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Development of Amperometric Sensor System for Measurement of Diarrheic Shellfish Poisoning (DSP) Toxin, Okadaic Acid (OA)

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An amperometric sensor system for measuring okadaic acid (OA), a diarrheic shellfish poisoning (DSP) toxin, has been developed, which is a combination of enzyme inhibition assay and flow injection analysis (FIA) utilizing immobilized pyruvate oxidase (PyOx). The sensor system consists of an oxygen electrode with immobilized PyOx, a recorder, an air pump, a HPLC pump and a buffer tank. The sensor is based on the principle that protein phosphatase 2A (PP2A) is inhibited by OA. OA concentration was determined from the amount of oxygen that corresponded to the amount of phosphate ion released from a phosphoprotein in the presence of PP2A, and that was consumed by PyOx. The optimum conditions for the proposed sensor system were as follows: 20 mM PIPES (pH 7.0) containing 1 mM TPP, 5 mM $MgCl_2$ and 1 mM pyruvic acid at final concentrations, a flow rate of 0.15 ml min^{-1} and an injection volume of 50 μl. The results obtained by the sensor system and the enzyme-linked immunosorbent assay (ELISA) were compared and agreement was observed at OA concentrations ranging from 0.1 to 4 ppb. The detection limit of the proposed sensor was 0.1 ppb, which was approximately 50 times more sensitive than that of ELISA.

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1. Introduction

Diarrheic shellfish poisoning (DSP) is a gastrointestinal disease caused by the ingestion of shellfish contaminated by toxic dinoflagellates of the genus *Dinophysis*. The main symptoms of DSP are diarrhea, vomiting and abdominal pain. The symptoms occur shortly after shellfish ingestion; approximately 90% or more of patients develop the symptons within 4 h after ingestion of contaminated shellfish.[1] The toxins involved in DSP are divided into three groups on the basis of their chemical structures: the okadaic acid group (OA group), the pectenotoxin group (PTX group) and the yessotoxin group (YTX group).[2] Toxins belonging to the OA group [OA and the dinophysis toxins (DTXs)] are the most intensively studied because of their diarrheogenic effects.[3] The widespread distribution of DSP has posed a serious problem to both public health and the shellfish industry, particularly in Japan and Europe.[4,5] Therefore, regulations and monitoring systems for DSP have been established in many countries that promote the shellfish industry.

The most widely used screening method for DSP is the mouse bioassay[6] in which the acceptance level is set at 4–5 mouse units (MUs)/100 g mussel meat. One MU is defined as the minimum amount of toxin capable of killing a 20 g mouse within 24 h after intraperitoneal injection (i.p.) and corresponds to 4 μg of OA.[2,7,8] However, the mouse bioassay is hampered by ethical and economic concerns and gives false positives with respect to the risk of diarrhea depending on the extraction procedure, particularly the choice of the final extraction solvent.

Much effort has been made to develop analytical methods for determining the level of DSP contamination in seafood in order to assess its potential toxicological risk. Conventional immunological tests on microwell plates are widely used in diagnostics for the detection of characteristic pathological compounds. Commercial enzyme-linked immunosorbent assay (ELISA) kits for the detection of such molecules are readily available. On the other hand, the detection of small toxic molecules for environmental and farm-produce safety rarely employs such immunological methods and instead, the classic and well known high-performance liquid chromatography (HPLC) is preferred. Not only are these two methods time consuming and expensive, they require technical skills as well. In addition, immunological methods have low sensitivity and HPLC has low sensitivity for the detection reagent used. On the other hand, biosensor methods are expected to be simple, selective and economical. The use of immunosensors for OA detection has been proposed.[9,10]

In this study, we proposed an amperometric sensor system for the measurement of a DSP toxin, OA, which is a combination of the protein phosphatase 2A (PP2A) inhibition assay and flow injection analysis (FIA) utilizing pyruvate oxidase (PyOx) immobilized on the tip of an oxygen electrode set into a flow cell. This sensor is based on the principle that PP2A is inhibited by OA. OA concentration was determined from the amount of oxygen that corresponded to the amount of phosphate ion released by a phosphoprotein in the presence of PP2A, and that was consumed by PyOx.

2. Materials and Methods

2.1 *Substances*

Pure okadaic acid (98%) and phosvitin (from egg yolk) were obtained from Sigma-Aldrich, Japan (Tokyo, Japan). Protein phosphatase 2A was obtained from Upstate Biotechnology, Inc. (New York, U.S.A.). Pyruvate oxidase (PyOx, E.C.1.2.3.3 from *Aerococcus viridans*) was obtained from Toyobo (Osaka, Japan). Thiamin diphosphate (TPP) and sodium pyruvate were purchased from Wako Pure Chemicals (Osaka, Japan). Photocrosslinkable poly(vinyl alcohol) bearing stilbazolium groups (PVA-SbQ) was obtained from Tokyo Gosei Kogyo (Chiba, Japan). The ELISA kit OA-Check was manufactured by Yatoron Co., Ltd. (Tokyo, Japan). Other reagents and solvents were of analytical grade.

2.2 *Sample preparation for PP2A inhibition assay*

The PP2A inhibition assay was carried out in a microtube. Each tube contained 450 μl of 20 mM PIPES buffer (pH 7.0), 350 μl of phosvitin (15 mg/ml final concentration) and 100 μl of OA standard or samples and the reaction was started by the addition of 100 μl of PP2A (0.05–0.2 U/ml) and conducted for 60 min at room temperature. Conventionally, the PP2A inhibition assay was conducted in an alkaline medium and *p*-nitrophenylphosphate (*p*-NPP) was used as substrate.[11] In the case of the alkaline medium, however, the solutions had to be injected into the enzyme sensor system and the lifetime of the enzyme sensor was short. Therefore, various substrates active at neutral pH were examined and phosvitin was found to be the most suitable. The above reaction mixture was used for the determination of the enzyme inhibitory activity of OA.

2.3 *Construction of amperometric sensor system and procedure for measurement of DSP toxin, OA*

An immobilized PyOx membrane was prepared from a mixture of PVA-SbQ at a final concentration of 2–5% (w/v) and PyOx (200 U). The mixture was left to dry on a plate at 4°C for 24 h in the dark. Both sides of the dried membrane were irradiated for 10 min with a fluorescent lamp (27 W). The distance between the light source and the membrane was 5 cm. The membrane was stored at 4°C. The oxygen electrode (cathode diameter, 3 mm; DG-5G, ABLE, Tokyo) was a Clark-type electrode consisting of a platinum cathode, a lead anode, an alkaline electrolyte (KOH) and an oxygen-permeable Teflon membrane. The PyOx membrane (5 × 5 mm) was placed on the Teflon membrane and covered with a rinsed dialysis membrane (PyOx electrode). The FIA system consisted of the PyOx electrode fixed in a flow cell, a recorder (U-1, Rikadenki, Co., Tokyo), an air pump (α4000, Nihon Suisou Kougyou Co., Tokyo), a HPLC pump (PU-980, Jasco, Co., Tokyo) and a buffer tank, as shown in Fig. 1. After the steady state of the output current was obtained, two samples, namely, added and not added OA, were injected. The maximum decreases in the output current were obtained from the recorder. OA concentration corresponding to the difference between the maximum decrease obtained from two samples was determined from a standard curve.

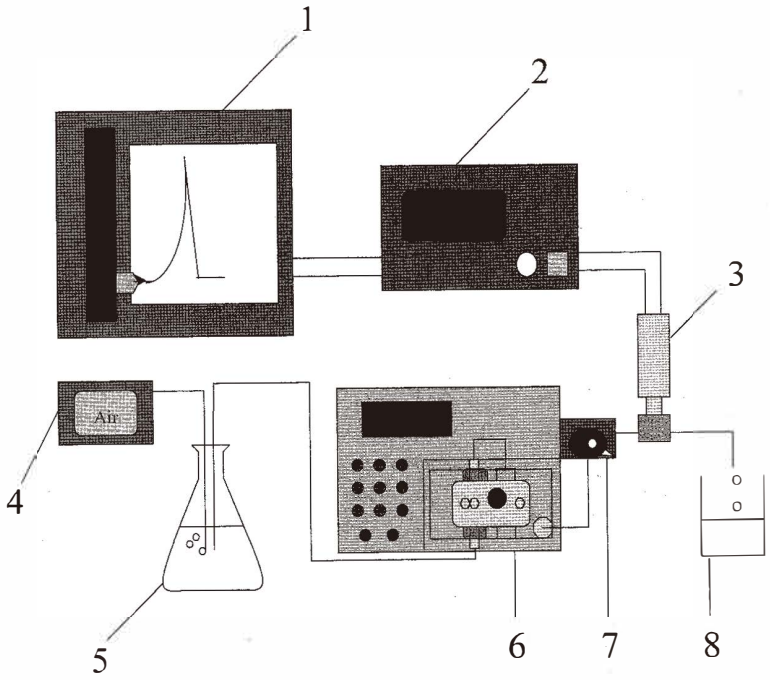

Fig. 1. Schematic of the sensor system. 1. recorder, 2. electrometer, 3. oxygen electrode, 4. air pump, 5. carrier buffer, 6. HPLC pump, 7. injection port and 8. waste.

2.4 *ELISA assay*

Standard OA was diluted with ethanol and used for the assay with a commercial ELISA kit (DSP-Check). The extraction procedure was carried out according to Tubaro, *et al.*[11] Mussel digestive glands (DGs) were separated and homogenized in 10 times volume of methanol. The homogenate was centrifuged for 15 min at 15,000 × *g* and the supernatant was washed with hexane. The lower phase was separated and diluted with water to about 50% methanol and subsequently extracted twice with the same volume of ethyl acetate. The organic phase was recovered and taken to dryness on a rotary evaporator and the solvent was removed under nitrogen. All samples were stored at –20°C. Immediately before testing, the residue was dissolved in an appropriate amount of ethanol. The precision of extraction and analysis had been confirmed using the known amount of the standard OA.

3. Principle of Proposed Sensor System

The sensor system is based on the following principles: 1) PP2A is inhibited by OA; 2) PP2A catalyzes the dephosphorylation of phosphoprotein (reaction 1); and 3) PyOx

catalyzes the oxidation of pyruvic acid in the presence of Pi (inorganic phosphate) (reaction 2).

$$\text{Phosphoprotein} + H_2O \rightarrow \text{Decomposed Protein} + \text{Pi} \quad (1)$$
$$\text{Protein phosphatase2 <PP2A> (EC3.1.3.16)}$$

$$\text{Pyruvate} + O_2 + H_2O + \text{Pi} \rightarrow CH_3COO\text{-}PO_3H_2 + CO_2 + H_2O_2 \quad (2)$$
$$\text{Pyruvate oxidase < PyOx > (EC1.2.3.3)}$$

Therefore, OA concentration was determined from the output of the oxygen electrode corresponding to the oxygen consumed by PyOx, which was equal to the Pi released by PP2A.

4. Results and Discussion

4.1 *Optimum conditions for proposed sensor system*

Effect of pH on PyOx electrode output: The sensor output was determined in the pH range of 5.0 to 9.0. The maximum output was obtained at pH 7.0. Then, different buffer solutions at pH 7.0 were investigated for maintaining stable output. A comparison of Tris-HCl, PIPES [piperazine-1,4-bis(2-ethanesulfonic acid)], TES [2-hydroxy-*N*-tris(hydroxymethyl) methyl-3-aminopropanesulfonic acid] and MOPS (3-morpholinopropanesulfonic acid) at pH 7.0 revealed that the output was most stable when PIPES buffer was used (data not shown). In addition, the effect of PIPES buffer at concentrations ranging from 20 to 100 mM on the sensor output was examined. No significant change in output was observed at those concentrations. On the basis of the results described above, 20 mM PIPES buffer (pH 7.0) was used as the optimum buffer.

Effect of pyruvic acid concentration on PyOx electrode output: The effect of pyruvic acid at concentrations ranging from 0.1 to 5 mM as the substrate for PyOx was investigated. Almost the same outputs were obtained at concentrations ranging from 0.5 to 5 mM. Therefore, 1 mM pyruvic acid was used in subsequent experiments. The concentrations of the activated cofactors of PyOx, TPP and Mg^{2+} were also investigated. TPP at 1 mM and Mg^{2+} at 5 mM were determined to be the optimum concentrations.

Effect of buffer flow rate on sensor response: In general, when the flow rate of the transport solution was too low, the response curve was broadened due to diffusion of the sample solution. On the other hand, when the flow rate was too high, part of the sample solution did not react with the enzyme on the tip of the Pt electrode and flowed out of the system. The maximum output of the proposed sensor was obtained at a flow rate of 0.15 ml min^{-1}.

Effect of injection volume on sensor output: The effect of injection volume in the range of 10 to 100 μl on sensor output was investigated. The outputs obtained at the minimum injection volume of 10 μl and the maximum injection volume of 100 μl were 1150 and 400

nA, respectively, and the difference between them was 2.9-fold. On the basis of the results in which the effect of solvent volume on sensor output was noted, the injection volume was fixed at 50 μl.

4.2 *Calibration curve and sensitivity*

An example of the response curve is shown in Fig. 2. One assay was completed within 10 min. The coefficient of variation (CV) of the output was 10% in five successive measurements. Two calibration curves for the phosphate ion sensor for the monitoring OA were obtained according to OA concentration. A linear relationship between OA concentration and sensor output was obtained in the range of 0.1 to 4.0 ppb OA. In the OA concentration range of 0.1 to 0.8 ppb, the correlation coefficient was 0.985 using 0.05 U/ml PP2A, as shown in Fig. 3(A). In the OA concentration range of 0.8 to 4.0 ppb, a linear

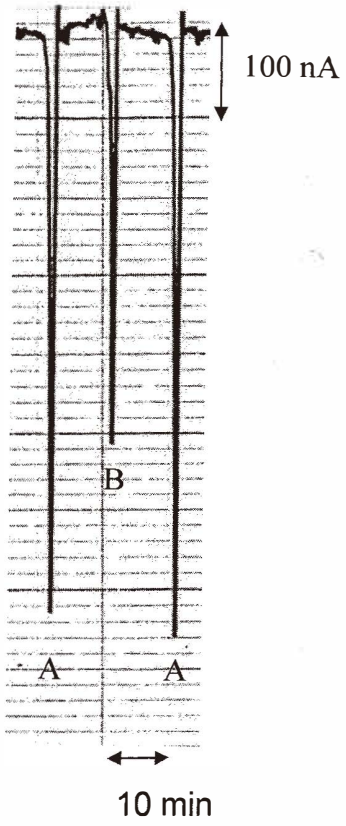

Fig. 2. Response curve of the proposed sensor system. Sensor conditions: 20 mM PIPES (pH 7.0); 1 mM TPP; 5 mM $MgCl_2$; 1 mM pyruvic acid; flow rate, 0.15 ml min^{-1}; injection volume, 50 μl. A: not added OA; B: added OA (0.2 ppb).

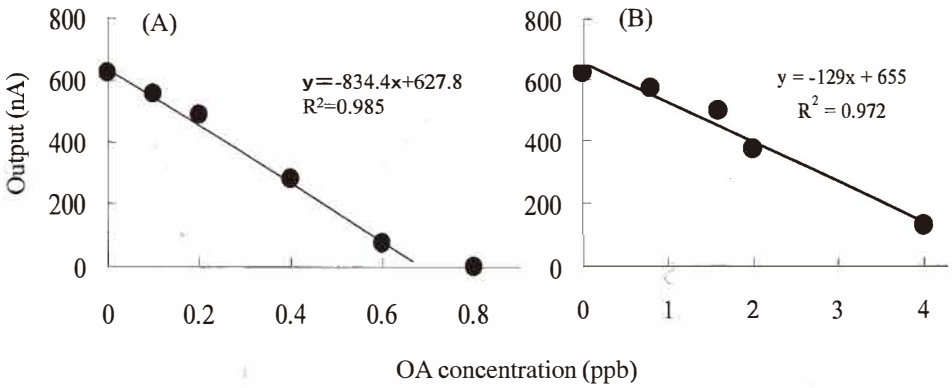

Fig. 3. OA calibration curve obtained by the proposed sensor system.
At (A) 0.05 U/ml PP2A and (B) 0.2 U/ml PP2A. Assay conditions: room temperature (24–25°C); 20 mM PIPES (pH 7.0); 1 mM TPP; 5 mM $MgCl_2$; 1 mM pyruvic acid; flow rate, 0.15 ml min^{-1}; injection volume, 50 μl.

relationship with a correlation coefficient of 0.972 was obtained at 0.2 U/ml PP2A (Fig. 3B). The coefficient of variation (CV) of the output was 10% in five successive measurements. The proposed sensor system was much more sensitive than the other immunosensor methods described in the Introduction, the lowest OA concentration detected being 2 ppb.

4.3 *Comparison of proposed sensor system with ELISA*

The OA standard curve obtained in the concentration range of 5 to 300 ppb using conventional ELISA is shown in Fig. 4. The detection limit of the proposed sensor system was 0.1 ppb, whereas that of ELISA was 5 ppb. The proposed sensor system appears to be approximately 50 times more sensitive than ELISA. OA inhibited PP2A activity in a dose-dependent manner at concentrations ranging from 0.1 to 4.0 ppb (Fig. 3). The OA concentrations required to reduce PP2A activity by 50% (IC_{50}) were 0.33 ppb for 0.05 U/ml PP2A, 0.9 ppb for 0.1 U/ml PP2A, and 3.2 ppb for 0.2 U/ml PP2A. Good agreement was observed between approximately (1/100 to 1/10) diluted samples determined by the proposed sensor system and original samples by conventional ELISA. In our preliminary experiments, the OA content in 50 blue mussels (*Mytilus galloprovincialis*) collected from Oarai Bay, Ibaraki Prefecture, was determined using the proposed sensor system and ELISA. OA was detected at concentrations ranging from 8.2 to 280 ng/g in 10 samples analyzed by ELISA, but not in any of the samples analyzed by the proposed sensor system. Therefore, the possibility that ELISA detected DSP toxins other than OA was suggested. It has also been reported that an anti-OA antibody of DSP-Check shows a cross-reaction with DTX1 (dinophysistoxin-1) and YTXs,[12] but not with DTX2 (dinophysistoxin-2).[13] Therefore, it was suggested that OA determination by ELISA was ambiguous and the proposed sensor system could selectively detect OA compared to ELISA. In the initial phase of our

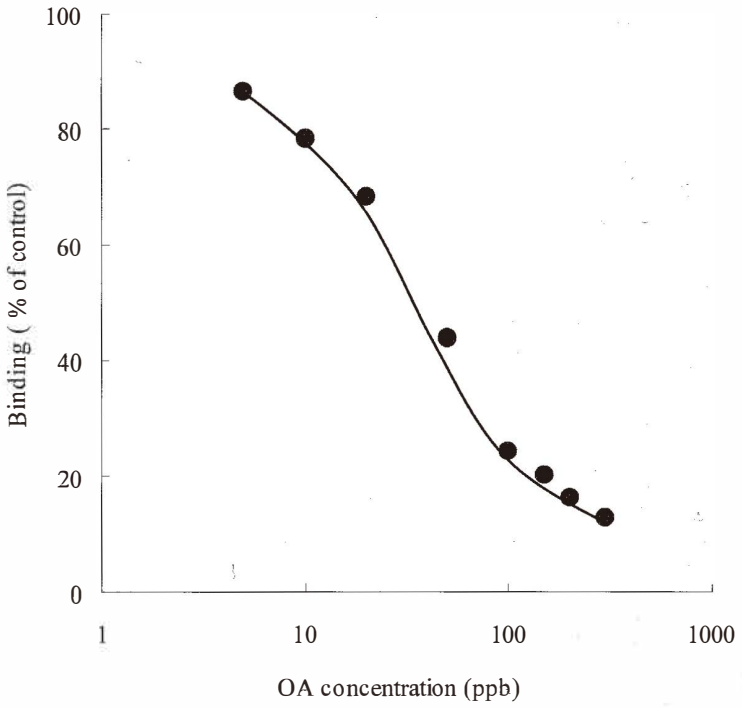

Fig. 4. Standard curve for OA obtained by ELISA.

study, we aimed at realizing the continuous determination of OA by immobilizing PP2A and PyOx. However, PP2A could not be immobilized because of its solubility in glycerol. It is surmised that glycerol inhibits the immobilization of PP2A. On the other hand, when glycerol was removed, the enzyme activity was lost. The immobilization of PP2A will be a subject of future studies.

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