cm was selected as a compromise between the peak area and the analysis time. In Fig. 2 is shown the dependence of peak area upon the concentration of NADPH in C_2. The peak area steeply increased to 0.02 mM in accordance with the increase of the concentration of NADPH, and decreased gradually at concentrations of more than 0.02 mM. Interestingly, it seemed that NR was inhibited by NADPH, a coenzyme of NR. Thus, 0.02 mM was used in the following experiments as the concentration of NADPH in C_2. The effect of flavin adenin dinucleotide (FAD), which was used as a supplementary electron carrier in the catalysis of NR in citation 13, was also investigated. However, FAD in

Table 1. Effect of Buffer Type (0.1 M, pH 7.5) Used as a Carrier Medium on Peak Area

Buffer	Relative peak area
Phosphate	1
Tris	0.63
PIPES	1.19
HEPES	0.67
Triethanolamine	0.45
Imidazole	0.13

Peak area is shown as the relative area to that when phosphate buffer was used as a medium of carriers. Carrier, C_2, contained 0.02 mM NADPH. Other conditions were the same as described in the legend of Fig. 2.

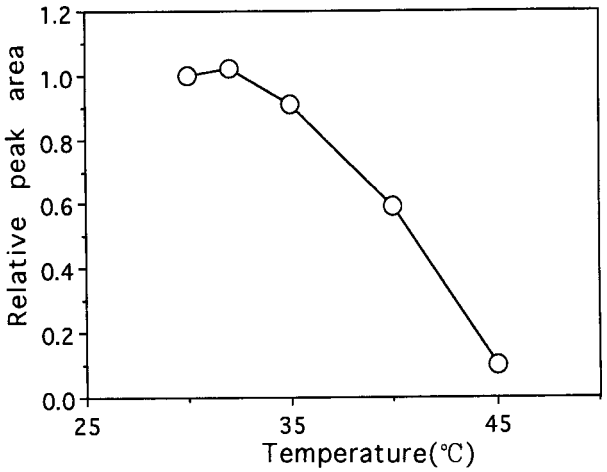

Fig. 4. Dependence of Peak Area on Temperature of the Water Bath

Peak area is shown as the relative area to that obtained when temperature of the water bath was 30°C. Carrier medium used was 0.1 M phosphate buffer (pH 7.5) and C_2 contained 0.02 mM NADPH. Injected sample was a 100 μM nitrate solution.

the concentration range from 0.5 to 200 μM in C_2 scarcely affected the peak area. Thus, FAD was not contained in a carrier. Figure 3 shows the effect of pH of the phosphate buffer used as a medium of carriers in the range from 6.5 to 8.0, indicating clearly that the optimum pH was 7.5. Table 1 shows the dependence of peak area upon the buffer type (0.1 M, pH 7.5) used as the medium of carriers. Of the buffer types investigated, PIPES buffer afforded the greatest peak area. In expectation of the enhancement of the enzyme activity, enzymatic reaction was examined at elevated temperatures. The dependence of the peak area on the temperature of the water bath in which RC_3 was immersed is shown in Fig. 4. At the elevated temperatures reduced peak areas were observed, suggesting that NR was unstable to heat. Maximum peak area was observed at 32°C.

Under the optimum condition, a linear calibration curve ($r = 0.996$) was obtained in the nitrate concentration range from 5 to 100 μM and the detection limit ($S/N = 3$) was 1.8 μM. The relative standard deviation of the peak area at 20 μM nitrate was 4.2% ($n = 7$). The detection limit in the present method (1.8 μM) is a little lower than that in the batchwise method[13] using NR (5 μM). In the latter method more than 45 min was required for analysis, in contrast to the present method in which the analysis of nitrate could be accomplished in several minutes. The sensitivity to nitrate in the present method would be comparable with that in ion chromatography detection at 210 nm, judging from the molar absorptivity of NADPH (6300 at 340 nm) and that of potassium nitrate (about 7800 at 210 nm).

Table 2. Nitrate Content in Natural Water Samples (μM)

	Present method	JIS method
River water 1	182	175
River water 2	213	210
Rainwater	50	50
Well water 1	359	373
Well water 2	255	248
Lake water	85	89

River water and well water samples for analysis were prepared by 10 fold dilution of the original water, and rainwater and lake water by 2 fold dilution.

The present method was applied to the analysis of nitrate in various natural water samples, and Table 2 shows the results. The relative standard deviations of the peak areas of 10 fold diluted samples of river water 1 and well water 1 were 4.3 and 3.1% ($n = 7$), respectively. The nitrate content obtained by this method agreed well with that by another method (JIS method),[14] in which nitrate was reduced to nitrite by copperized-cadmium, and nitrite was detected at 540 nm after the reaction with sulphanilamide and *N*-(1-naphthyl)ethylenediamine.

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