Determination of Phosphate Using Immobilized Enzyme Reactor—Chemiluminescence Detector by FIA

Akiko Noguchi*, Toyoaki Aoki* and Toshihisa Oshima**

** College of Engineering, University of Osaka Prefecture, 1-1 Gakuen-cho, Sakai 593, Japan*

*** Department of Chemistry, Kyoto University of Education, Fukakusa, Fushimi-ku, Kyoto 612, Japan*

Abstract

Phosphate in water was determined by FIA with an immobilized enzyme reactor which contained purine nucleoside phosphorylase and xanthine oxidase. In this reactor, purine nucleoside phosphorylase catalyses the phosphorolysis of inosine to hypoxanthine, which is oxidized by xanthine oxidase to generate hydrogen peroxide. Hydrogen peroxide was detected by chemiluminescence in reaction with bis[2-(3,6,9-trioxadecanyloxycarbonyl)4-nitrophenyl]oxalate. The concentration of phosphate was determined down to the level of 0.1 μM and the time required for the analysis of one sample was 3 min.

1. Introduction

Phosphate is one of the essential nutrients and a limiting factor for phytoplankton-bloom in natural waters. The determination of phosphate in water is necessary for the prevention of high nutrient level in aquatic environment. The most general method for the determination of phosphate in water is the molybdenum blue method[1] which , however, requires pretreatment such as concentration because its sensitivity is not high enough to determine low level of phosphate in natural waters .

Phosphate is a substrate for many enzyme reactions. Some of these reactions have been utilized in chemical analysis for the anion. Purine nucleoside phosphorylase (PNP) catalyses the phosphorolysis of inosine to ribose-1-phosphate and hypoxanthine (eq. 1), which is oxidized by xanthine oxidase (XOD) to generate hydrogen peroxide (eq. 2).

$$\text{phosphate} + \text{inosine} \xrightarrow{\text{PNP}} \text{hypoxanthine} + \text{ribose-1-phosphate} \qquad (1)$$

$$\text{hypoxanthine} + 2H_2O + 2O_2 \xrightarrow{\text{XOD}} \text{uric acid} + 2H_2O_2 \qquad (2)$$

This reaction has been used for the determination of phosphate. Yao *et al.* [2] reported an amperometric flow injection system with an immobilized enzyme reactor. However, other electrochemical-active species interfered with amperometric detection. Kawasaki *et al.* [3] reported a flow injection method with the same immobilized enzymes and chemiluminescence (CL) detection by

the luminol—microperoxidase system which, however, is likely to receive interference from heavy metals.

The CL reaction of hydrogen peroxide with peroxyoxalate[4] has been known to give high selectivity and CL efficiency. This paper describes a novel FIA for the determination of phosphate with an immoblized enzyme reactor and peroxyoxalate CL detection.

2. Experimental

2.1. Reagents

PNP and XOD were purchased from Funakoshi (Japan) and Sigma (St. Louis, USA), respectively. Bis[2-(3,6,9-trioxadecanyloxycarbonyl)4-nitrophenyl]oxalate (TDPO) was purchased from Wako Pure Chem. (Japan). All other chemicals were purchased from Wako Pure Chem. and of analytical grade. A stock solution of hydrogen peroxide was prepared from 31 % hydrogen peroxide and was standardized by a titration with permanganate. A stock solution of phosphate was prepared from sodium dihydrogenphosphate dihydrate. These stock solutions were stored in a refrigerator. Working solutions were prepared daily by appropriate dilution of the stock solutions. Doubly distilled water was used for the preparation of all solutions.

2.2. Immobilization of enzymes

Immobilization was carried out according to the method described by Yao *et al.* [2] and Hayashi *et al.* [5] PNP and XOD were immobilized by the glutaraldehyde method on aminopropyl-controlled pore glass beads (aminopropyl-CPG, 500 A, 200/400 mesh) which were previously packed in a column (5 mm x 4 mm i.d.). When not in use, the immobilized enzyme reactor was stored in 3.2 M ammonium sulfate in a refrigerator.

2.3. Flow injection apparatus and procedure

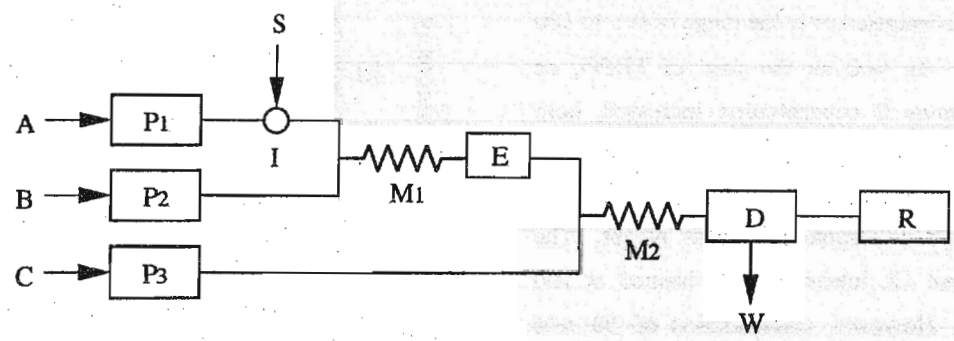

Figure 1. Schematic diagram of a flow system for the determination of phosphate. A; distilled water, B; inosine—EDTA solution, C; TDPO—rhodamine B / acetone solution, E; immobilized enzyme reactor, P_1, P_2, P_3; double-plunger pumps, S; sample, I; sample injector, M_1, M_2; mixing coils, D; CL detector, R; recorder, W; waste.

A schematic diagram for the present FIA method is shown in Figure 1. Three double-plunger pumps were used to propel solutions through teflon tubing (0.5 mm i.d.). A sample (250 μl) was injected into a distilled water stream (A) by a teflon rotary valve (Rheodyne 5020, California, USA), merged with an inosine—EDTA stream (B) and passed through an immobilized enzyme reactor (E). Phosphate in the sample was converted to hydrogen peroxide in this reactor and then the stream cotained hydrogen peroxide was merged with a TDPO—rhodamine B stream (C). CL emission was detected with a CL detector (D: S-3400, Soma, Japan) eqipped with a spiral flow cell (100 μl) and a photomultiplier tube (R268, Hamamatsu photonics, Japan). The signal from the CL detector was recorded on a chart recorder (R: Chromatopac C-R18, Shimadzu, Japan).

3. Results and discussion

3.1. Optimization of the CL system

Concentrations, pH and flow rates of reagent solutions were optimized for the CL system by injecting standard solution of hydrogen peroxide (1 μM H_2O_2) from a rotary valve. In this case, inosine was not added to the EDTA stream and an immobilized enzyme reactor was removed from the system shown in Figure 1.

The effect of TDPO concentration on CL intensity was investigated over the range of 1 to 500 μM. As TDPO concentration increased, both CL for background and for hydrogen peroxide increased. CL intensity shown in Figure 2 corresponded to peak height obtained from baseline. The maximal CL intensity was obtained at 500 μM. However, it was levelling off around 500 μM. From the standpoint of flow-analysis, TDPO was expensive. Therefore, 100 μM TDPO was used in the subsequent experiments. The effect of rhodamine B concentration on CL intensity was investigated over the range of 0.1 to 100 μM. As well as the case of TDPO, as rhodamine B concentration increased, both CL for background and for hydrogen peroxide increased. CL intensity shown in Figure 3 corresponded to peak height. The maximal CL intensity was obtained at 100 μM. However, concentration of 50 μM was chosen as the optimum concentration since rhodamine B did not dissolve easily in acetone. Triton X-100 (0.01%) was added to TDPO—rhodamine B solution to prevent rhodamine B from depositing on teflon tubes

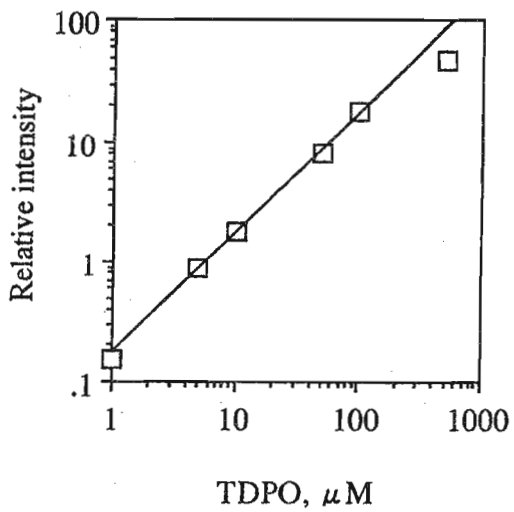

Figure 2. Effect of TDPO concentration on CL intensity for 1 μM hydrogen peroxide.

in the system. Addition of Triton X-100 did not influence CL intensity.

Effect of pH on CL intensity was studied over the range of pH 6—9. The maximal CL intensity was obtained at pH 8.5. Therefore, EDTA solution was ajusted to pH 8.5 in subsequent experiments.

Optimum flow rates of distilled water, 1mM EDTA (pH 8.5) solution and TDPO—rhodamine B solution were determined as 0.5, 0.5 and 1.0 ml/min, respectively. The ratio of these three flow rates was kept at 1 : 1 : 2 to achieve good mixing at T-pieces.

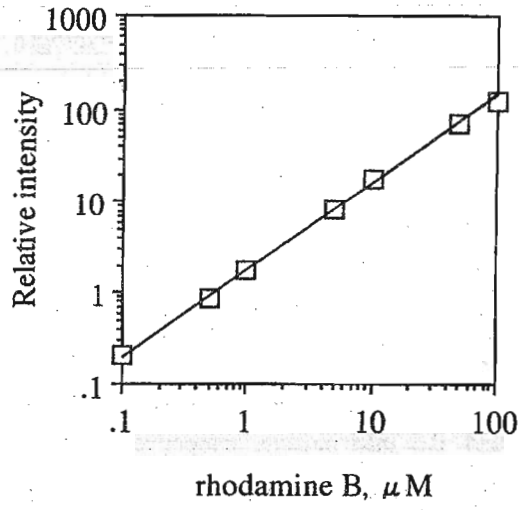

Figure 3. Effect of rhodamine B concentration on CL intensity for 1 μM hydrogen peroxide.

Under the conditions indicated above, calibration curve for hydrogen peroxide was linear over the range of 0.1—2.0 μM. The concentration of hydrogen peroxide was determined down to the level of 0.1 μM. The relative standard deviation were 1.69 % and 2.19 % (n=6) for 1 μM and 0.5 μM hydrogen peroxide, respectively.

3.2. Optimization of the present system

Concentrations, pH and flow rates of reagent solutions were optimized for the present system by injecting phosphate solution of 1 μM and 0.5 μM from a rotary valve.

Effect of pH on CL intensity was studied over the range of pH 6—9. The maximal CL intensity was obtained at pH 7.5 as shown in Figure 4. Therefore, inosine—EDTA solution was ajusted to pH 7.5 in subsequent experiments.

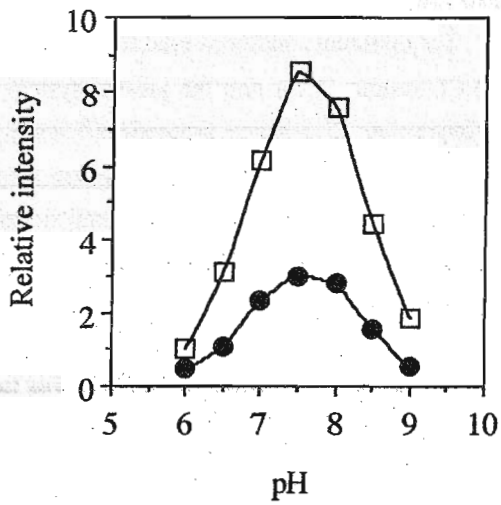

Figure 4. Effect of pH of inosine—EDTA solution on CL intensity. □; 1 μM phosphate, ●; 0.5 μM phosphate.

The effect of EDTA and inosine concentrations on CL intensity was investigated. As the result shown in Figure 5, CL intensity increased as inosine

concentration increased in the range of 0—0.5 mM. In this range, generation of hydrogen peroxide seems to increase with the increase in inosine concentration according to eq. 1 and eq. 2. However, CL intensity decreased in the range of 0.5—5 mM. Probably inosine might prevent the second enzyme reaction (eq. 2) or CL reaction in high level of inosine concentration. Consequently the maximal CL intensity was obtained at 2 mM EDTA and 0.5 mM inosine, respectively. These concentrations were used in the following experiments.

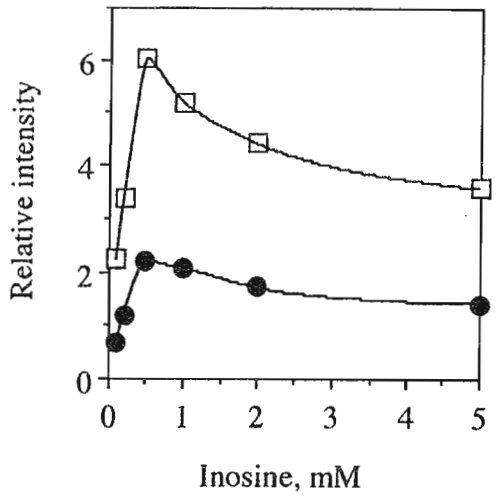

Figure 5. Effect of inosine concentration on CL intensity. □; 1 μM phosphate, ●; 0.5 μM phosphate.

The total flow rate was varied over the range of 1.2—2.8 ml/min. The total flow was composed of distilled water, inosine—EDTA solution and TDPO—rhodamine B solution, and the ratio of these three flow rates was kept at 1 : 1 : 2 described above. The maximal CL intensity was obtained at the lowest rate, 1.2 ml/min. The lower the flow rate becomes, the longer the sample retains in the enzyme reactor. As the result, more hydrogen peroxide was generated. When the total flow rate was adjusted to less than 1.2 ml/min, the background was not stable. Therefore, 1.2 ml/min was selected as the optimum total flow rate.

The optimum conditions established above are shown in Table 1. The optimum values of pH for the CL system (3.1.) and the present system were 8.5 and 7.5, respectively. This indicates that peroxyoxalate CL reaction proceeds efficiently at pH 8.5 and enzyme reaction dose at pH 7.5. The optimum flow rates for the present system were lower compared with those for the CL system. As the flow rates become slower, the retention time of substrates in enzyme reactor becomes longer and generation of hydrogen peroxide proceeds efficiently. Consequently, enzyme reaction, rather than CL reaction, seems to control the total system.

Table 1. Optimum conditions for the system

TDPO concentration	100 μM
rhodamine B concentration	50 μM
EDTA concentration	2.0 mM
inosine concentration	0.5 mM
pH of inosine—EDTA solution	7.5
flow rate of TDPO—rhodamine B solution	0.6 ml / min
flow rate of inosine—EDTA solution	0.3 ml / min
flow rate of distilled water (sample carrier)	0.3 ml / min

3.3. Caliblation curve and sensitivity

Under the optimum conditions, the calibration curve for the determination of phosphate was represented in Figure 6. The calibration curve was linear over the range of 0.1—2.0 μM. In defining the detection limit as 3 x σ, which is an abbreviation of standard deviation, the detection limit for this system was 3.9 x 10^{-8} M. The relative standard deviations were 1.41 % and 2.62 % (n=6) for 1 μM and 0.5 μM phosphate, respectively. The time required for the analysis of one sample was 3 min.

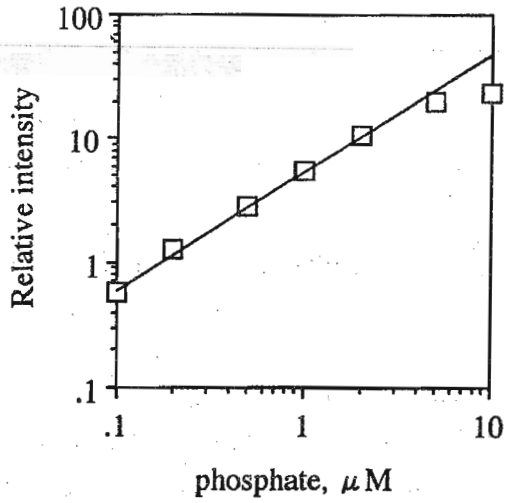

Figure 6. Calibration curve for the determination of phosphate.

4. Conclusions

The proposed FIA system with immobilized enzyme reactor and CL detection offers high sensitivity and precision for the determination of phosphate. To apply this method to natural waters, interference studies should be considered; these are now under investigation.

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固定化酵素と化学発光検出法を用いたFIAによる水中の微量リン酸の定量

野口晶子[*] ・ 青木豊明[*] ・ 大島敏久[**]

[*]大阪府立大学工学部：〒593 堺市学園町1－1

[**]京都教育大学理学科：〒612 京都市伏見区深草藤森町1

固定化酵素と化学発光検出法を用いたFIAによる水中のリン酸の定量法の開発を行った．担体としてアミノプロピルーCPGをカラムに充填し，グルタルアルデヒドによる架橋法で，プリンヌクレオシドホスホリラーゼ(PNP)とキサンチンオキシダーゼ(XOD)の2つの酵素を固定化したリアクターを用いた．本定量法はFIA方式であり，流れに注入されたサンプル中のリン酸は，リアクター内の酵素反応で過酸化水素に変換される．続いて，生成した過酸化水素は，過シュウ酸エステルと反応し，発光する．この発光を検出することで，水中の極微量のリン酸を定量した．本法では，シュウ酸エステルとしてTDPO，蛍光物質としてローダミンBを用いた．本法で，リン酸濃度0.1 μMまで定量が可能であり，1 μM，0.5 μMにおける繰り返し測定（n＝6）の相対標準偏差は，それぞれ1.41%，2.62%であった．定量に要する時間は，1サンプルにつき約3分であった．

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