Flow Injection Determination of Orthophosphate Over the Wide Concentration Range Using a Dual Enzyme Electrode

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Abstract

A dual enzyme electrode with and without amplification for orthophosphate is proposed for the determination of orthophospate over the wide concentration range. The calibration graph obtained at the sensing part without amplification was linear over 5×10^{-6} – 2×10^{-3} M for orthophosphate, while at the sensing part with amplification it was linear over 2×10^{-7} – 5×10^{-5} M. As a result, orthophosphate can be determined over the wide concentration range of 2×10^{-7} – 2×10^{-3} M from the FIA current signals obtained at two sensing parts. The detection limit was 8×10^{-8} M (S/N=3) for a 20-μl injection.

Keywords Dual enzyme electrode, orthophosphate, amplification, substrate recycling, FIA

1. Introduction

The determination of orthophosphate is especially important in the field of environmental [1] and food analysis [2], because increased orthophosphate concentration leads to eutrophication of lakes and rivers and an excess diet of orthophosphate containing in foodstuffs affects human health. In addition, the determination of orthophosphate over the wide concentration range is desirable in such analytical fields, because it can be carried out without dilution or concentration of sample solution. Many research groups have proposed the enzyme sensors for the specific detection of orthophosphate. Engblom [3] has recently reviewed the characteristics of the biosensors developed for the determination of orthophosphate. Most of these enzyme sensors are based either on the inhibitory effect of orthophosphate on alkaline phosphatase reaction [4,5], or the use of purine nucleoside phosphatase [6,7], maltose phosphorylase [8,9] and pyruvate oxidase [10,11] which require orthophosphate as a cosubstrate.

In this paper, we used the combination of maltose phosphorylase (MP), mutarotase (Mut), and glucose oxidase (GOD) to fabricate an orthophosphate biosensor. By three successive enzymatic reactions, orthophosphate can be converted to electroactive hydrogen peroxide as an end product, which is detected amperometrically. In addition, the additional combination of acid phosphatase (AcP) to three enzymes permits the recycling of orthophosphate in the presence of excess of maltose [12]. As a result, this gives an amplified signal for orthophosphate. We describe here the utility of a novel dual enzyme-electrode consisted of two sensing parts with and without amplification for orthophosphate, because it can detect selectively orthophosphate over the wide concentration range from the current signals obtained at two sensing parts. The analytical performance of the dual enzyme electrode is assessed under flow injection conditions, because handling is minimized, reproducibility is enhanced, and an additional detector is not necessary.

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2. Experimental

2.1. Reagents

The enzymes used, AcP (EC 3.1.3.2, 5.1 U mg^{-1} of solid from potato), GOD (EC 1.1.3.4, 182 U mg^{-1} of solid from *Aspergillus niger*), and Mut (EC 5.1.3.3, 3,300 U mg^{-1} of solid from porcine kidney) were obtained from Sigma and MP (EC 2.4.1.8, 570 U ml^{-1} of solution from bacteria) was from Oriental Yeast. Bovine serum albumin (BSA, 96-99 % albumin), glutaraldehyde (20 % solution), ascorbic acid, uric acid, L-cysteine, maltose, and 1,2-diaminobenzene were obtained from Wako. All other chemicals were of analytical reagent grade. They were used as received. Acetate buffers were prepared from sodium acetate. Phosphate standard solution (10 mM) was prepared from sodium dihydrogen phosphate. Distilled water purified with the use of a Millipore Milli Q system was used throughout.

2.2. Construction of the dual enzyme electrode

A BAS cross-flow electrochemical flow-cell was used for the surface modification of the electrode. The electrode assembly consisted of a dual electrode with two platinum disks (3 mm in diameter) as a working electrode, a silver-silver chloride reference electrode, and a stainless steel as an auxiliary electrode. Prior to the enzyme coating, the surface of the platinum disks was polished with 6 μm diamond particles (BAS) and then 0.05 μm alumina particles (BAS), then rinsed with distilled water, and allowed to air-dry. Both the platinum disks were then modified by cross-linking enzymes and BSA using glutaraldehyde. The method was similar to that described previously [13] and was as follows. The MP (5.7 U), Mut (660 U), GOD (73 U), and 20 μl of 10 % (w/v) aqueous BSA were added to 30 μl of 0.1 M sodium phosphate buffer (pH 7.0). Similarly, the AcP (7.7 U), Mut (660 U), GOD (73 U), and 20 μl of 10 % (w/v) BSA were added to 30 μl of the same phosphate buffer. A 8 μl portion of a 4 % (v/v) solution of glutaraldehyde was added to each solution and the combinations mixed well. A 4 μl aliquot of the resulting solutions was carefully spread out on each of two platinum

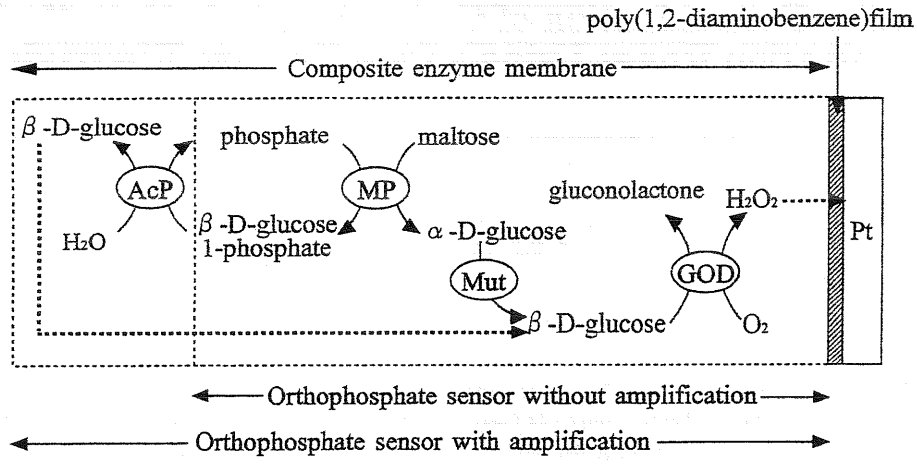

Fig. 1 Working principle of dual enzyme electrode with two sensing parts for orthophosphate.
AcP: acid phosphatase; MP: maltose phosphorylase; Mut: mutarotase; GOD: glucose oxidase.

disks. The membranes were allowed to form for half a day at room temperature, open to the air. In this way, each of two platinum disks of the dual electrode was modified with the MP-Mut-GOD or AcP-MP-Mut-GOD composite membrane cross-linked with BSA by glutaraldehyde. Then the dual electrode was assembled into the BAS electrochemical flow-cell and washed with 0.1 M glycine buffer (pH 7.5) to remove the excess of enzymes and the residual aldehyde groups onto the enzyme membranes.

Furthermore, the electropolymerization to coat with a thin film of poly(1,2-diaminobenzene) was subsequently carried out by holding the dual enzyme electrode at 1.0 V vs. Ag/AgCl with a Fuso HECS 966 multichannel potentiostat for 45 min, in a flowing stream of a solution of 1,2-diaminobenzene (10 mM) in 0.2 M, pH 5.5, sodium acetate buffer, by a procedure similar to that described by Sasso *et al.* [14]. After the electropolymerization, the dual enzyme electrode was washed exhaustively with the same acetate buffer. The completed electrode was stored in acetate buffer (0.1 M, pH 5.5) at 4-5℃ when not in use.

2.3. Apparatus and procedures

The apparatus for the FIA measurements was similar to that described previously [15]. The FIA system consisted of an Eicom double-plunger micropump, an injector (Rheodyne 7125) with a sample loop of 100 μl, a BAS cross-flow electrochemical flow-cell with a dual enzyme electrode, a Fuso HECS 966 multichannel potentiostat, and a multipen recorder (Nippon Denshi Kagaku U-638). The dual electrode was arranged in parallel for the carrier flow direction and connected to the outlet of the injector with an 100 cm length of PTFE coil (0.5 mm i.d.). The 0.1 M sodium acetate buffer (pH 5.5) was pumped as a working carrier solution, at a constant flow rate. Sample solutions (20 μl) were injected with a microsyringe.

3. Results and Discussion

3.1. Working principle of the dual enzyme electrode

The reactions that occur in two sensing parts of the dual enzyme electrode are shown schematically in Fig. 1.

In the sensing part with a MP-Mut-GOD composite membrane, the injection of orthophosphate into the flow line results in the production of hydrogen peroxide as an end product by three successive enzymatic reactions during diffusion through the composite enzyme membrane, in the presence of sufficient maltose in the carrier buffer. The hydrogen peroxide produced can be amperometrically detected at 0.6 V *vs.* Ag/AgCl.

On the other hand, in the sensing part with an AcP-MP-Mut-GOD composite membrane, coupled enzymes (AcP and MP) permit the recycling of orthophosphate in the presence of excess of maltose. The β-D-glucose-1-phosphate produced in the MP-catalysed reaction is converted back to orthophosphate by the AcP. The orthophosphate produced can be reconverted to β-D-glucose-1-phosphate by the MP, and so on. Thus, for every orthophosphate molecule a number of maltose molecules are consumed to produce a large amount of α-D-glucose and β-D-glucose. These glucoses produced can be converted to electroactive hydrogen peroxide according to the following enzyme reactions. Therefore, this sensing part responses sensitively to orthophosphate, because a large amount of hydrogen peroxide is produced from orthophosphate as a result of substrate recycling.

3.2. Arrangement of dual enzyme electrode

Two sensing parts of the dual enzyme electrode can be arranged in series or in parallel for the carrier flow direction. However, when two sensing parts were arranged in series as upstream and downstream electrodes, respectively, some fractions of the hydrogen peroxide generated enzymatically at the upstream electrode were detected at the downstream electrode, as reported before [15]. Therefore, the in parallel arrangement was selected in this work.

3.3. Hybridization of poly (1,2-diaminobenzene) film to improve the sensor selectivity

At an applied potential of 0.6 V *versus* Ag/AgCl, two sensing parts coated with enzyme membrane alone responded to orthophosphate, but gave fairly large responses to the electroactive interferentes such as ascorbate, urate, and cysteine, as shown in Table1. In contrast, the hybridization of poly(1,2-diaminobenzene) film to enzyme membranes was effective to block the access of such electroactive compounds to the platinum electrode surface, by the size-exclusion function

Table 1 Effect of poly(1,2-diaminobenzene) film hybridized to two sensing parts of dual enzyme electrode

Substrate (0.1 mM each)	Sensing part without amplification[a]		Sensing part with amplification[b]	
	without film[c]	with film[c]	without film[c]	with film[c]
Ascorbic acid	232	3	216	2
Uric acid	208	3	192	3
Cysteine	186	2	175	2
Orthophosphate	189	108	3750	2150

a. Sensing part with a MP-Mut-GOD membrane.
b.Sensing part with an AcP-MP-Mut-GOD membrane.
c.Response (peak current, nA) obtained by dual enzyme electrode without and with poly(1,2-diaminobenzene) film.

of poly(1,2-diaminobenzene) film as described before [16]. As shown in Table 1, two sensing parts with poly(1,2-diaminobenzene) film gave FIA signals to orthophosphate without interferents due to electroactive species such as L-ascorbate, cysteine, and urate. In addition, the sensitivity of the sensing part with a AcP-MP-Mut-GOD composite membrane to orthophosphate was about 20 times that of the sensing part with a MP-Mut-GOD composite membrane. This means that the substrate recycling occurs effectively for orthophosphate during diffusion through the composite membrane.

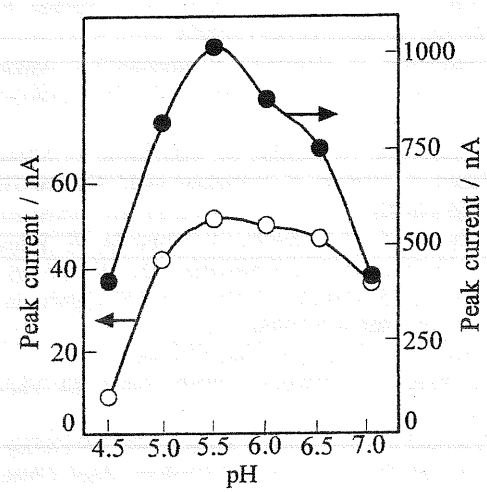

Fig. 2 Effect of carrier pH on the sensor responses. (○) No amplified current and (●) amplified current obtained at the sensing parts with a MP-Mut-GOD and an AcP-MP-Mut-GOD composite membranes of dual enzyme electrode, respectively. A 20-μl aliquot of 5×10^{-5} M orthophosphate solution was injected in the flow line with the dual enzyme electrode. Carrier solution: 0.1 M sodium acetate buffer containing 5 mM maltose; and carrier flow rate: 0.2 ml min^{-1}.

3.4. Analytical characteristics of dual enzyme electrode

Experiments were carried out to establish the optimum conditions for the orthophosphate detection using the dual enzyme electrode with poly(1,2-diaminobenzene) film, especially for the pH and the maltose concentration of the carrier solution. Sodium acetate buffers (0.1 M) at various pH values, containing 5 mM maltose, were tested as the carrier solution. Maximum response to orthophosphate was observed at pH 5.5 at both sensing parts, as shown in Fig. 2. In particular, the amplified current obtained at the sensing part with amplification decreased sharply at pH values above and below 5.5.

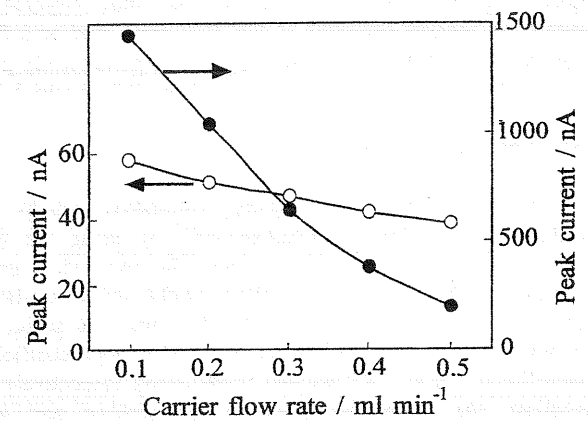

Fig. 3 Effect of carrier flow rate on the sensor responses for 5×10^{-5}M orthophosphate. The symbols are as in Fig. 2. Carrier solution: 0.1 M, pH 5.5, sodium acetate buffer containing 5 mM maltose.

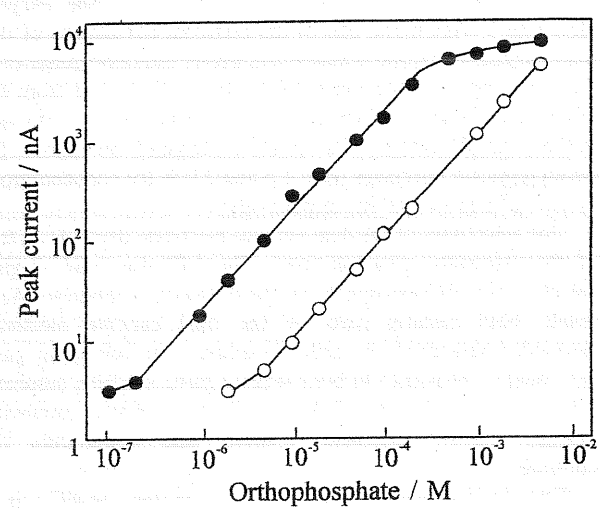

Fig. 4 Calibration graphs for orthophosphate. (○) No amplified current at the sensing part with a MP-Mut-GOD composite membrane and (●) amplified current at the sensing part with a AcP-MP-Mut-GOD composite membrane of dual enzyme electrode. A 20-μl aliquot of orthophosphate standard solutions was injected into the carrier stream (pH 5.5, 0.1 M sodium acetate buffer) pumped at 0.2 ml min^{-1}.

Without maltose in the carrier solution, no response to orthophosphate was observed at both sensing parts. As the maltose concentration in the carrier was raised from 0.1 to 5 mM, the sensitivity to orthophosphate increased as expected; the increase was slight at higher concentrations, probably because of oxygen limitation in the carrier solution. In particular, the increase was drastic at the sensing part with amplification. From these results, 0.1 M sodium acetate buffer at pH 5.5 containing 5 mM maltose was selected as the carrier solution.

The carrier flow rate is related to the residence time of the sample zone onto the dual enzyme electrode. Therefore, the signal intensity increased with decreasing flow rate, as shown in Fig. 3. In particular, the sensitivity of the sensing part with amplification to orthophosphate increased markedly with decreasing the flow rate. However, the time required to determine became excessive at low flow rates, and so a flow rate of 0.2 ml min^{-1} was selected. This provided relatively good sensitivity and reasonable sample throughput (60 samples h^{-1}).

3.4. Sensitivity and selectivity

Figure 4 shows two log-log calibration graphs for orthophosphate obtained simultaneously by using the dual enzyme electrode in a FIA system. The calibration graph obtained at the sensing part without amplification was linear over 5×10^{-6} – 2×10^{-3} M for orthophosphate; the slope, the current response intercept and the linear correlation coefficient were1.02 μA mM^{-1}, 3.9 nA and 0.998 (n=8), respectively. In contrast, the calibration graph at the sensing part with amplification was linear over the concentration range of 2×10^{-7} – 5×10^{-5} for orthophosphate; the slope, the intercept and the linear correlation coefficient were 21.9 μA mM^{-1}, 4.8 nA and 0.998 (n=7), respectively. The detection limit was 8×10^{-8} M based on a signal-to-noise ratio of 3. In these linear ranges, the sensing part with amplification was amplified by a factor of about 20. This result indicates that substrate recycling can be a useful method for increasing the sensitivity of the enzyme electrode. Furthermore, one of the principal advantages of this dual enzyme electrode is that it can detect orthophosphate over the wide concentration range of 2×10^{-7} - 2×10^{-3} M from the FIA current signals obtained at two sensing parts with and without amplification. The relative standard deviations for seven replicate injections were 1.5 and 2.9 % for concentrations of 1×10^{-4} and 1×10^{-6}M, respectively.

The selectivity of the dual enzyme electrode depends on that of the enzymatic reaction used. The MP was itself highly selective for orthophosphate in the presence of maltose. As a result, both sensing parts of the dual enzyme electrode responded selectively to orthophosphate. The following gave zero relative responses at both sensing parts; sulphite, sulphate, nitrite, nitrate, chlorate, iodate, carbonate, lactate, pyruvate, tartrate, citrate, succinate, oxalate, and ammonium (as chloride).

When the dual enzyme electrode was stored at 4℃ in optimum acetate buffer, it retained most of its activity for five weeks. However, the operational stability was inferior to that of the storage stability; after the repetitive use of three weeks (2 h per day) the sensitivity to orthophosphate of both sensing parts decreased to 40 –55% of their original values, because of the inactivation of enzymes during the period of the use.

In conclusion, the proposed dual enzyme electrode was found to be useful as a specific detector of orthophosphate over the wide concentration range of 2×10^{-7} – 2×10^{-3} M.

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(Received September 3, 2002)
(Accepted September 27, 2002)