AN ENZYME ELECTRODE FOR DETERMINATION OF AROMATIC DIAMINES AND AMINOPHENOLS BASED ON CROSS-LINKED CERULOPLASMIN

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The determination of aromatic amines and phenols by an enzyme pO_2 electrode containing cross-linked ceruloplasmin in the reaction layer was examined. The electrode shows at pH 5·5 a marked selectivity for derivatives with $—NH_2$ or NR_2, resp., and —OH-groups in *para* and *ortho* position while its sensitivity for aromatic diamines is better than its sensitivity for aminophenols and polyphenols. *p*-Phenylenediamine and its N,N-dialkyl derivatives are the best analytes which can be detected starting from concentrations of 10^{-5} mol l^{-1} and higher; the reproducibility is 5—7% at an electrode response time of 2—5 min.

Ceruloplasmin is a copper-containing blue glycoprotein occurring in relatively high quantities in blood (up to 0·63 g per liter of human blood[1]). The ability of ceruloplasmin to catalyze the oxidation of aromatic diamines and polyphenols has lead us to utilize this enzyme as a biological transducer in the reaction layer of an enzyme electrode based on a pO_2 electrode of the Clark type. The newly assembled sensor gave a well measurable response to the addition of several compounds of technical importance[2]; we investigated therefore in the present study its applicability to the detection of various aromatic amines, aminophenols, and phenols. A similar sensor based on immobilized polyphenol oxidase for a rapid determination of low concentrations of phenol, *p*-cresol, catechol, and pyrogallol has been described by us earlier[3].

EXPERIMENTAL

Ceruloplasmin (EC 1.16.3.1) has been isolated from the dialyzed fraction of porcine plasma (30—60% saturation with ammonium sulfate) at 4°C on a column of DEAE-cellulose by stepwise elution with 30 mmol l^{-1} phosphate buffer, pH 7·0, containing 10 mmol l^{-1} benzylamine hydrochloride (elution of monoamine oxidase) and by 0·1 mol l^{-1} phosphate buffer, pH 7·0 (elution of ceruloplasmin). After rechromatography of the concentrated preparation on a column of DEAE-Sephadex A-50 (gradient of 0—0·3 mol l^{-1} NaCl in 10 mmol l^{-1} phosphate buffer, pH 7·0) and repeated concentration by ultrafiltration and evaporation, a blue solution of $A_{610}/A_{280} = 0·070$ was obtained, containing 817 μg of copper[4] and 298 mg of protein[5] per ml;

these values correspond to 277 mg of ceruloplasmin of a purity 93·6% and activity 16 ncat/mg (pH 5·5, 30°C, 10 mmol l^{-1} of *p*-phenylenediamine as substrate).

The measuring part of a pO_2 electrode (Radiometer E 5047, Pt–Ag/AgCl system, 25 μm polypropylene membrane) was coated with a reaction layer obtained by evaporation of 18 μl (3·3 mg) of ceruloplasmin and 4 μl of 2% glutaraldehyde (Fluka) on a circular 40 mm^2 area of polyamide webbing (25 mesh/mm^2, fibers 40 μm thick). The sensor was linked through an amplifier to a recorder (TZ 4100, Laboratorní přístroje, Prague) and polarized at −650 mV. The speed chart was 0·1 mm s^{-1}. The analyses were carried out at 30°C with stirring by a magnetic stirrer in a thermostated reaction vessel containing 3·0 ml of 0·1 mol l^{-1} acetate buffer, pH 5·5. After the output current had stabilized, 2—40 μl of the aqueous substrate solutions (or alcoholic solutions in the case of benzidine and its derivatives) were injected into the reaction vessel. The recorded electrode signal (decrease of current) was converted into the corresponding decrease of oxygen pressure in kPa.

RESULTS AND DISCUSSION

The advantage of ceruloplasmin from the viewpoint of analytical relevance is the fact that it can be isolated relatively easily from a cheap source and that it retains its oxidizing activity for many years. Likewise, the preparation of the reaction layer of the ceruloplasmin sensor is easy and can be accomplished in one hour at room temperature. We obtained a sensor of optimal characteristics as regards its sensitivity and stability at a reasonable response time when 8·3 mg of ceruloplasmin (24·5 μg of Cu; 132·7 ncat) was cross-linked by 10 μl of 2% glutaraldehyde, calculated per 1 cm^2 of membrane area; this corresponds to a weight ratio of 41 : 1 of both components. As in earlier work[3,6] a fine polyamide webbing was used to advantage as a support of the reaction layer. An admixture of an inert protein was unnecessary. The dry ceruloplasmin membranes were stored at 4°C. They were still active after one year.

The electrodes with a freshly prepared ceruloplasmin reaction layer show at first an inadequately strong response to the first addition of aromatic diamine; the properties of the electrode stabilize in the course of prolonged use. We have been able to stabilize the membranes in 0·1 mmol l^{-1} *p*-phenylenediamine in 0·1 mol l^{-1} acetate buffer, pH 5·5 for about an half-hour. The active zone blackens during this period because of the cumulation of polymer reaction products, similarly to membranes with cross-linked polyphenol oxidase[3]. The dark-colored products do not affect the proper function of the electrode. One and the same membrane was used 14 days without a substantial loss of its catalytic properties even though it was in contact with the substrate 18 h at 30°C; the rest of the period the membrane was kept in acetate buffer, pH 5·5.

A typical response to a series of successive additions of *p*-phenylenediamine into the same medium is shown in Fig. 1. The oxidation of the substrate in the ceruloplasmin membrane leads to a decrease of oxygen concentration around the active part of the pO_2 electrode which manifests itself by a drop of the current. The calibration curve

can be constructed[7] either from the slope of the time profile of current change (rate method) or from the total current change (steady-state method), as shown in Fig. 1. The second procedure is more time-consuming since the stabilization of the current may last with some of the substances tested up to 10 min (Table I), yet the error of the manual evaluation is smaller.

Because of the high content of ceruloplasmin in the membrane the electrode response is relatively little dependent on the pH of the medium (Fig. 2). The biggest decrease of current in acetate buffer can be shown by both procedures to occur at pH 5·5. A phosphate or phosphate-citrate buffer was found less suitable.

The plot of electrode response versus analyte concentration is linear over the range $10^{-5}-2{\cdot}5\,.\,10^{-4}$ mol l^{-1} at least; it is shown for three selected amines in Fig. 3. The reproducibility of the analyses expressed by the variation coefficient for 10 replicates of 0·12 μmol N,N-dimethyl-*p*-phenylenediamine and *p*-phenylenediamine was 4·7 and 7·0%, resp. as determined by the steady-state method and 7·6 and 8·3%, resp. by the rate method.

The selectivity of the enzyme electrode with ceruloplasmin in the reaction layer was tested with isomers of aromatic diamines, aminophenols, phenols, and benzidine derivatives. The results are given in Table I showing the relative sensitivity calculated from the slopes of the calibration curves for the range $10^{-4}-10^{-5}$ mol l^{-1}. The electrode gives marked preference to the *p*-derivatives over the *o*-derivatives; it is practically insensitive to *m*-substituted derivatives (*m*-phenylenediamine, *m*-aminophenol, resorcinol) and to monofunctional compounds (phenol, guaiacol, aniline). Aromatic diamines are generally better analytes than aminophenols and polyphenols. When the content of enzyme in the reaction layer was lowered to one third practically no electrode response towards polyphenols has been observed. This indicates a possibility of enhancing the electrode selectivity in favor of aromatic amino compounds.

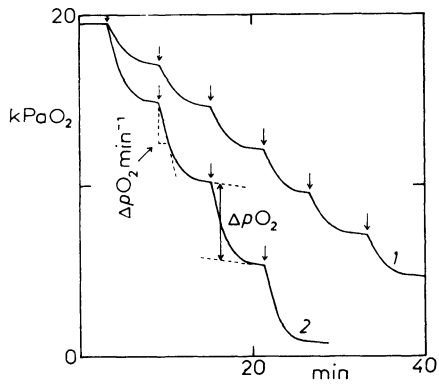

FIG. 1

Response of ceruloplasmin electrode to stepwise addition of *p*-phenylenediamine. Each arrow indicates the addition of 4 or 8 μl of substrate solution to the measuring cell, enhancing the concentration by 40 *1* and 80 *2* μmol l^{-1}, respectively. Response reading from initial change of current (rate method) or from total change of current (steady-state method) is also shown

TABLE I

Sensitivity of ceruloplasmin pO_2 electrode to aromatic amines and phenols (0·1 mol l^{-1} acetate buffer, pH 5·5, 30°C)

Substance	Relative electrode response, %		Steady-state response time min
	rate method	steady-state method	
N,N-Dimethyl-*p*-phenylenediamine . H_2SO_4	100	100	2—4
N,N-Diethyl-*p*-phenylenediamine . H_2SO_4	60	71	3—5
p-Phenylenediamine . 2 HCl	70	84	3—5
o-Phenylenediamine	32	27	2—4
p-Aminophenol . HCl	34	51	4—7
o-Aminophenol	23	47	5—10
Pyrogallol	22	38	4—7
Catechol	13	32	4—10
Hydroquinone	8	10	3—7
Benzidine	35	84	6—9
Dianisidine	37	42	3—4
3,3'-Diaminobenzidine.4 HCl	49	53	3—5

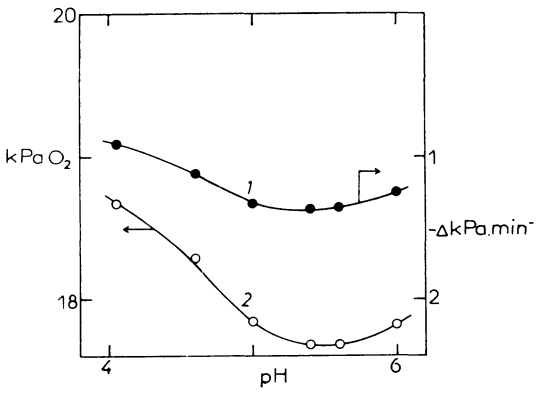

FIG. 2

Sensitivity of ceruloplasmin electrode as function of pH with *p*-phenylenediamine as substrate. A 0·2 mol l^{-1} sodium acetate buffer was used. Response to the addition of 0·12 μmol of substrate was evaluated by the rate method *1* and by the steady-state method *2*

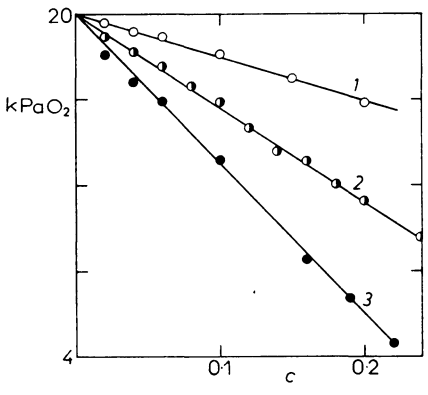

FIG. 3

Relationship between steady-state electrode response (pO_2 decrease) and concentration of analyte at pH 5·5 and 30°C. *1* *p*-Aminophenol, *2* *p*-phenylenediamine, *3* N,N-dimethyl-*p*-phenylenediamine; concentration (*c*) in mmol l^{-1}

The results obtained show that the newly assembled ceruloplasmin electrode could receive practical application, because of its simple preparation, stability, and sensitivity in the field of rapid analyses of the concentration of *p*-phenylenediamine, its asymmetric N-dialkyl derivatives, alternatively also of *o*- and *p*-aminophenol in colored or turbid media.

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REFERENCES

1. Musil J.: *Glykoproteiny*, p. 131. Avicenum, Prague 1978.
2. Jílek M.: *Thesis*. J. E. Purkyně University, Brno 1980.
3. Macholán L., Schánĕl L.: This Journal *42*, 3667 (1977).
4. Peisach J., Levine W. G.: J. Biol. Chem. *240*, 2284 (1965).
5. Kalckar H. M.: J. Biol. Chem. *167*, 461 (1947).
6. Toul Z., Macholán L.: This Journal *40*, 2208 (1975).
7. Nanjo M., Guilbault G. G.: Anal. Chim. Acta *73*, 367 (1974).

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