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A bienzyme electrochemical probe for flow injection analysis of okadaic acid based on protein phosphatase-2A inhibition: An optimization study

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ABSTRACT

A bienzyme electrochemical probe has been assembled and used to monitor the inhibition of the enzyme protein phosphatase-2A (PP2A) by okadaic acid (OA), taking advantage of the particular characteristics of a biochemical pathway in which PP2A is involved. This enzyme has significant activity toward glycogen phosphorylase *a* (PHOS *a*), which in turn catalyzes the conversion of glycogen to glucose-1-phosphate (G-1-P). In addition, PP2A is strongly inhibited by OA and its derivatives. Due to this combination of properties, PP2A was employed to develop an assay system involving a preliminary phase of off-line enzymatic incubations (OA/PP2A, PP2A/PHOS *a*, PHOS *a*/glycogen + phosphate). This off-line step was followed by the electrochemical detection of H_2O_2, which is the final product of two sequential enzymatic reactions: G-1-P with alkaline phosphatase (AP) producing glucose, then glucose with glucose oxidase (GOD) producing hydrogen peroxide. These two enzymes were coimmobilized on a nylon net membrane that was placed over an H_2O_2 platinum probe inserted into a flow injection analysis (FIA) system. During a first phase of the study, all analytical parameters were optimized. During a subsequent phase, the inhibition of PP2A enzyme by OA was evaluated. The calibration of the system shows a working range for detection of OA between 30 and 250 pg ml^{-1}. The total analysis time is the sum of 50 min for the off-line enzymatic incubations and 4 min for the biosensor response.

Okadaic acid (OA)[1] is a polyether derivative of a C38 fatty acid produced by toxigenic dinoflagellates (*Dynophysis* and *Prorocentrum*) and is implicated in massive fish kills as well as diarrheic shellfish poisoning (DSP), a gastrointestinal disturbance due to the human ingestion of contaminated shellfish [1,2]. The DSP group includes four structural classes—OA and its derivatives dinophysistoxins (DTXs), pectenotoxins (PTXs), yessotoxins (YTXs), and azaspiracids (AZAs)—which present different toxicological effects and different mechanisms of action. The Commission of the European Community, on the basis of the toxicity to humans, has established different tolerance limits for the presence of these toxins in the live bivalve molluscs; OA, DTXs, and PTXs must not exceed 160 μg of OA equivalent/kg, whereas YTXs must not exceed 1 mg of YTX equivalent/kg. On the other hand, the mouse bioassay has been indicated as the official method for DSP analysis [3], and a death time of 24 h was selected as a criterion for shellfish prohibition. The major problems of this bioassay are limited sensitivity, high cost, variability, and lack of specificity. For instance, this method does not differentiate among the various DSP toxins. In addition, interference from unsaturated free fatty acid can lead to false-positive results [4]. For these reasons, and to avoid or minimize the use of living animals, there is a need for the development of more suitable and practical methods to evaluate the edibility of mussels.

OA, along with its derivatives (especially DTX1), is the major compound responsible for DSP [5,6] and, as a specific inhibitor of protein phosphatases-1 and -2A (PP1 and PP2A, respectively, two of the four major serine/threonine protein phosphatases involved in cellular regulation) [7–9], it has also been indicated as a powerful tumor promoter [7].

Numerous methods are reported in the literature for the detection of OA alone or in combination with DTXs. These methods can be divided into five main groups: cytotoxicity tests [10–13], high-performance liquid chromatography (HPLC) [14–19], liquid chromatography–ion spray mass spectrometry (LC–MS) [5,6,20–22], immunological assay [23–26], and protein phosphatase (PP1 and PP2A) inhibition assay with colorimetric and fluorimetric detection

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[1] *Abbreviations used:* OA, okadaic acid; DSP, diarrheic shellfish poisoning; DTX, dinophysistoxin; PTX, pectenotoxin; YTX, yessotoxin; AZA, azaspiracid; PP1, protein phosphatase-1; PP2A, protein phosphatase-2A; HPLC, high-performance liquid chromatography; LC–MS, liquid chromatography–ion spray mass spectrometry; p-NPP, *p*-nitrophenylphosphate; MUP, 4-methylumbelliferyl phosphate; PHOS *a*, phosphorylase *a;* G-1-P, glucose-1-phosphate; FIA, flow injection analysis; AP, alkaline phosphatase; GOD, glucose oxidase; Pi, inorganic phosphate; EGTA, ethyleneglycoltetraacetic acid; DTT, dithiothreitol; MWCO, molecular weight cutoff; ABD, amperometric biosensor detector; EDTA, ethylenediaminetetraacetic acid; CH_2Cl_2, methylene chloride; TOTFB, triethyloxonium tetrafluoroborate; DW, distilled water; G-6-P, glucose-6-phosphate.

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using artificial substrates [27–33] such as *p*-nitrophenylphosphate (*p*-NPP) and 4-methylumbelliferyl phosphate (MUP). The protein phosphatase assay and LC–MS-based methods have been considered to have the greatest potential to replace the mammalian assay and to detect levels of OA group toxins below the current European Union regulatory limit (as reported in a document of the European Food Safety Authority, http://www.efsa.europa.eu/EFSA, question number EFSA-Q-2006-065A). Until now, however, these methods still have not been completely validated.

Serine/Threonine protein phosphatases (PP1 and PP2A) are enzymes that dephosphorylate the α-subunit of phosphorylase kinase. Of note, PP1 and PP2A also have significant activity toward glycogen phosphorylase *a* (PHOS *a*) [34], which catalyzes the conversion of glycogen to glucose-1-phosphate (G-1-P).

The aim of the current work is to exploit the metabolic approach for OA analysis, based on PP2A inhibition and on the use of PHOS *a* as PP2A substrate. The degree of PP2A inhibition is evaluated by measuring G-1-P, the final product of the biochemical cascade that involves PHOS *a* and PP2A enzymes, with the use of a new bienzyme electrode probe.

A partial automation of the OA analysis was achieved by running the biochemical pathway off-line and by measuring the produced G-1-P via a flow injection analysis (FIA) system in which the bienzyme electrode probe was inserted.

During a first phase of this study, all analytical parameters were optimized, including factors such as ionic strength and pH of the buffer, alkaline phosphatase (AP)–glucose oxidase (GOD) coimmobilization procedure, glycogen and inorganic phosphate (Pi) concentrations, amount of PHOS *a* and PP2A, incubation times, and the strategy to stop the enzymatic reactions prior to the injection into the FIA system. During a subsequent phase, different concentrations of OA (inhibitor) were added to the incubation solution containing PP2A.

Materials and methods

Reagents and materials

PP2A (from human red blood cells, enzyme formulation: 10 U in 100 µl of 20 mM Mops [pH 7.5] containing 50% glycerol, 150 mM $MgCl_2$, 60 mM 2-mercapthoethanol, 1 mM ethyleneglycoltetraacetic acid [EGTA], 0.1 mM $MnCl_2$, 1 mM dithiothreitol [DTT], and 0.1 mg ml^{-1} serum albumin) was purchased from Upstate Biotechnology (Lake Placid, NY, USA). OA was obtained from Alexis Biochemicals (Lausen, Switzerland).

Lyophilized GOD (EC 1.1.3.4, type VII, from *Aspergillus niger,* 197 U mg^{-1} of solid), lyophilized glycogen PHOS *a* (EC 2.4.1.1, from rabbit muscle, 10.2 U mg^{-1} of solid), AP (from bovine intestinal mucosa, 10.1 mg protein ml^{-1} of triethanolamine solution and 3920 U mg^{-1} of protein), glycogen, G-1-P, glucose, Mops, and all other chemical reagents were obtained from Sigma Chemical (St. Louis, MO, USA).

Polycarbonate membrane (12 µm pore size) was obtained from Nucleopore (Pleasanton, CA, USA), and nylon net membrane (mesh 56 µm, 81 µm thick, 100 treads cm^{-1}) was obtained from SaatiTech (Como, Italy). Cellulose acetate membrane (~100 Da molecular weight cutoff [MWCO]) was prepared as reported previously [35]. A precision gauge tool (Precision Gage and Tool, Dayton, OH, USA) was used for casting the cellulose acetate membrane.

Apparatus

An amperometric biosensor detector (ABD), a hydrogen peroxide-based platinum probe, and a flow-through cell (Universal Sensors, Metairie, LA, USA) were used for amperometric biosensor measurements. The current output was recorded with an L 250 E *x-t* recorder (Linseis, Selb, Germany). A Minipuls 3 peristaltic pump (Gilson, Villiers-le-Bell, France) and a model 5020 Teflon injection valve (Rheodyne, Cotati, CA, USA), equipped with 100-µl loops, was used for FIA.

Principle of the method

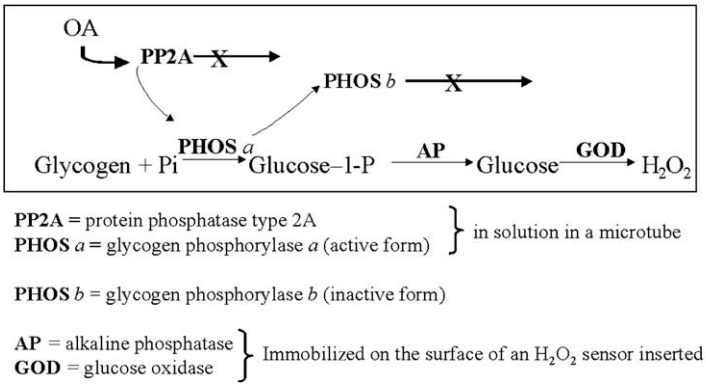
To better understand the principle of the proposed method, the enzymatic reactions involved are summarized in Scheme 1, whereas the overall scheme of the assay system is presented in Fig. 1.

The basic reaction of this system is the one catalyzed by the PHOS *a* that converts glycogen and Pi into G-1-P, which in turn produces glucose through the use of AP. The glucose is then converted by GOD into H_2O_2, the final product, which is electrochemically oxidized at the platinum electrode.

As already explained, the PP2A enzyme is able to block this reaction chain because it converts the PHOS *a* (the active form) into PHOS *b* (the inactive form), which is not able to catalyze the conversion of glycogen to G-1-P. In this control situation, with little G-1-P produced, there is consequently little production of glucose by AP. In turn, the absence of substrate for GOD results in negligible production of H_2O_2 (low current signal).

In the presence of OA, however, the PP2A is inhibited (to differing degrees depending on toxin concentration) and the higher residual levels of PHOS *a* lead to an increase in the current signal proportional to the toxin concentration.

To use this set of enzymatic reactions to construct a semiautomatic assay system for OA, the "marker enzymes" (AP and GOD) were immobilized onto the surface of an H_2O_2 probe, whereas



Scheme 1. Summary of enzymatic reactions involved in the proposed method.

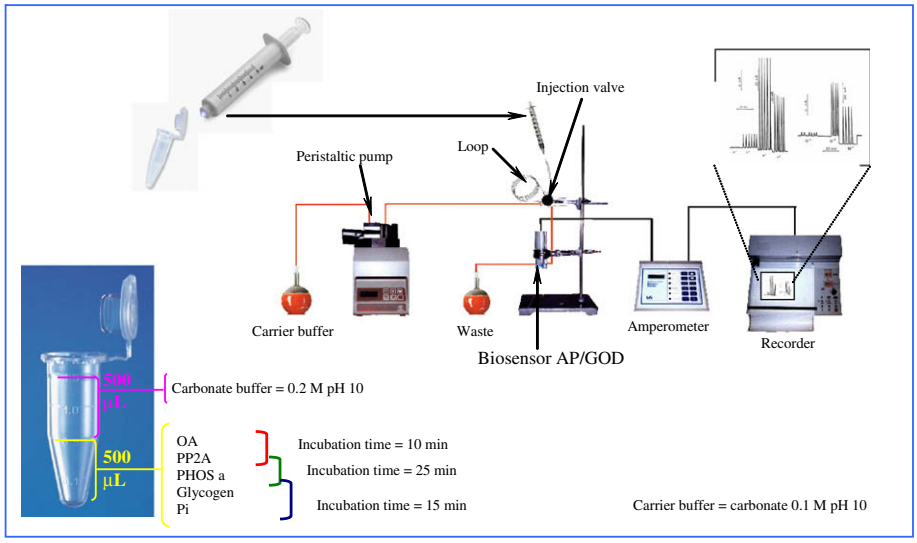


Fig. 1. Scheme of the system used for OA analysis. The reactions OA/PP2A, PP2A/PHOS *a*, and PHOS *a*/glycogen + Pi were performed off-line in an Eppendorf tube. At the end, the PHOS *a* reaction was stopped by the addition of carbonate buffer (0.2 M, pH 10.0) and the contents of different microtubes (with different concentrations of OA) were injected consecutively into the FIA system where a GOD–AP biosensor was inserted.

the other reactions involved in the biological cascade (OA/PP2A, PP2A/PHOS *a*, and PHOS *a*/glycogen + Pi) were allowed to proceed in a microtube test using 40 mM Mops (+0.1 mM ethylenediaminetetraacetic acid [EDTA] + 1.4 mM $MgCl_2$, pH 7.4) as buffer solution. After a set interval, the PHOS *a* reaction was stopped by the addition of carbonate buffer (0.2 M, pH 10.0) and the contents of different microtubes (controls plus those containing different concentrations of OA) were injected consecutively into the FIA stream.

The various steps in the assembly of the biosensing system are detailed in the following sections.

GOD–AP immobilization procedure on nylon net membrane

Prior to the immobilization, 100 µl of AP solution was dialyzed (using Spectra/Por Micro DispoDialyzer, 25 kDa MWCO, 100 µl volume) against three changes of 1 L of 40 mM carbonate buffer + 1 mM $MgCl_2$ (pH 7.0) for 24 h at 4 °C under magnetic stirring to remove triethanolamine that could interfere with the immobilization procedure. After dialysis, the collected volume (~200 µl) was subjected, along with GOD, to the AP–GOD coimmobilization performed by adapting a previously reported procedure for covalent binding of a single enzyme [36]. At room temperature, a 1-cm^2 nylon net membrane was immersed in methylene chloride (CH_2Cl_2) for 10 min and then in a 0.1-M triethyloxonium tetrafluoroborate (TOTFB) solution prepared in CH_2Cl_2 for 5 min. The membrane was then washed three times with freshly prepared iced methanol and immersed in an aqueous solution of 5% (w/v) polyethylenimine for 3.5 h at room temperature. After a three-cycle washing step in distilled water (DW), the nylon net membrane with immobilized polyethylenimine was allowed to react with 1% (v/v) glutaraldehyde in 0.1 M carbonate buffer (pH 10.0) for 40 min. After several washing steps in DW, the enzyme coimmobilization was performed as follows. After 10 mg of GOD was dissolved in 500 µl of carbonate buffer (0.1 M, pH 7.0), 47 µl of this solution (180 U) was mixed, in a porcelain well, with 50 µl of the dialyzed AP (~990 U). The mixture was spread onto the surface of the activated nylon net membrane and incubated overnight at 4 °C. After immobilization, the membrane was washed with a 0.5 M glycine solution for 30 min. When not in use, the membrane was stored in 0.1 M carbonate buffer (pH 7.0) containing 0.1% (v/v) Kathon as an antibacterial agent.

Biosensor assembly

The probe was assembled by placing membranes on an inverted electrode jacket in the following order: cellulose acetate, enzyme membrane, and then a polycarbonate membrane that protects the enzyme from large molecules or bacteria. The membranes were then secured with an O-ring. The electrode jacket was filled with 0.1 M potassium chloride (supporting electrolyte) and then placed onto the platinum base sensor and screwed down until the tip of the platinum was firmly in contact with the membranes. Finally, the assembled biosensor was inserted into a flow-through cell.

Procedure for optimization of glycogen and Pi concentrations

The concentrations of glycogen and Pi were optimized using a fixed amount of PHOS *a*. For this purpose, we added in tubes the following solutions: 420 µl of buffer A (40 mM Mops + 0.1 mM EDTA + 1.4 mM $MgCl_2$, pH 7.4) + 25 µl of PHOS *a* (1 U/ml prepared daily in buffer A starting from a stock solution [20 U ml^{-1}] stored at −20 °C) + 50 µl of glycogen (1%, 2%, 5%, 10%, and 20%) + 5 µl of NaH_2PO_4 (100, 500, and 1000 mM). After 5 min of incubation at 30 °C between PHOS *a* and its substrates, the enzymatic reaction was stopped by adding 500 µl of 0.2 M carbonate buffer (pH 10.0). Finally, a few microliters of each mixture were withdrawn and injected via the automatic valve loop (100 µl) consecutively into the FIA system, where an appropriate carrier buffer (0.1 M carbonate buffer, pH 10.0) was flowed by a peristaltic pump (at a rate of 300 µl min^{-1}) through the electrochemical biosensor cell and a transient current variation (current peak) was recorded.

For further experiments, 50 µl of 10% glycogen + 5 µl of 500 mM NaH_2PO_4 were chosen, as explained in Results and Discussion.

Procedure for optimization of the concentration and incubation time of PHOS a

Once the optimal concentrations of glycogen and Pi were determined, different amounts of PHOS *a* were incubated at 30 °C with these substrates at different times as per the following procedure: 420 µl of buffer A + 25 µl of PHOS *a* (1 U ml^{-1}, 0.5 U ml^{-1}, and 0.25 U ml^{-1} prepared daily in buffer A) + 50 µl of 10% glycogen + 5 µl of 500 mM NaH_2PO_4. After 10, 15, and 20 min of incubation at 30 °C, the enzymatic reaction was stopped and FIA was

performed as reported in the previous section (“Procedure for Optimization of Glycogen and Pi Concentrations”).

An incubation time of 15 min and 25 μl of 1 U ml^{-1} PHOS *a* were selected, as explained in Results and Discussion.

Procedure for optimization of the concentration and incubation time of PP2A

The procedure adopted for the optimization of PP2A enzyme in terms of concentration and incubation time involved the following incubations: 410 μl of buffer A + 10 μl of PP2A (10 U ml^{-1}, 5 U ml^{-1}, and 2.5 U ml^{-1} prepared daily in buffer A by dilution of the commercial stock solution divided into aliquots frozen at −20 °C) + 25 μl of PHOS *a* (1 U ml^{-1} prepared daily in buffer A). After incubation times of 5, 10, 15, and 30 min at 30 °C between PP2A and PHOS *a,* 50 μl of 10% glycogen + 5 μl of 500 mM NaH_2PO_4 were added consecutively to each Eppendorf tube and the reaction catalyzed by the residual PHOS *a* was allowed to proceed for 15 min at 30 °C. After this interval, the enzymatic reaction was stopped by adding 500 μl of 0.2 M carbonate buffer (pH 10.0). Additional microtubes containing 490 μl of buffer A + 10 μl of PP2A (10 U ml^{-1}, 5 U ml^{-1}, and 2.5 U ml^{-1}) + 500 μl of 0.2 M carbonate buffer (pH 10.0) were used as blanks to evaluate the electrochemical interferences due to some components of the PP2A formulation (2-mercaptoethanol, glycerol, and DTT), although this enzyme had already been diluted several-fold. Finally, FIA was performed as reported in a previous section.

An incubation time of 25 min and 10 μl of 5 U ml^{-1} PP2A were selected, as demonstrated in Results and Discussion.

OA analysis procedure

Standard solutions of OA (3–100 ng ml^{-1}) were prepared in ethanol starting from a stock solution (0.1 mg OA ml^{-1} of ethanol).

The procedure for the analysis of OA (Fig. 1) is as follows. To each microtube, 405 μl of buffer A, 5 μl of OA standard solutions, and 10 μl of PP2A (5 U ml^{-1} prepared daily in buffer A) were added. After an incubation time of 10 min at 30 °C, 25 μl of PHOS *a* (1 U ml^{-1} prepared daily in buffer A) was added, followed by waiting for 25 min (at 30 °C). To measure the residual activity of PHOS *a,* 50 μl of 10% (w/v) glycogen and 5 μl of 500 mM NaH_2PO_4 solutions were pipetted consecutively into each microtube. Then, after an incubation time of 15 min (at 30 °C), the reaction catalyzed by PHOS *a* was stopped by adding 500 μl of 0.2 M carbonate buffer (pH 10.0). An additional microtube, prepared by adding 485 μl of buffer A + 5 μl of pure ethanol + 10 μl of PP2A (5 U ml^{-1}) + 500 μl of 0.2 M carbonate buffer (pH 10.0), was used as a blank to evaluate the electrochemical interferences due to the combined action of ethanol and some components of the enzyme formulation. Also in this case, FIA was carried out as reported in a previous section.

Results and discussion

Preliminary study

The biosensor used in this work (an AP + GOD/H_2O_2-based probe) for the detection of G-1-P, which is the final product of the biochemical cascade that involves PHOS *a* and PP2A enzymes, can be considered as a new biosensor. In fact, although several biosensors based on the use of GOD coupled with alkaline or acid phosphatase are reported in the literature, they are employed only for the detection of glucose-6-phosphate (G-6-P) [37–39]. Regarding the interaction between these enzymes and phosphorylated glucose, during preliminary experimental studies we demonstrated that the determination of G-1-P is more difficult than that of G-6-P. First, it is not possible to use acid phosphatase because this enzyme does not interact with G-1-P. Second, when the GOD–AP biosensor was assembled by using common preactivated membranes (e.g., Immobilon Affinity membrane [Millipore, Bedford, MA, USA], Immunodyne ABC membrane [Pall BioSupport, Port Washington, NY, USA]) as immobilization support, only very high concentrations of G-1-P could be detected. So, with the objective of developing a sensitive biosensor for G-1-P, we needed to use several strategies: a very active AP enzyme (see “Reagents and Materials” section), an effective immobilization procedure using a nylon net membrane, and the use of a biosensor working in carbonate buffer at pH 10.0. Concerning the choice of the carrier buffer, Mops (pH 6–8), Tris (pH 7–9), glycine (pH 8–10), and carbonate (pH 8–10) buffer were tested using a fixed concentration of G-1-P. Negligible current variation was recorded at pH levels less than 7.5, and only carbonate buffer gave a low and reproducible background current. Therefore, this buffer was selected and the optimal pH for the detection of G-1-P turned out to be 10.0.

After optimization of the GOD–AP biosensor, a calibration curve for determination of G-1-P was performed. Standard solutions of this analyte (0–50 mM) were prepared in 500 μl of buffer A, and prior to injection into the FIA system an equal volume of 0.2 M carbonate buffer (pH 10.0) was added. A linear range between 10^{-5} and 10^{-2} M and a detection limit of 5×10^{-6} M were obtained.

The choice of buffer A as working solution was due to the fact that the subsequent experiments with PHOS *a* and PP2A will be performed in this buffer (suitable for both enzymes), whereas carbonate buffer is the same as that used as carrier for FIA measurements.

Because the reaction catalyzed by PHOS *a* needs Pi, which is also a product of the reversible AP reaction, the influence of the Pi on the biosensor (AP–GOD) response was also evaluated. Different amounts of Pi (0, 10, 20, and 50 mM) were added to a fixed concentration of G-1-P. The solutions were then injected into the FIA stream, and in all cases the same current variation was recorded, demonstrating that Pi does not affect the biosensor signal.

Optimization of glycogen and Pi concentration

Because the reaction catalyzed from PHOS *a* generates a current signal that represents the 100% response of our system, the concentrations of glycogen and Pi were optimized using a fixed amount of PHOS *a* and following the procedure reported in the corresponding section.

Fig. 2 shows the experimental results where the amounts of the two substrates are reported as the concentrations that really interact with PHOS *a* (concentrations in Eppendorf tubes prior to the addition of 500 μl of carbonate buffer). As shown, the best current signal was obtained by using 1% glycogen and 5 mM Pi.

Optimization of the concentration and incubation time of PHOS a

Once the optimal concentrations of glycogen and Pi were fixed, different amounts of PHOS *a* were incubated at 30 °C with these substrates at different times following the procedure reported in the corresponding section.

As shown in Fig. 3, for each incubation time there is a linear relationship between the PHOS *a* concentration and the rate of the enzymatic reaction measured as current signal. Also in this case, we have reported the concentration of PHOS *a* in the Eppendorf tubes prior to the addition of 500 μl of carbonate buffer. A concentration of enzyme equal to 0.05 U ml^{-1}, which gave the highest current signal, and an incubation time of 15 min were chosen for the next experiments. We did not select a longer incubation period given the fact that other time-consuming steps were necessary to perform the entire procedure for the determination of OA; therefore, this period was the best compromise between a reasonable analysis time and good sensitivity.

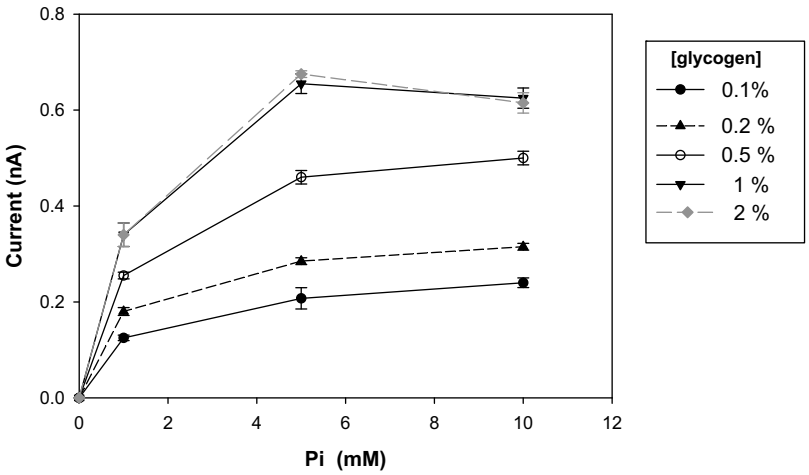


Fig. 2. Optimization of glycogen and phosphate concentrations. PHOS *a* = 0.05 U ml^{-1} in buffer A; incubation time = 5 min. All of the concentrations reported are those realized off-line in buffer A prior to the addition of the blocking solution.

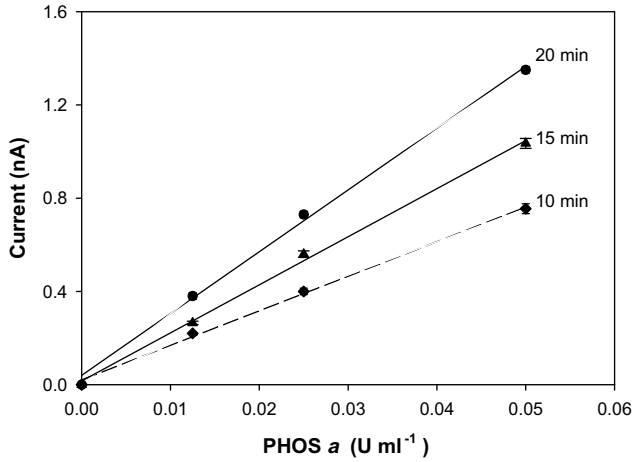


Fig. 3. Optimization of PHOS *a* concentration and incubation time. Different concentrations of PHOS *a* were tested at three incubation times using 1% glycogen + 5 mM Pi as substrates. The concentrations reported are those realized off-line in buffer A prior to the addition of the blocking solution.

Study of enzyme stability

To evaluate the stability of PHOS *a,* several aliquots of a stock solution of PHOS *a* (20 U ml^{-1} prepared in buffer A) were prepared, stored at −20 °C, and tested occasionally. Results showed that the residual activity of the PHOS *a* under these conditions was 70%, 60%, and 50% after 1, 2, and 3 weeks, respectively. Because the sensitivity of the entire analytical method is strictly dependent on the activity of the PHOS *a* enzyme, we decided to limit the storage period of the PHOS *a* stock solution to 1 week; this will guarantee that the analytical performance of the method will not be affected by the limited stability of PHOS *a*.

With regard to PP2A, to minimize the electrochemical interferences due to some components of the enzyme formulation (high concentrations of 2-mercaptoethanol, glycerol, and DTT used as stabilizing agents), we prepared a solution of 5 U ml^{-1} daily using only buffer A as solvent.

The stability of the bienzyme GOD–AP probe was tested each day before and after measurements of G-1-P. After 2 months of regular measurements, the activity of the two enzymes remained nearly the same and the performance of the probe as H_2O_2 sensor was unchanged.

Optimization of the concentration and incubation time of PP2A

The objective of the next experiments, carried out following the procedure reported in the corresponding section, was to determine the concentration of PP2A enzyme able to inactivate PHOS *a* to approximately 80% of its initial activity and to select the best incubation time. The experimental data are shown in Fig. 4, where the residual activity of PHOS *a* is reported as a function of the incubation time that occurs between different concentrations of PP2A and the selected amount of PHOS *a*.

An incubation time of 25 min and a concentration of 0.1 U ml^{-1} PP2A (concentration in Eppendorf tubes prior to the addition of the stop solution) were selected to obtain residual activity of PHOS *a* of approximately 20%. We chose 0.1 U ml^{-1} PP2A rather than a higher amount because it is known from the literature [32] that a lower concentration of PP2A corresponds to greater inhibition due to the OA.

Ethanol effect on the assay system

It has been reported that ethanol, used to solubilize OA, has an inhibitory effect on PP2A [29]. Before attempting to verify the ef-

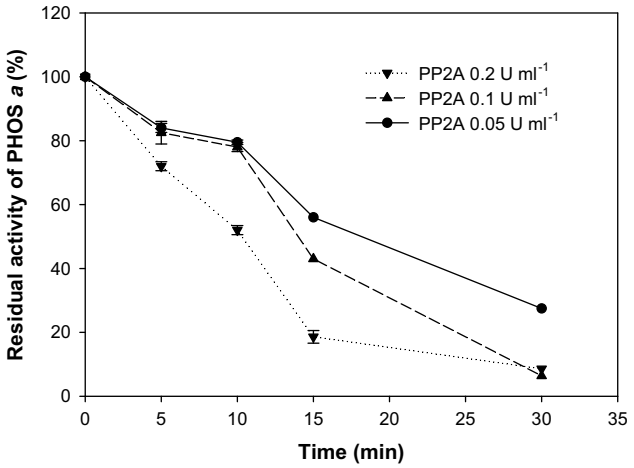


Fig. 4. Optimization of PP2A concentration and incubation time. Percentage of residual activity of PHOS *a* reported as a function of the incubation time between different concentrations of PP2A and PHOS *a* = 0.05 U ml^{-1}. The concentrations reported are those realized off-line in buffer A prior to the addition of the blocking solution.

fect of various concentrations of ethanol on the activity of the PP2A and on other enzymes used in our procedure, it was necessary to ascertain whether this solvent could be oxidized directly on the electrode surface. Experiments carried out using 500 μl of standard solutions of ethanol (0.5%, 1%, 2%, 5%, and 10%) prepared in buffer A mixed with an equal volume of 0.2 M carbonate buffer (pH 10.0) and injected into the FIA system showed an oxidation pick proportional to the concentration of ethanol. This is because the cellulose acetate membrane (employed for the biosensor assembly) prevents the permeation, toward the platinum electrode, of electroactive substances with a molecular weight greater than 100 Da.

Then, to evaluate the ethanol effect on GOD–AP and PHOS *a* enzymes, standard solutions of ethanol (prepared as reported above) were used as blanks to subtract their current values. No influence of the ethanol was observed on the response of the GOD–AP biosensor toward G-1-P or on the activity of PHOS *a* toward its substrates.

With regard to the effect of ethanol on the PP2A, if this solvent inhibits the enzyme, an increased current signal would be observed because a lesser amount of PHOS *a* will be converted in PHOS *b*. During a first phase, the selected PP2A concentration (0.1 U ml^{-1}) was incubated for 5 min with different percentages of ethanol and the residual activity of PHOS *a* was measured under the optimized experimental conditions. In this case, blank solutions were obtained with standard solutions of ethanol (prepared as reported above) containing 0.1 U ml^{-1} PP2A. Results of this test are reported in Table 1. As shown, only the highest concentrations of the solvent (5% and 2%) significantly inhibited the enzymatic activity of PP2A, as demonstrated by the observed increase in the percentage of residual activity of PHOS *a*. In all subsequent experiments, then, the concentration of ethanol was kept at 1%. Once this concentration was fixed, different incubation times between PP2A and ethanol were tested. The experimental results are presented in Table 2. As can be observed, by using 1% ethanol, no inactivation of PP2A was recorded for an incubation time of 10 min. Longer times showed a consistent inhibitory effect of the ethanol on the PP2A enzyme, which in turn determined an increase in the percentage of residual activity of PHOS *a*. These results indicated that ethanol, and therefore OA (solubilized in this solvent), could be incubated with PP2A for a maximum time of 10 min. Thus, for OA analysis, three different incubation times (0, 5, and 10 min) between OA (1 ng ml^{-1}) and PP2A (0.1 U ml^{-1}) were tested during a preliminary phase.

OA analysis

Because the experimental results indicated that this inhibition is time dependent, an incubation time of 10 min was selected and a calibration curve, using different concentrations of OA, was carried out following the procedure reported in the corresponding section. The experimental data (Fig. 5) were fitted using a four-parameter logistic equation:

$$f(x) = \frac{a-d}{1+(x/c)^b} + d,$$

where *a* and *d* are the asymptotic maximum and minimum values, respectively, *c* is the *x* value at the inflection point, and *b* is the slope. As can be seen, OA ranging from 30 to 250 pg ml^{-1} dose-dependently inhibits the PP2A enzyme, causing an increase in the percentage of residual activity of PHOS *a*. In particular, in the absence of OA this percentage was 23 ± 3 ($n = 3$), whereas for OA = 30 and 250 pg ml^{-1} the percentages of residual activity of PHOS *a* were found to be 34 ± 4 and 84 ± 1 ($n = 3$), respectively. The concentration of OA needed to produce a 50% increase in the signal (inflection point) was 70 pg ml^{-1}.

Ethanol, used to solubilize the toxin, did not interfere with the enzymatic activity at the concentration and for the time used in the assay (1% ethanol and 10 min of incubation between 1% ethanol and 0.1 U ml^{-1} PP2A).

Table 1
Effect of ethanol on PP2A activity measured as a percentage of residual activity of PHOS *a*

Enzyme(s) used	Residual activity of PHOS *a* (% ± SD, $n = 3$)
Only PHOS *a*	100 ± 1
PHOS *a* + PP2A	23 ± 3[a]
PHOS *a* + PP2A (+5% CH_3CH_2OH)	68 ± 2[b]
PHOS *a* + PP2A (+2% CH_3CH_2OH)	40 ± 2[b]
PHOS *a* + PP2A (+1% CH_3CH_2OH)	23 ± 3[b]
PHOS *a* + PP2A (+0.5% CH_3CH_2OH)	23 ± 3[b]

Note. Ethanol–PP2A incubation time = 5 min.

[a] For the calculation of residual activity of PHOS *a*, the following mixture was used as blank solution: 500 μl of buffer A containing 0.1 U ml^{-1} PP2A + 500 μl of 0.2 M carbonate buffer (pH 10.0).

[b] For the calculation of residual activity of PHOS *a*, the following mixtures were used as blank solutions: 500 μl of buffer A containing 0.1 U ml^{-1} PP2A and the different percentages of ethanol + 500 μl of 0.2 M carbonate buffer (pH 10.0).

Table 2
Effect of 1% ethanol on PP2A activity, in function of the incubation time, measured as a percentage of residual activity of PHOS *a*

Enzyme(s) used	Incubation time PP2A–CH_3CH_2OH (min)	Residual activity of PHOS *a* (% ± SD, $n = 3$)
Only PHOS *a*		100 ± 1
PHOS *a* + PP2A		23 ± 3[a]
PHOS *a* + PP2A (+ 1% CH_3CH_2OH)	5	23 ± 3[b]
	10	44 ± 2[b]
	20	60 ± 2[b]
	30	23 ± 3[b]

[a] For the calculation of residual activity of PHOS *a*, the following mixture was used as blank solution: 500 μl of buffer A containing 0.1 U ml^{-1} PP2A + 500 μl of 0.2 M carbonate buffer (pH 10.0).

[b] For the calculation of residual activity of PHOS *a*, the following mixtures were used as blank solution: 500 μl of buffer A containing 0.1 U ml^{-1} PP2A and 1% ethanol + 500 μl of 0.2 M carbonate buffer (pH 10.0).

Conclusions

This work has reported an optimization study for the development of an electrochemical biosensor system for the detection of

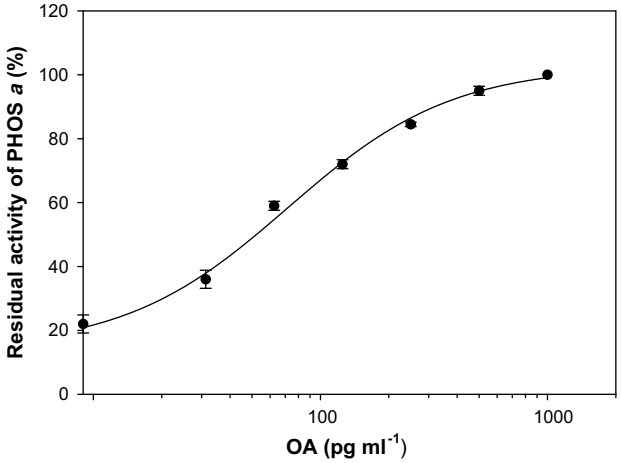


Fig. 5. Calibration curve obtained using standard solutions of OA. PP2A = 0.1 U ml^{-1}; PHOS *a* = 0.05 U ml^{-1}; glycogen = 1%; Pi = 5 mM. Total incubation time = 50 min, where OA/PP2A = 10 min + PP2A/PHOS *a* = 25 min + PHOS *a*/glycogen/Pi = 15 min. Biosensor response time = 4 min. The concentrations reported are those realized off-line in buffer A prior to the addition of the blocking solution.

OA based on the inhibition of the enzyme PP2A that is involved in a naturally occurring enzyme cascade. The proposed assay involved a preliminary phase of off-line enzymatic processes (OA/PP2A, PP2A/PHOS *a*, and PHOS *a*/glycogen + Pi) that mimic metabolic events of eukaryotic cells; this step was followed by injection of the mixture into an FIA system where a very stable bienzyme amperometric probe (GOD–AP) was inserted and used as signal transducer.

Although the optimization phase turned out to be rather laborious because it was necessary to control numerous parameters, the final assay can be considered to provide a novel and practicable method for the determination of OA. In fact, the whole procedure can be summarized in a few steps (see "OA Analysis Procedure" section for the correct optimized concentrations):

1. Add OA (standard or sample) into an Eppendorf tube containing buffer A and PP2A enzyme and wait for 10 min of incubation.
2. Add to the same Eppendorf tube the PHOS *a* enzyme and wait for 25 min.
3. Add glycogen and Pi and wait for 15 min, then block the reaction by adding carbonate buffer (pH 10.0).
4. Inject the final solution into the sample loop of the FIA system.

As can be seen, the procedure is simple and relatively fast and could allow the automation of OA analysis.

Our next work will be the analysis of DTXs and the application of the biosensor in a large number of mussel samples so as to demonstrate the suitability of the method as a screening tool for the detection of OA and its derivatives in shellfish tissue samples.

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