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Development of a new amperometric biosensor based on polyphenoloxidase and polyethersulphone membrane*

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Abstract: An amperometric biosensor based on the enzyme polyphenoloxidase (PPO), which makes the bioelectrocatalysis of phenolic compounds, was developed and optimized using cathecol as substrate. Polyethersulphone membranes were used for enzyme immobilization. Polyphenoloxidase oxidizes monophenols (cresolase activity) and diphenols (catecholase activity) into the corresponding *o*-quinones; the *o*-quinones formed in the enzymatic catalysis are then reduced back to cathecol at –200 mV (vs. Ag, AgCl) at a platinum electrode. The polyphenoloxidase immobilized was from commercial origin or extracted from mushrooms. *p*-Cresol and phenol substrates were also tested. Reproducibility, response time, linearity, sensitivity, and stability of the biosensor were studied.

INTRODUCTION

Phenolic compounds that have deleterious effects on health are found in the environment, and thus, their determination is of great importance [1].

The detection of mono- and polyphenols is usually done by HPLC and/or spectrometry. However, these techniques are expensive, reagent, and time-consuming.

Biosensors are attractive alternative techniques, due to their unique characteristics such as selectivity, the relative low cost of realization and storage, the potential of miniaturization and easy automation, and the rapid operation [2]. Numerous biosensors have been proposed for the detection of phenolic compounds based on the enzyme polyphenoloxidase [1–12].

Polyphenoloxidase (tyrosinase) is a bifunctional, copper-containing oxidase having catecholase and cresolase activity. It is responsible for browning reactions through the phylogenetic scale [13]. The enzyme has a molecular weight of 128 000 daltons. It is a tetramer containing four atoms of copper per molecule and two binding sites for aromatic compounds including phenolic substrates. There is a distinct binding site for oxygen. The optimum pH range is 6–7. PPO converts phenolic compounds into *o*-quinones [3] in the presence of molecular oxygen in two steps. In the first step, the phenolic compound is oxidized to catechol. In a second step, catechol is oxidized to *o*-quinone. The *o*-quinone can be reduced back to catechol in an electrode at an appropriate potential (cf., Fig. 1).

The stabilization of enzymes is a very important field mainly in applied enzymology. Enzymes are very prone to lose activity, and attempts to prevent that are done by stabilization strategies [14–16]. Polyhydric alcohols and carbohydrates, such as glycerol and sucrose, have the ability to increase the stability of proteins and enzymes in solution [17]. Immobilization to solid phases, and stabilizers such

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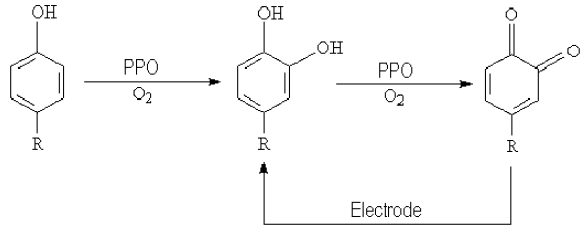

Fig. 1 Oxidation and reduction reactions occurring at an electrode based on polyphenoloxidase [3].

as gelatin, bovine serum albumin, [18], polyvinylalcohols, PAN/Coat^{TM} [19], are also some of the methods successfully used to stabilize enzymes.

MATERIALS AND METHODS

Polyphenoloxidase (622 ua/mg DW) was purchased from Worthington (38J2162W, 3792). A solution of PPO 16.7 mg/mL in a phosphate buffer of pH 7 was freshly prepared and 30 µL of this solution (0.5 mg) was immobilized on each membrane. Fresh mushrooms were bought at a local supermarket. The enzymatic extraction was made in a phosphate buffer pH 7 containing PVP-40 Sigma (109H0201) or PVP-360 (Sigma, 28F-0302) stabilizers. Glycerol was from BDH chemicals (Ref.lot.10118). Catechol was from Merck, *p*-cresol from M&B, and phenol was from Riedel-de Häen. Stock solutions from the substrates 10^{-2}mol/L in phosphate buffer pH 7 were daily prepared.

L-DOPA (Sigma) was freshly prepared in a 10 mM concentration in phosphate buffer pH 7, for the enzymatic activity determination of PPO in the enzymatic extract.

A 1 mg/mL stock BSA solution (Sigma) was freshly prepared for the determination of the calibration curve, and the total protein contained in the mushrooms extract was obtained by the Bradford [20] method. The diluted BSA solutions used in the determination of the calibration curve were made in 0.1 g of Coomasie blue G-250 (Sigma), 50 mL of ethanol, and 100 ml of H_3PO_4 (up to 1 L distilled water).

The acrylamide/Bis was from Sigma, the Tris from Riedel-de Häen, the ammonia persulfate (PSA) from Sigma (37H0572), the TEMED from Riedel-de Häen (63082, 23-72), the sodium dodecyl sulfate (SDS) 10% was from Merck (359360), and the 2-mercaptoethanol and the Coomasie blue were from Sigma.

Polyethersulphone membranes (Ultrabind, US450 0.45 µm) were from Gelman, and discs of 1.90 mm diameter were cut.

Extraction of PPO from mushrooms

Twenty-five grams of fresh mushrooms were homogenized in a liquefier with 100 mL of phosphate buffer pH 7 containing 2.5 g of PVP-360 stabilizer during 2 min. The suspension was filtered through one layer of a Whatman filter in a bückner funnel on ice and centrifuged at 3000 rpm for 30 min at 4 ºC; it was stored at this temperature in a refrigerator and utilized as the enzymatic source in the determination of the PPO activity and total protein procedures.

Measurement of the PPO activity

The activity of soluble PPO in the crude extract was determined in triplicate by measurement of the absorbance at 475 nm due to dopachrome produced in the reaction between 100 µL of supernatant solution and 3 mL of 10 mM L-DOPA solution in phosphate buffer (pH 7) at 25 ºC.

Total protein determination

The total protein concentration was determined in triplicate by a standard Bradford [20] method using bovine serum albumin as standard.

Electrophoresis

The denaturing electrophoresis was carried out using acrylamide/Bis 30% from Sigma, Tris-HCl pH 8.8 (resolving gel) or pH 6.8 (stacking gel), PSA, and TEMED, in the presence of sodium dodecyl sulfate (SDS) 10%, using a Biorad electrophoresis apparatus. The gel was loaded with 90 μg of commercial PPO, and 20 μL of a 0.045 μg/μL enzymatic extract two and three times concentrated in a buffer containing Tris-HCl pH 6.8, glycerol, SDS 10%, and 2-mercaptoethanol from Sigma, and run at 200 V during about 45 min. Then the electrophoresis gel was stained with Coomasie blue during 2 h and destained with an acetic acid/methanol solution until the proteins could be observed on the gel.

Preparation of the biosensor

Thirty microlitres of the crude extract (2.73 μg as determined in the total protein determination experiment) or 30 μL of a 16.7 mg/mL commercial PPO solution (0.5 mg) were added on several membranes of polyethersulphone with a micropipette. The enzyme was allowed to react with the membranes for 2 h at 25 ºC. The membranes were then used.

Amperometric detection

The membrane side containing the enzyme was applied on the internal membrane of the electrode stem in contact with the platinum electrode, and the biosensor was dipped in 5 mL of phosphate buffer saline (PBS). Then, a voltage of –0.200 V was applied from an amperometric biosensor detector (US), the background current was allowed to reach a steady value, and 50 μL of the substrate were added to the solution, under stirring. The transient current-time was registered until the system was stable again. The variation of the current was calculated, and this variation is proportional to the substrate concentration. In the study of the linearity of response, the same procedure was done repeating the addition of 50 μL of the substrate to the solution after stabilization of the current response.

It is a well-known fact [2] that quinones formed either by the electrochemical or enzyme reaction are very unstable in water and eventual polymers formed can foul the electrode. We found this problem when the first addition of catechol was done. The problem was circumvented by discarding the first readings. The second ones were reproducible, and the enzyme and electrochemical reactions would cycle the redox reactions, as shown on Fig. 1.

Stabilization studies

Stabilization was studied under the following conditions: membranes with immobilized PPO stored dry at room temperature and at 4 ºC, in glycerol 30% (v/v) at room temperature and at 4 ºC.

RESULTS AND DISCUSSION

Commercial samples and enzyme extracted from mushrooms by us, were used in the preparation of biosensors.

Parameters such as reproducibility, linearity range, sensitivity, and limit of detection were studied. Glycerol was tested for stabilization purposes.

Reproducibility of the biosensor preparation

Reproducibility studies were done in sets of five membranes prepared under the same conditions, as described in the "preparation of the biosensor" section. Biosensors prepared with the enzyme containing membranes were dipped in 5 mL of PBS, and the steady current obtained after injection of 50 μL of 10^{-2} or 10^{-3} mol/dm^{-3} catechol, *p*-cresol or phenol in PBS was recorded. Catechol, being the study substrate, was repeated several times, and 5 different assays done on different days are reported. Values obtained are summarized in Table 1.

Responses of the order of 2 min were found for 10^{-5} mol/L catechol solution. Responses were longer for phenol, indicating that a different mechanism of response was taking place. A lag time in the monophenolase activity of enzymes from several sources using monophenols have also been observed by other authors [18].

Table 1 Steady-state currents (average of five membranes) and corresponding standard deviations obtained with the amperometric biosensor based on commercial PPO.

	I/nA		
Conc./mol dm^{-3}	Catechol	*p*-Cresol	Phenol
10^{-5}	–0.92 ± 0.046	–0.67 ± 0.058	–
10^{-4}	–1.90 ± 0.100	–	–0.89 ± 0.113
	–2.06 ± 0.247		
	–2.08 ± 0.368		
	–2.21 ± 0.430		
	–2.22 ± 0.280		

Linearity

Linearity studies were performed under two different circumstances. In one set of experiments, subsequent linearity studies were done on three separate membranes of the same batch, on the same conditions. On the other set, the internal membrane was renewed every time an external membrane was substituted. Similarly to what was done for the reproducibility studies, 50 μL of 10^{-3} mol/L catechol were added to 5 mL of PBS, and the addition was repeated after stabilization of the current response.

Figures 2 and 3 show the results obtained.

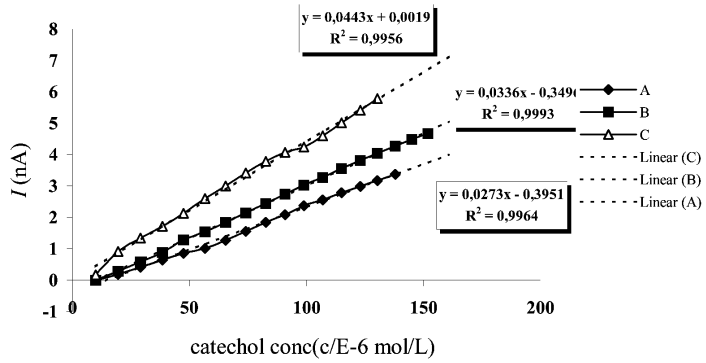

Fig. 2 Linearity study of the amperometric biosensor, without substitution of the internal membrane.

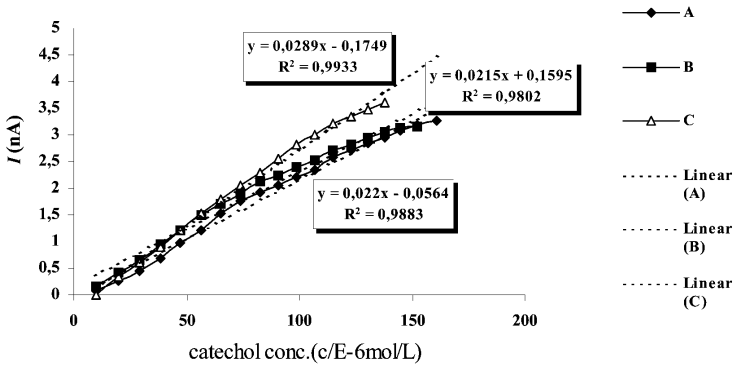

Fig. 3 Linearity study of the amperometric biosensor, with substitution of the internal membrane.

The response was linear in the range 9.09×10^{-6}–138×10^{-6} mol/L for membrane A with sensitivity 2.73×10^{-5} AxL/mol, when no substitution of the internal membrane was done. Sensitivities were found to increase for subsequent experiments. The substitution of the internal membrane resulted in sensitivities not varying much from membrane to membrane. It seems, thus, that the products formed accumulate on the internal membrane, activating the enzyme.

Extracts from mushrooms

In order to make the biosensor more available than the one depending on commercial enzymes, we prepared extracts from mushrooms obtained from a local supermarket. A simple method of extraction was used, in order to turn the procedure as easy as possible. The extracts prepared were immobilized on the same lot of membranes as the commercial enzyme, by the same procedure. Those that were used on the biosensor on the same day as extracted yielded answers to catechol of the order of 1.5 nA for 10^{-6} mol/L catechol in one case, whereas another sample gave the same magnitude of current when the concentration was 10^{-5} mol/L catechol. As expected, the response was different for different sources of mushrooms. The responses sharply decreased after a couple of days of storage at 4 ºC. An SDS-PAGE gel electrophoresis run on the extracts and on the commercial enzyme showed the presence of proteins, probably proteases, that might have caused the rapid decrease in activity of the PPO. It was found that our enzyme extracts gave a better limit of detection than the commercial sample used, when the extracts were used on the biosensor on the same day as prepared. However, reproducibilities and life time were better for the commercial enzyme, and parameters such as linearity and stabilization effects were studied with the commercial enzyme.

Enzyme stabilization

It is reckoned that "a general problem for many biosensors is the lack of the necessary operational and storage stability needed for commercial exploitation, and is currently a major obstacle to solve in the biosensor area" [2].

Our experiments involved the storage of membranes done at room temperature and at 4 ºC either dry or in 30% (v/v) glycerol. The enzyme lost activity, with no amperometric answer being obtained, after two days, when the membranes were kept dry at room temperature. Membranes stored dry, at 4 ºC, kept the activity (1.05 relative activity) after 37 days. Membranes stored in 30% (v/v) glycerol kept the activity, at room temperature, for 9 days. Relative activities, as obtained by the amperometric response ratio are shown on Fig. 4.

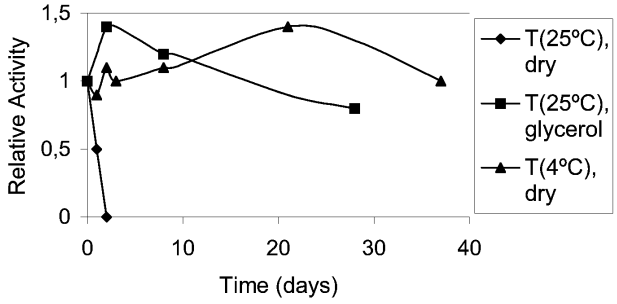

Fig. 4 Relative activity of the biosensor as a function of time (days) for membranes stored dry at 4 ºC, dry at room temperature and in glycerol 30% at room temperature (25 ºC). Each point is the average of 5 membranes. Catechol 10^{-4} mol/L was the substrate used. Internal membranes were substituted.

It was observed that the immobilization on the polyethersulphone membranes, such as used in the present work, was effective in keeping the activity of the enzyme, when stored at 4 ºC, for more than 30 days (up to 37 days were measured). Storage at room temperature failed to maintain the activity of the enzyme for the membranes without stabilizer. Stabilization for 9 days, at room temperature was successful, using glycerol 30% as stabilizer.

CONCLUSIONS

An amperometric biosensor based on an easy and stable immobilization procedure was developed. A derivatized polyethersulphone membrane provided the solid support and immobilization ability for the polyphenoloxidase enzyme used. Both a commercial source and extracts from mushrooms obtained by us were used as the biological selective element of the biosensor. Catechol was the study substrate used, although *p*-cresol and phenol were also detected. The bioelectrochemical reaction of catechol and *p*-cresol was rapid and reproducible. Reproducibilities ca. 5% were obtained for catechol (-1.90 ± 0.100 nA for a solution 10^{-4} mol/L catechol). An easy and rapid method of extraction of the enzyme was found to give rise to a quick way of preparing a biosensor, although the enzyme lost 46% of its activity in 4 days. A linearity range of 9.09×10^{-6} mol/dm^3 -1.38×10^{-4} mol/dm^3 in catechol was obtained for one membrane, the span being of the same order of magnitude for the other membranes. This is of similar order of magnitude than reported in [3] and larger than the values reported by others [12]. The biosensor kept its activity for 37 days when the membranes where stored dry, at 4º. This remarkable stabilization effect of the membrane we used, is larger than other ones reported [1–2,12]. Storage in 30% glycerol, at room temperature, maintained the activity of the biosensor for 9 days, which makes it possible to use the biosensor in circumstances where storage at room temperature is required.

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