# MUSHROOM TYROSINASE-MODIFIED CARBON PASTE ELECTRODE AS AN AMPEROMETRIC BIOSENSOR FOR PHENOLS

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A biosensor for a variety of substances could be readily prepared by the adsorption of partially purified mushroom tyrosinase at a carbon paste electrode, producing an enzyme layer which was protected with a dialysis membrane. The electrode poised at −100 mV (vs an Ag/AgCl reference electrode) functions on the basis of a reversible electrochemical reduction of the *o*-quinones formed from phenols in the tyrosinase reaction. The response times were 40 s for *o*-diphenols and 2 min for monophenols; the reproducibility for 10 consecutive assays of 3 μmol $l^{-1}$ phenol solutions was better than 4%. The detection limit was 10 nmol $l^{-1}$ (catechol as substrate) and the steady state responses were linear up to a concentration of 200 μmol $l^{-1}$. The optimum pH values were 6·0 for phenol and 6·5 for catechol. The shapes of the pH-response curves indicated the presence of at least two tyrosinase isoenzymes with different pH optima.

The determination of phenols in waste and surface waters is important in environmental protection. This analysis can be usefully carried out using amperometric biosensors, whose functioning is based on the use of the biological recognition component, mushroom tyrosinase (EC.1.14.18.1). The first types of these biosensors were based on the Clark oxygen electrode and an enzyme membrane containing tyrosinase cross-linked with glutaraldehyde[1]. They were followed by various types of bioelectrodes based on tissue sections from beet roots[2], champignons[3], bananas[4] and other nontraditional sources[5].

Research is now being carried out on sensors with simpler designs based on various types of modified carbon electrodes. A graphite electrode has been described for the determination of cresol, working in organic solvents[6], along with several types of paste and composite electrodes[7,8]. The chemically modified electrodes include a paste electrode with hexacyanoferrate bonded to poly(4-vinylpyridine)[9] and a graphite electrode modified with tetracyanoquinodimethane[10].

This work describes a simple design for a phenol enzyme electrode with a wide linear response range, based on the electrochemical reduction of the quinoid products of the tyrosinase oxidation of phenols.

## EXPERIMENTAL

*Materials and methods*: Tyrosinase from champignons purified to degree 6 according to Kertesz and Zito[11], specific activity 830 ncat $mg^{-1}$, was prepared in our department. L-Tyrosine was the product of Calbiochem (U.S.A.), the other chemicals were of p.a. purity and were the products of Lachema (Brno). Work was carried out using water deionized on the Milli-Q apparatus (Millipore, U.S.A.).

*Preparation of enzyme electrodes*: Four types of carbon electrode were prepared. Spectrographic graphite rods (1 cm long) or glassy carbon rods were fixed in glass tubes with suitable diameter using an adhesive and the conductor lead was attached to the rod using a suitable conductive adhesive. The electrode working areas were 29 $mm^2$ for graphite and 13 $mm^2$ for glassy carbon.

In the preparation of the carbon paste electrodes (CPE), the conductor lead was attached to the glass tube so that a 2 mm deep free space remained between the uninsulated end of the conductor and the edge of the tube; this space was filled with carbon paste. The carbon paste was obtained by thorough mixing either of graphite powder and paraffin oil in a ratio of 1 : 0·5 (w/w) or of conductive carbon black and mineral oil in a ratio of 1 : 0·8. In both cases, the working area was 33 $mm^2$.

The surface of the paste electrode was smoothed using a smooth paper brush; the graphite and glassy carbon electrodes were polished with fine emery paper and rinsed with water. Then 10 µl of 50 mmol $l^{-1}$ of phosphate buffer, pH 7·3, containing 100 ncat tyrosinase was transferred to the electrode (only half as much for glassy carbon). The enzyme layer was then covered with a dialysis membrane fixed with an O-ring.

*Apparatus*: The amperometric measurements were carried out in a three-electrode arrangement. The reference electrode was an Ag/AgCl electrode and the auxiliary electrode was a Pt wire. The potentiostat was the amperometric detector ADLC 2, with a TZ 4200 recorder (both from Laboratorní přístroje, Prague) with a digital voltmeter. The cyclic voltammograms were measured on an OH 105 polarograph (Radelkis, Hungary).

## RESULTS

### *Comparison of Various Types of Enzyme Electrodes*

The sensitivity and response times were compared for four experimental enzyme electrodes. Measurements were carried out in 50 mmol $l^{-1}$ phosphate buffer at pH 7·3 and 30°C, with a working electrode potential of −200 mV. The sensitivity was determined as the slope of the calibration curve ($dI/dc$) for pyrocatechol, for a series of 5 additions in the concentration range 5−250 µmol $l^{-1}$; correction was made for the electrode active surface area. The velocity of the sensor response was characterized by parameter $\tau_{95}$, corresponding to the time required to attain 95% of the total current value at steady state. It can be seen from Table I that the best results were obtained using the paste electrode containing conductive carbon black. Thus, this type of electrode was used in the subsequent investigations.

### *Cyclic Voltammetry*

The electrochemical parameters of the biosensor were studied using two basic tyro-

sinase substrates – pyrocatechol and phenol. The cyclic voltammograms of these substances were measured both at a tyrosinase-modified CPE and also at a CPE without the enzyme layer (Fig. 1). Under the given conditions (50 mmol $l^{-1}$ phosphate, pH 7·3, substrate concentration 250 μmol $l^{-1}$, 30°C, scan rate 8·3 mV $s^{-1}$), the voltammograms of phenol and pyrocatechol at the enzyme electrode are practically identical, while the voltammetric curves of these two substances differ at the unmodified CPE. When the voltammograms are measured in a solution freed of

TABLE I

Parameters of biosensors based on various types of carbon electrodes[a]

| Type of electrode | Sensitivity[b] μA $mol^{-1}$ l $mm^{-2}$ | $\tau_{95}$ s |
|---|---|---|
| CPE/conductive carbon black | 15·5 | 40 |
| CPE/graphite powder | 3·09 | 50 |
| Graphite rod | 13·6 | 130 |
| Glassy carbon rod | 0·215 | 300 |

[a] Measuring conditions: 50 mmol $l^{-1}$ phosphate, pH 7·3, temperature 30°C, working electrode potential −200 mV vs Ag/AgCl reference electrode. [b] Slope of the calibration curve for pyrocatechol in the concentration range 5 to 250 μmol $l^{-1}$.

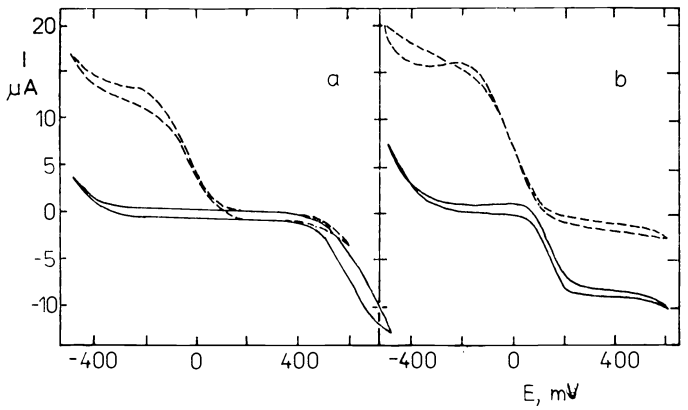



FIG. 1

Cyclic voltammograms for the unmodified CPE (———) and tyrosinase-modified CPE (— — —) in the presence of 250 μmol $l^{-1}$ phenol (*a*) or pyrocatechol (*b*). Measured in 50 mmol $l^{-1}$ phosphate, pH 7·3, 30°C, three-electrode arrangement (Ag/AgCl reference electrode and Pt wire auxiliary electrode), scan rate 8·3 mV $s^{-1}$

oxygen by bubbling for 20 min with nitrogen, the voltammograms at the enzyme electrode approach the shape of those at the unmodified CPE.

*Optimization of Operating Conditions*

Figure 2 depicts the dependence of the sensitivity of the tyrosinase-modified CPE on the working electrode potential (in 50 mmol $l^{-1}$ phosphate, pH 7·3, 30°C, pyrocatechol as substrate). A potential of −100 mV (vs the Ag/AgCl reference electrode) was selected as optimum. A slight increase in the sensitivity can be attained at more negative potentials but is accompanied by a large increase in the background (3·5 nA at −100 mV, 8·6 nA at −200 mV and 25·2 nA at −300 mV).

The dependence of the sensor sensitivity on the pH (Fig. 3) has a different shape for phenol and for pyrocatechol, with pH optima for their determination at 6·0 and 6·5, respectively. The ionic strength of the medium does not greatly affect the sensor functioning for 50 to 500 mmol $l^{-1}$ phosphate buffer, pH 7·3 (sensitivity from 0·301 to 0·240 A $mol^{-1}$ l); a marked decrease occurs only at higher buffer concentrations (for 1 mol $l^{-1}$ phosphate, the sensitivity is only 0·126 A $mol^{-1}$ l). The temperature dependence is negligible in the range 15 to 30°C (from 0·256 to 0·265 A $mol^{-1}$ l). The maximum sensitivity is attained at about 38°C (0·332 A $mol^{-1}$ l), where $\tau_{95}$ is decreased to 34 s, but the sensor lifetime is decreased. A further increase in the temperature leads to rapid loss of enzyme activity. The sensor was always washed

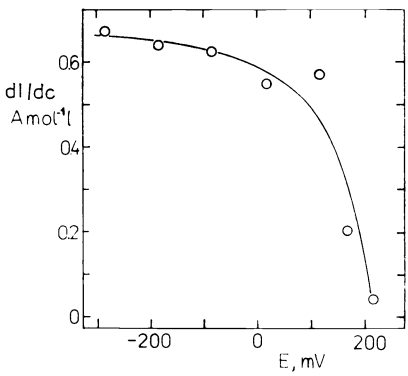



FIG. 2

Dependence of the sensitivity of the tyrosinase-modified CPE on the working electrode potential. Measuring conditions: 50 mmol $l^{-1}$ phosphate, pH 7·3, 30°C. Potential referred to Ag/AgCl reference electrode

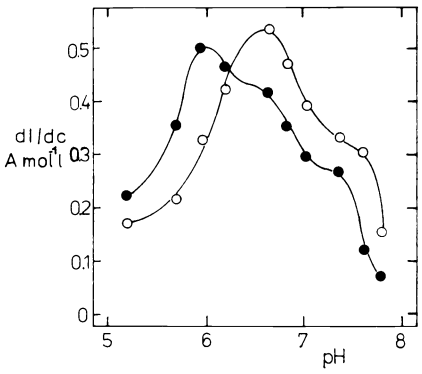



FIG. 3

Dependence of the sensitivity of the tyrosinase-modified CPE on the pH for phenol (●) and pyrocatechol (○). Measured in 50 mmol $l^{-1}$ phosphate, pH determined at the end of the determination. Potential −100 mV vs Ag/AgCl reference electrode

with water, dried and stored at 4°C after use. When not used, the sensitivity after 5 months is equal to 85% of the original value. No change was observed in the sensitivity during the first 30 measurements; however, recalibration is then necessary. More than 150 determinations can be carried out with a single electrode.

*Sensor Response for Various Phenolic Substrates*

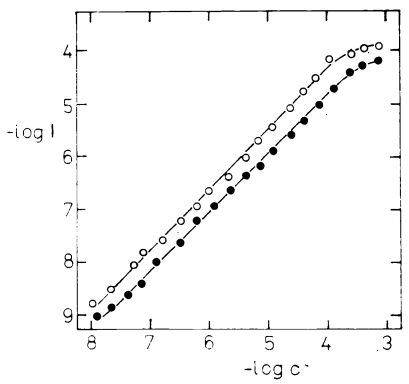
The response of the sensor to phenol and pyrocatechol under optimum conditions (50 mmol $l^{-1}$ phosphate, pH 6·0 for phenol and 6·5 for pyrocatechol, 30°C, working potential −100 mV) yields a linear calibration curve over four concentration orders (Fig. 4). The depicted dependences of log $I$ on log $c$ have a slope very close to 1 (the ideal case). The lowest concentrations that can be detected using this sensor are 10 nmol $l^{-1}$ for pyrocatechol and 13 nmol $l^{-1}$ for phenol. The upper limit for linearity of the calibration curves for both substrates is 200 μmol $l^{-1}$ (limited by the

TABLE II
Sensitivity of CPE modified with tyrosinase for various phenolic substrates

| Substrate | Sensitivity A $mol^{-1}$ l | Substrate | Sensitivity A $mol^{-1}$ l |
|---|---|---|---|
| Phenol | 0·475 | Pyrogallol | 0·0256 |
| 4-Cresol | 0·265 | L-Tyrosine | 0·00447 |
| 2-Cresol | 0 | Tyramine | 0·0106 |
| 4-Nitrophenol | 0 | 3,4-Dihydroxyphenylalanine | 0·00872 |
| Catechol | 0·525 | Gallic acid | 0 |
| Hydroquinone | 0·00621 | Chlorogenic acid | 0·0134 |

FIG. 4

Response of the tyrosinase-modified CPE to phenol (●) and pyrocatechol (○) given as the dependence of the logarithm of the sensor current at steady state ($I$, A) on the logarithm of the concentration ($c$, mol $l^{-1}$). Measuring conditions: 50 mmol $l^{-1}$ phosphate, pH 6·0 (phenol) or 6·5 (pyrocatechol), 30°C, measured at a potential of −100 mV vs Ag/AgCl reference electrode. The slope of the log $I$ vs log $c$ plot for phenol equals 1·06 (correlation coefficient $r = 0{\cdot}986$), for pyrocatechol, 1·13 ($r = 0{\cdot}999$)



solubility of oxygen in the working medium, equal to 221 μmol $l^{-1}$). The sensor sensitivity is equal to 0·52 A $mol^{-1}$ l for pyrocatechol and 0·47 A $mol^{-1}$ l for phenol. The relative standard deviation for a series of 10 determinations of 3 μmol . . $l^{-1}$ phenol is 4%, for pyrocatechol under the same conditions, 3%.

The senssor can be used to determine various phenolic substrates (Table II). They can be divided into two groups – monophenols and substances containing two hydroxyls in the *o*-position. These groups have different response times of the sensor; for the first, $\tau_{95}$ is equal to about 2 min and for the second, only 40 s.

## DISCUSSION

The main criterion for evaluation of the determination of traces of phenols in drinking water is the lowest possible detection limit. (The highest permissible concentration according to the Czechoslovak standard ČSN 83 061 is 0·05 mg $l^{-1}$, corresponding to 0·53 μmol $l^{-1}$).

When using sensors based on measuring a decrease in the oxygen concentration, the greatest limitation arises from the necessity to determine very small differences in the large initial current value corresponding to an almost saturated solution of air. Consequently, the detection limit attainable with this type of sensor without preconcentration is greater than 1 μmol $l^{-1}$ (ref.[1]). An increase in the sensitivity of up to five-fold can be attained by recycling the quinones formed in the enzyme reaction in the sensor membrane to phenols using hydrazine[12].

Another approach involves the determination of phenols after extraction into an organic phase. A simple sensor has been designed for measurements in chloroform, with a detection limit of 1 μmol $l^{-1}$ (ref.[6]). The functioning of this sensor is based on the cathodic reduction of *o*-quinones, the products of the oxidation of phenols by tyrosinase.

Measurements in flowing solutions, where the quinones were reduced by hexacyanoferrite bonded to poly(4-vinylpyridine) in carbon paste, permitted the determination of down to 14 ppb (150 nmol $l^{-1}$) of phenol[9]. This sensor had the disadvantage of low current response – only 733 nA $mol^{-1}$ l. Another sensor was based on the discovery that, in the presence of phenols, the tetracyanoquinodimethane ion can function as a reduction equivalent donor in the oxidation of phenol by tyrosinase; the oxidized mediator is then again reduced cathodically[10]. The detection limit was 230 nmol $l^{-1}$ with high current responses of up to 2 A $mol^{-1}$ l.

The sensor described here has a detection limit of up to an order lower – 10 nmol . . $l^{-1}$. It is necessary in the measurement of such low concentrations to wait about 5–10 min for stabilization of the current background; however, the time between measurements at concentrations above 100 nmol $l^{-1}$ is less than 2 min.

It can be seen (Fig. 1) that, in spite of the simple design, tyrosinase is capable of converting high concentrations of substrate (250 μmol $l^{-1}$) completely to *o*-quinone

in the enzyme layer. This is then electrochemically reduced at the CPE. Thus, the formation of products of polymerization of *o*-quinones is curtailed; these products would decrease the lifetime of the enzyme layer.

The sensor can be employed to determine a number of phenolic substances (Table II); the sensitivities for phenol and pyrocatechol are almost the same. This property is useful when the sensor is used as a phenol detector in liquid chromatography[7], but can lead to difficulties in the analysis of unknown samples. Thus the occurrence of tyrosinase in various mushrooms, plants and insects was screened, yielding enzymes with much lower affinity for phenol[8].

It can be seen from the different shapes of the pH dependence of the response of the sensor for pyrocatechol and phenol (Fig. 2) that the tyrosinase obtained from champignons contains two to three isoenzymes with different affinity for the substrates. Thus, attempts could be made to improve the selectivity of the detector by purification of homogeneous isoenzymes.

The ability of the sensor to work in a wide concentration range of phenols (Fig. 4) is important. It is preferable to construct the calibration curve over only two concentration orders, according to the expected phenol content in the sample. The sensor stability during storage is satisfactory; however, the preparation is fast and simple and it is preferable to store the CPE's and prepare the biosensor immediately prior to the measurement (preparation time, 5 min). The sensor can be used immediately after being prepared.

Applications include the sensitivity determination of phenols in water, the determination of inhibitors of tyrosinase similarly as in ref.[12] and determination of the activity of some hydrolytic enzymes using various phenol conjugates[13].

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