ENZYME ELECTRODE WITH IMMOBILIZED POLYPHENOL OXIDASE FOR DETERMINATION OF PHENOLIC SUBSTRATES

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An active enzyme membrane has been prepared by insolubilization of a mixture of potato or champignon polyphenol oxidase and an inert protein by means of glutaraldehyde on the surface of polyamide netting. After the enzyme membrane had been stretched over the hydrophobic membrane of a Clark oxygen cell, a sensor was obtained which can monitor phenolic substrates according to the oxygen uptake in the enzymic reaction. The pH-dependence of the electrode response and the effect of various amounts of the enzyme or serum albumin on the membrane lifetime were studied. The polyphenol oxidase electrode can be employed for a rapid determination of *p*-cresol, phenol, pyrocatechol, and pyrogallol in quantities of 20–200 nmol per 3 ml of reaction mixture. Dihydroxyphenylalanine, tyrosine, and chlorogenic acid are bad substrates for both immobilized polyphenol oxidases. The membranes with the immobilized mushroom enzyme were used for a simple detection of phenols in waste water.

Electrodes with immobilized enzymes can selectively monitor specific metabolites. After Updike and Hicks[1] had constructed the basic functional model for determination of glucose and introduced the term of enzyme electrode, several types of these electrodes were described[2–4] and theoretical analyses of their function were also performed[4–6]. The most widespread application has received the original sensor for oxidase substrates consisting of a Clark oxygen cell whose outer surface has been coated with a thin layer of enzyme immobilized by physical or chemical methods. The decrease of oxygen concentration in the enzyme membrane permits the substrate uptake during the enzymic reaction to be easily determined electrochemically. This analytical technique has been employed as yet for the determination of substrates of glucose oxidase, L-amino acid oxidase, diamine oxidase, uricase, and alcohol oxidase.

In this study a biochemical sensor with insolubilized champignon or potato polyphenol oxidase (*o*-diphenol oxidase, EC 1.14.18.1) was used for rapid and technically simple determination of phenolic substrates by measurement of uptake of oxygen as cosubstrate of the enzymic reaction.

EXPERIMENTAL

Material. All chemicals were of analytical purity. Aqueous 25% glutaraldehyde solution was from Merck-Schuchardt (FRG). Bovine serum albumin was a preparation of Mann Res. Laboratories, U.S.A. Commercial polyamide netting (silon, 25 mesh/mm^2) served as a matrix for immobilization of polyphenol oxidase. Waste water containing phenol was obtained from the VŽKG coke plant, Ostrava-Vítkovice. Potato polyphenol oxidase, specific activity 9·0 U per mg of protein was isolated according to Balasingam and Ferdinand[7] with the exception that the final chromatography was effected on a Sephadex G-150 column. Polyphenol oxidase from commercial champignons (Agaricus bisporus) was purified to degree 6 according to Kertesz and Zito[8] (specific activity 46·5 U per mg of protein). Both enzymes were osmotically concentrated before use by polyethylene glycol (Aquacid III) in dialyzing tubing to a value of 902 U/ml (enzyme from potatoes) or 1075 U/ml (enzyme from champignons).

The activity of soluble polyphenol oxidase was measured at 30°C by an oxygen electrode in terms of initial rate of oxygen uptake in 3 ml of reaction mixture containing 0·1M potassium phosphate buffer, pH 7·0, and 20 mM pyrocatechol as substrate. The reaction mixture was saturated with air. One enzyme unit (U) is defined as the quantity of enzyme which catalyzes the uptake of 1 μgatom of oxygen per min. The activity of the enzyme membranes was determined in an analogous manner: the active layer was cut to pieces which were subsequently suspended in the reaction mixture with intensive magnetic stirring. The measurements were made on two types of analyzers of dissolved oxygen. The first one was an instrument manufactured by the Instrument Development Workshops, Czechoslovak Academy of Sciences (electrode system Au—Ag/AgCl, polypropylene membrane), the second one was Model 52 of Yellow Springs Instruments, Ohio (Pt—Ag electrode, teflon membrane). The cathode of the oxygen electrode was polarized by a constant potential of −0·7 V. Scale range 250 mm, chart speed 1 cm/min.

Preparation of electrode. Type A: The active artificial membrane was prepared as described earlier[6] for the system diamine oxidase-polyamines, *i.e.* by insolubilization of the enzyme by glutaraldehyde on polyamide netting which ensures good mechanical stability of the membrane. A circular area of about 25 mm^2 was outlined on the polyamide netting by means of molten paraffin. Both sides of this area were coated with a mixture of 6 μl of 10% bovine serum albumin, 4 μl of concentrated polyphenol oxidase solution, and 4 μl of 1% glutaraldehyde. The membranes were allowed to dry in a refrigerator (3 h, 4°C) and stored in 0·1M potassium phosphate buffer, pH 7·0, at +2°C. Before use the enzyme membrane was stretched over the outer surface of the hydrophobic membrane of the oxygen electrode and fixed by a rubber ring.

Type B: A mixture of 25 mg of bovine serum albumin, 2 ml of polyphenol oxidase (60 U), and 0·1 ml of 25% glutaraldehyde in 2 ml of 0·1M potassium phosphate buffer, pH 7·0, was rapidly frozen in a mixture of dry ice and acetone (−30°C), transferred to a refrigerator, and allowed to thaw slowly at 4°C. The spongy copolymer was filtered off by suction, washed with 0·1M phosphate, pH 7·0, disintegrated mechanically, and stored in the refrigerator until used. A small portion of the product was applied to the surface of the hydrophobic membrane of the oxygen cell, coated by thick polyamide netting, and fixed by a rubber ring. The electrode was stored at 4°C in 0·1M potassium phosphate buffer, pH 7·0.

Determination of concentration of phenolic substrates. The oxygen electrode with the attached enzyme membrane was placed in a thermostated (30°C) reaction vessel containing 3 ml of 0·1M buffer saturated with air. After the electrode current became constant increasing volumes (5—25 μl) of 10 mM substrate solution were added at 1-min intervals (magnetic stirring); the unknown sample was added between the measurements. Each addition of substrate manifested itself on the record by a wave indicating the decrease of the electrode current; the height of this

wave was read at the site of the dividing point[6]. The concentration of the unknown sample was determined with the aid of a calibrating curve expressing the dependence of the height of electrode response (in mm) on the quantity of the phenolic standard used for the individual additions.

RESULTS

Response of Polyphenol Oxidase Electrode

In the course of amperometric measurement of substrate oxidation oxygen is the only component of the reaction mixture which penetrates through the hydrophobic barrier of the oxygen cell to the cathode where it undergoes a four-electron reduction at a potential of $-0{\cdot}7$ V:

$$O_2 + 2\,H_2O + 4\,e^- \rightarrow 4\,OH^-.$$

The oxygen uptake during the enzymic reaction manifests itself by a continuous decrease of the electrode current. If the outer surface of the oxygen electrode is coated with an enzyme membrane, then the electrode response measured corresponds to the decrease of oxygen concentration in the enzyme membrane while an insubstantial decrease of oxygen and hence also of substrate takes place in the surrounding solution. We have obtained proof showing that at an electrode response corresponding to 90% of the scale range the original oxygen concentration in the reaction mixture dropped by 6–7% only. The electrode can be usually used even at such substrate concentrations at which almost all of the oxygen diffusing into the membrane is taken up.

Since the oxygen uptake depends on the diffusion flow of substrate into the membrane, the electrode current, which rapidly decreases at the beginning, levels off during 1–2 min at a value corresponding to the given substrate concentration. Therefore each substrate addition manifests itself on the record by a wave from which can be read the initial change in electrode current (rate method) and the wave height of steady state response at the site of dividing point[6] (steady-state method). The values of both parameters are proportional to the concentration of the phenolic substrate added to the reaction mixture. It is, however, more convenient to evaluate the wave height since it is difficult to read accurately the slope of steep lines characterizing the initial phases of electrode response at high substrate concentrations. When the substrate concentration is fixed then the wave height (sensitivity) decreases with the gradual loss of activity of the enzyme membrane; this can be eliminated by careful calibration.

The dependence of electrode response on the pH of the reaction mixture was investigated with seven substrates in 0·1M potassium phosphate and acetate buffers. The effect of concentration of hydrogen ions on the magnitude of electrode response was very little marked with most substrates in the pH-range 5·0–8·5 commonly

used; a slight indication of pH-optimum at 7·0 was found in the case of pyrocatechol. The measurements were carried out therefore mostly at pH 6·5–7·0 with the exception of chlorogenic acid which yielded a more pronounced response in the acid pH-range.

TABLE I

Effect of Albumin on Lifetime of Polyphenol Oxidase Membrane

The membrane was prepared by drying a mixture of 4 µl (3·6 U) of potato polyphenol oxidase, 2–6 µl of 10% bovine serum albumin, and 4 µl of 1% glutaraldehyde on an area of 25 mm^2.

Albumin content mg	Electrode response to addition of 0·2 µmol of pyrocatechol		Number of additions of reproducible wave weight
	initial rate mm/s	wave height mm	
0	5·4	44·1 ± 3·1	15
0·2	4·7	47·1 ± 6·6	28
0·4	3·8	43·6 ± 3·2	70
0·6	3·3	42·8 ± 3·0	84

TABLE II

Effect of Quantity of Components of Polyphenol Oxidase Membrane on Its Lifetime and Electrode Response

The membrane was prepared by insolubilization of champignon polyphenol oxidase (1 U/µl) on an area of 25 mm^2.

Polyphenol oxidase, µl (1% glutaraldehyd, µl)	Electrode response to addition of 0·2 µmol of pyrocatechol		Number of additions of reproducible wave height
	initial rate mm/s	wave height mm	
2 (2)	2·2	27·0 ± 0·4	3
2 (2)[a]	1·6	30·0 ± 1·5	11
4 (4)	2·4	23·6 ± 1·2	9
4 (4)[a]	1·8	38·8 ± 1·9	90
6 (6)	2·8	23·6 ± 1·2	17
6 (6)[a]	2·4	39·7 ± 2·4	48

[a] Albumin, 0·6 mg.

Activity and Lifetime of Membranes

The membranes prepared by immobilization of purified champignon or potato polyphenol oxidase only loose their enzyme activity relatively quickly upon repeated use. Their lifetime depends on the quantity of enzyme incorporated and becomes considerably protracted if an inert protein has been added during the insolubilization by a cross-linking reagent (Table I and II). The direct measurement of the activity of the membrane cut to pieces showed that it retains in active state 4–5% only of the enzyme originally applied. This is most likely an orienting value only which may be affected by specific properties of the membrane as regards the diffusion of the substrate.

A high enzyme concentration can be obtained by mechanical attachment of the copolymer prepared beforehand to the oxygen electrode (Type B). This two-step preparation, however, does not guarantee a homogeneity of the active layer whose reproducible activity can be achieved only with difficulties. An advantage of the oxygen electrode of type B is that it permits the determination of even such substrates of membrane-attached polyphenol oxidase which are difficult to oxidize (see below). To obtain well reproducible analytical results membranes prepared by immobilization of a mixture of the enzyme and bovine serum albumin directly on the polyamide matrix (Type A) were used exclusively in this study. The first measurement with a fresh membrane showed as a rule a worse electrode response than the subsequent measurements which were well reproducible. The variation coefficient calculated from, *e.g.* five subsequent determinations of 0·2 μmol of pyrocatechol was 4·9–5·5%. The highest number – up to 90 – of reproducible values obtained with one membrane was obtained with a membrane prepared by insolubilization of a mixture of 4 μl (3·6–4 U) of enzyme and 0·6 mg of albumin by 4 μl of 1% glutaraldehyde (Table I and II). As soon as the electrode response to the same addition of substrate started decreasing or, alternatively, as soon as the calibration curve was not linear enough, it was necessary to replace the membrane.

Freshly prepared membranes can be stored for long periods at +2°C or frozen in 0·1M phosphate buffer at pH 7·0. In our experiments the steady-state electrode response decreased after 1-month storage at +2°C of membranes containing potato polyphenol oxidase by 6–10% as judged by *p*-cresol, pyrocatechol, pyrogallol, and dihydroxyphenylalanine as substrates.

Effect of Concentration of Enzyme and Substrate

If a small quantity (about 1 U) of polyphenol oxidase is used for the preparation of enzyme membrane, the electrode response to a repeated addition of the same quantity of phenolic substrate to the the reaction mixture gradually decreases.

By plotting the total decrease of electrode current from the original value down to the corresponding wave versus the corresponding final substrate concentration

in the reaction mixture, a hyperbolic profile reminding of the Michaelis–Menten saturation curve was obtained. The double reciprocal plot of this dependence gave a straight line (Fig. 1). It was observed that the substrate concentration, read at the intersection of this line with the abscissa did not differ substantially from the value of the apparent Michaelis constant found with the free enzyme at fixed oxygen concentration in the reaction mixture (saturated with air). Thus. *e.g.* when pyrocatechol

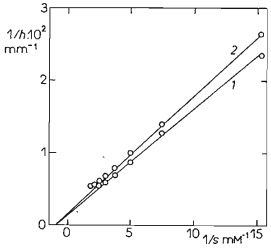


FIG. 1

Double Reciprocal Plot of Steady-State Electrode Response (h) *versus* Pyrocatechol Concentration (*s*)

The measurements were made with a little active membrane containing immobilized champignon polyphenol oxidase (1 U) at 30°C in 0·1M potassium phosphate buffer, pH 7·0. See text for details *1* second measurement, *2* third measurement with the same membrane.

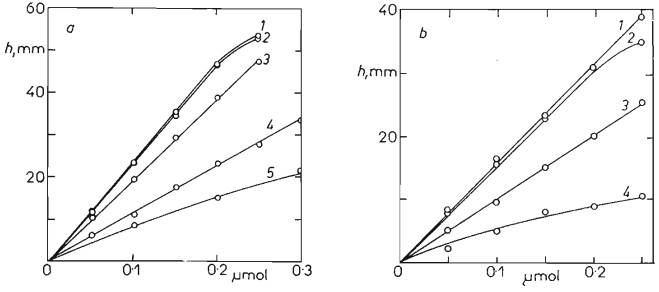


FIG. 2

Calibration Curves for Various Polyphenol Oxidase Substrates and Initial pO_2 150 mm Hg

The diagram shows a plot of wave height of electrode response in steady state (h) *versus* quantity of substrate added to 3 ml of 0·1M potassium phosphate buffer, pH 7·0—7·1 at 30°C; *a* membrane with champignon polyphenol oxidase (4 U); *1* *p*-cresol, *2* phenol, *3* pyrocatechol, *4* pyrogallol, *5* dihydroxyphenylalanine; *b* membrane with potato polyphenol oxidase (3·6 U); *1* pyrocatechol, *2* *p*-cresol, *3* pyrogallol, *4* dihydroxyphenylalanine. Pyrogallol was determined at pH 6·5.

was used as substrate a value of 1·0 mM was found with membrane bound potato polyphenol oxidase (Fig. 1) and a K_m-value of 2·9 mM with free potato polyphenol oxidase in 0·1M potassium phosphate buffer at pH 7·0 and 30°C. When the measurements with the same membrane were repeated the slope of the line increased (Fig. 1) as a result of the decrease of membrane activity (decrease of V_{max}).

The response of the enzyme electrode prepared from a membrane into which 3·6–4 U of polyphenol oxidase has been incorporated is a simple function of concentration of phenolic substrate in solution provided that this concentration is low with respect to the corresponding K_m-value. The kinetics of the enzyme reaction in the membrane approaches a first order reaction under these conditions. With most substrates which are readily oxidized the calibration curves were linear in the concentration range 6·6–66 μM for membrane-bound polyphenol oxidase from both sources (Fig. 2).

Substrate Specificity

Champignon or potato polyphenol oxidase immobilized by glutaraldehyde in the membrane is capable of oxidizing pyrocatechol, *p*-cresol, phenol, pyrogallol, chlorogenic acid, L-tyrosine, and D,L-dihydroxyphenylalanine. Hydroquinone and floroglucinol were practically not oxidized at all. Different magnitudes of steady-state electrode response measured with the same membrane and the same quantity (100 nmol) of individual substrates were obtained since these phenols differ in their affinity for the enzyme. If we put the wave height of pyrocatechol equal 100% then the following values are obtained with membranes containing the immobilized potato enzyme: *p*-cresol 97%, chlorogenic acid 62% (pH 5·0), pyrogallol 60%, and dihydroxyphenylalanine 31%. A different sequence of substrates was obtained when the same quantity of substrate (0·1 μmol) was measured with

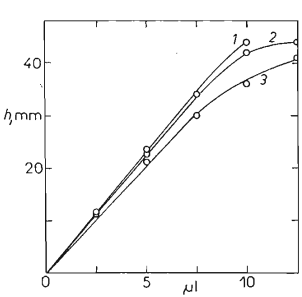


FIG. 3

Dependence of Electrode Response on Quantity of Coking Waste Water

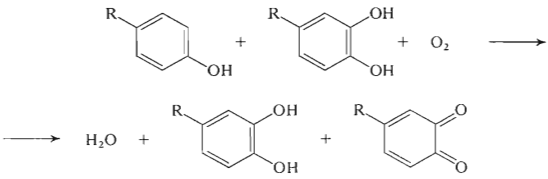
A membrane with immobilized champignon polyphenol oxidase (4 U) and waste water containing 1·3 g of phenols per liter were used for the measurements; *1* second measurement, *2* fifth measurement, *3* eighth measurement with the same membrane in 3ml of 0·1M potassium phosphate buffer, pH 7·0 at 30°C.

the membrane made of immobilized champignon polyphenol oxidase: *p*-cresol 121%, phenol 118%, pyrocatechol 100%, pyrogallol 56%, tyrosine 56%, and dihydrophenylalanine 43%.

The easy oxidizability of phenol was exploited for the analysis of coking waste water. The dependence of electrode response on the quantity of water was linear in the range of 1 – 10 μl and it was possible to use the membrane repeatedly (Fig. 3). The analysis of 2·5 – 10 μl of waste water showed the presence of 0·14 – 0·16% of phenol, determined by means of a calibration curve. The phenol content determined spectrophotometrically[9] was 0·13%.

DISCUSSION

Polyphenol oxidases catalyze the oxidation of mono- and diphenols by molecular oxygen without the formation of hydrogen peroxide:



o-Diphenol itself can also serve as an hydrogen donor. The arising quinone undergoes a series of consecutive reactions which proceed partly spontaneously and lead to dark colored melanine pigments. When the polyphenol oxidase electrode is repeatedly used melanine accumulates on the membrane, as obvious from the gradual blackening of the enzyme layer and this is clearly one of the causes of its limited lifetime.

The insolubilization of champignon and potato polyphenol oxidase leads to quantitative changes in its substrate specificity. These are – compared to the free enzymes[10–13] – the bad oxidizability of dihydroxyphenylalanine, tyrosine, and chlorogenic acid and the easy oxidizability of simple monophenols, without an induction period, especially by champignon polyphenol oxidase. This could be caused by a conformational change of the enzyme molecule during the insolubilization reaction even though the effect of specific diffusion properties of the membrane cannot be excluded either.

The easy oxidizability of phenol by membrane-bound champignon polyphenol oxidase can be of practical use, *e.g.* for the analysis of coking waste water without special treatment of the samples. Of the phenolic substances contained in this type of waste water the main portion is represented by phenol, cresol and pyrocatechol.

The enzyme electrode with insolubilized mushroom polyphenol oxidase can therefore serve for a rapid check-up of water pollution by industrial waste. The simple isolation procedure of the enzyme, and its stability in frozen state, as well as the possibility of long-term storage of the enzyme membranes are good prerequisites of such analyses. The measurement can be effected in a few minutes and the only chemical needed in addition to the standard is a phosphate buffer.

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