A SPECIFIC ENZYME ELECTRODE FOR SPERMINE AND SPERMIDINE

Lumír MACHOLÁN and Dagmar JÍLKOVÁ

Department of Biochemistry,
J. E. Purkyně University, 611 37 Brno

Received June 23rd, 1981

A sensor method has been worked out for the determination of spermine and spermidine in concentrations 0·02—0·5 mmol l^{-1}. The method is based on amperometric measurement of the consumption of oxygen and the formation of hydrogen peroxide in a reaction catalyzed by cross-linked polyamine oxidase from maize. One determination takes 1 to 2 min, with phosphate buffer as an only reagent. In the dry state the biocatalytic layer of the sensor was stable for at least 8 months. The optimum conditions for the analysis are described and the applicability of the sensor to urine samples is dealt with.

In view of the generally accepted significance of biogenic polyamines, spermine and spermidine, in syntheses of nucleic acids and proteins in early stages of development, differentiation and transformation[1-3], and as markers of the tumour kinetics[4,5], the problem of their determination is attracting more interest. The electrophoretic[5] and chromatographic[6-9] techniques, which are commonly used for biological materials, are largely time-consuming and demand expensive equipment. The invention of the biospecific electrochemical sensors, the so-called enzyme electrodes, introduces the possibility of a rapid and simple screening method for a large number of samples.

In a previous paper[10] we described an enzyme sensor based on the Clark oxygen cell coated with a layer of cross-linked pea diamine oxidase, which makes it possible to detect low concentrations of biogenic amines within 1 to 2 min. However, the enzyme used is not specific, since it is sensitive not only to polyamines, but also to putrescine, cadaverine, hexamethylenediamine and histamine[10]. The present paper describes a sensor based on a plant polyamine oxidase (EC 1.5.3.3) cross-linked with glutardialdehyde, which has proved to react specifically with spermidine and spermine. When the sensor is dipped into the solution to be analyzed the polyamine diffuses into the enzyme layer, where it gets oxidized by the dissolved oxygen in the very vicinity of the electrode, with the formation of hydrogen peroxide. The function of the sensor is based on amperometric detection of the oxygen being consumed in the enzyme layer, or of the formed hydrogen peroxide.

TABLE I

Stability of polyamine oxidase layer of the sensor. A stabilized sensor response to 1 μmol of spermidine at 30°C was measured by the oxygen method; the buffer was 0·1M K-phosphate of pH 6·5. The recorder scale was 250 mm

Time of storage days	Dry membrane			Membrane in the buffer		
	4°C		20°C	4°C		20°C
	mm	rel.%	mm	mm	rel.%	mm
2	70	100	–	57	100	–
8	88	125·7	–	76	133·3	–
15	108	154·3	108	91	159·6	95
22	122	174·3	127	100	175·4	108
30	134	191·4	–	102	178·9	111
42	134	191·4	152	107	187·7	128
51	147	210·0	139	113	198·2	121
65	148	211·4	136	115	201·7	118

EXPERIMENTAL

Materials

Spermine tetrahydrochloride and spermidine trihydrochloride were prepared from the bases (Koch-Light); 25% glutardialdehyde was from Fluka, bovine serum albumin from Mann Res. Laboratories; a commercial polyamide netting[10] had a density of 25 meshes/mm^2 and a thread thickness of 40 μm; plant polyamine oxidases isolated from sprouts of 7 days old etiolated germs of maize and oats, according to Suzuki's method[11,12], had specific activities of 35 ncat/mg and 144 ncat/mg of protein respectively (spermine as substrate, 30°C, pH 5·5). The enzymes were concentrated by ultrafiltration to an activity of *c.* 1 μcat/ml and stored at −20°C.

Procedure

Enzyme layers of optimum composition were prepared from a mixture of 4 μl of 10% serum albumin (in phosphate buffer, 50 mmol l^{-1}, pH 7·2), 2 μl of a polyamine oxidase and 2 μl of 2% glutardialdehyde. The mixture was spread over a ring area of *c.* 40 mm^2 of the polyamide netting and dried at 4°C. The moistened layer was stretched over the functional part of the electrode and fixed with a rubber ring. The sensor was held at a constant height in a glass vessel equipped with a water jacket and a magnetic stirrer. The vessel contained 3 ml of potassium phosphate buffer, 0·1 mol l^{-1}, which was kept at 30 ± 0·1°C with an ultrathermostat and saturated with air for 3 min. After the stirring was started and the initial electrode current had stabilized, several portions of a polyamine standard (2–10 μl, conc. 30 mmol l^{-1}) and the sample to be analysed were alternately added to the buffer with a syringe. Following each addition the changed current was recorded until it reached a constant value. The calibration line relates the magnitude of a stea-

dy state electrode response (the current change in mm or μA) to concentration of the standard. The electrode and the measuring systems were of two kinds.

a) the Clark pO_2 cell (7 mm disc Au cathode, Ag/AgCl anode, 2M-KCl) with a 20 μm polypropylene membrane, onto which the enzyme layer was applied on the side of the solution; the sensor was attached to an analyser of the dissolved oxygen, model MU-66, VD Czechoslovak Academy of Sciences, Prague, connected to a compensating recorder Messgerätewerk, Magdeburg (scale 250 mm, speed drive 1 cm/min). The cathode of the cell was polarised to a potential of −0·7 V.

b) A disc Pt-electrode (0·3 mm) coated with the enzyme layer and a cellophane membrane on the side of the solution. The reference saturated calomel electrode was placed in a side tube of the reaction vessel. The electrodes were attached to an universal recording polarograph OH-105 Radelkis, Budapest (scale 250 mm). A constant potential of +0·6 V *vs* S.C.E. was applied to the Pt electrode.

RESULTS AND DISCUSSION

Unlike other amine oxidases, polyamine oxidase, the flavoprotein enzyme of maize and other cereals, attacks one of the secondary amino group of spermidine and spermine. The reaction gives rise to hydrogen peroxide, 1,3-diaminopropane and Δ^1-pyrroline or 1-(3-aminopropyl)-pyrroline[13]. A phenomenon commonly observed after the addition of a substrate to the soluble enzyme is a rapid decrease of activity with time, which is not due to lability of the enzyme or to depletion of oxygen. This phenomenon, undesirable from the analytical point of view, is no longer observed if the enzyme in the reaction layer of the sensor has been cross-linked with albumin through glutardialdehyde. Such a reaction layer can then be used for tens of successive analyses.

The reticulation of the oxidase with an inert protein directly on a polyamide support[10,14] increases the mechanical firmness of the active layer, so that this layer can be taken off the electrochemical probe, stored separately in a buffer or in a dry state at low temperature, and mounted on again when it is needed. This extends its operation lifetime. The composition of the active layers was optimized by gradually changing the amounts of all the components. The best analytical properties were observed with layers obtained by drying dissolved serum albumin, polyamine oxidase and glutardialdehyde in a weight ratio of 10 : 2 : 1. The employed amount of the enzyme (2·7 ncat on an area of 40 mm^2) represents a five-fold of the activity needed for the sensor response to be maximum (Fig. 1). Although not all of the accessible active centres of the enzyme are in operation at first, the stock of the enzyme manifests itself markedly in a long-termed stability of the layers. The quality of the layers is also well reproducible under these conditions; the slopes of the calibration curves for 15 membranes differed within a range of only ±5·3%.

In using the enzyme layer combined with the Clark cell, the time needed for the response to stabilize was 1 to 1·5 min, with 90% and 95% of its value being reached

in 40 s and 50 s, respectively. The upper limit of linearity was observed at a concentration of $5 . 10^{-4}$ mol l^{-1} with spermidine and $7 . 10^{-4}$ mol l^{-1} with spermine (Fig. 2). The lower threshold of detection was at the concentration $2 . 10^{-5}$ mol l^{-1}. The root-mean-square deviation of a steady state sensor response for 20 equal additions of 0·18 μmol of spermidine was 24 ± 1·63 mm, the recorder scale being 250 mm. The sensor was relatively little sensitive to pH changes of the reaction medium with an excess of the enzyme in the reaction layer. It was only with low-activity layers that a more or less significant pH dependence was observed, with

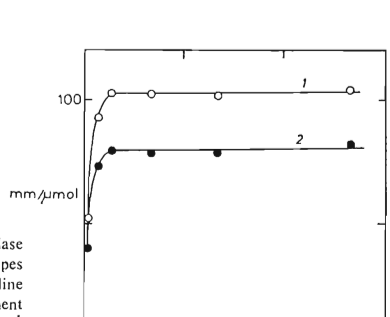

FIG. 1

Effect of amount of maize polyamine oxidase (n cat) in the sensor enzyme layer on slopes of the calibration curves for spermidine and spermine (mm/μmol). Measurement by the oxygen method in 0·1 mol l^{-1} K-phosphate buffer, pH 6·5, at 30°C. *1* spermidine, *2* spermine

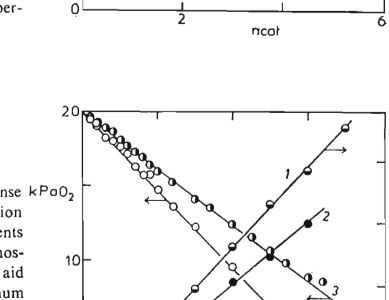

FIG. 2

Magnitude of steady state sensor response to spermidine and spermine in relation to their concentrations. The measurements were carried out in 0·1 mol l^{-1} K-phosphate buffer, pH 6·5, at 30°C with the aid of the Clark oxygen cell and a platinum disc anode (cellophane-coated) having the optimum layer of maize polyamine oxidase. The *y*-axis gives steady state sensor response in kPa of O_2 or μA (detection of H_2O_2), the *x*-axis concentrations of spermidine *1*, *4* and spermine *2*, *3* in mmol l^{-1}

a peak at about pH 6·5 for both the polyamines (Fig. 3) although the soluble polyamine oxidase exhibited[11,15] somewhat different pH optima for the oxidation of spermidine and spermine (6·3 or 6·0 and 5·5, respectively). With the maize enzyme, spermidine is a better substrate than spermine[11], which is also seen in unequal sensitivities of the enzyme sensor to both polyamines (Fig. 2). The results were analogous even if the catalytic layer of the sensor was prepared from cross-linked oat polyamine oxidase[16] of a higher specific activity.

Some of the experiments were done with a sensor consisting of a platinum anode and the same layer of a cros -linked polyamine oxidase as with the Clark pO_2 cell. Coating the enzyme layer with cellophane reduced the stirring effect and produced a stable noiseless response, even though at the expense of extending the time of the current response from 30 s to 60 s and reducing the original sensitivity to about a sixth to a tenth. Generally, it was not necesary to remove the oxide film on the electrode prior to its coating with the enzyme layer. With the optimized enzyme layer the pH profiles were as flat (Fig. 3) as in the detection of the oxygen consumption.

The calibration curves for spermidine and spermine at pH 6·5 are given in Fig 2, which shows that the lower limit of detection corresponds to a concentration of about 20 μmol l^{-1}. Consequently, the platinum anode with the cellophane coating of the enzyme layer had about the same sensitivity as the sensor made up of the oxygen cell. However, the upper limit of linearity was higher, evidently because the enzyme reaction is not limited by depletion of oxygen, which returns to the reaction in the anodic oxidation of the peroxide. The ratio of the responses to spermidine and spermine was 1·3, *i.e.* similar to that observed with the sensor based on the Clark

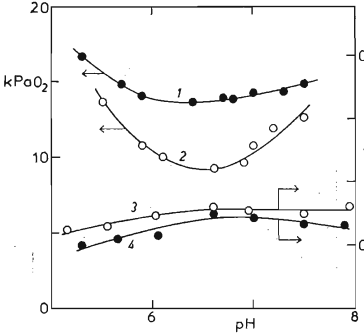

FIG. 3

Steady state response of the enzyme sensor to spermine and spermidine in relation to pH. The measurement was carried out in 0·1 mol l^{-1} K-phosphate at 30°C by the oxygen method using a low-activity enzyme layer and 1 μmol of a polyamine, and, for comparison, by the peroxide method with the optimum layer of maize polyamine oxidase without the cellophane coating, using 30 nmol of a polyamine. The *y*-axis gives steady state sensor response in kPa of O_2 or μA (detection of H_2O_2), the *x*-axis the final pH of the reaction medium. *1*, *4* spermine; *2*, *3* spermidine

cell (Fig. 3). In the presence of 1 mol l^{-1} sodium chloride, which considerably stimulates the oxidation of spermine by oat polyamine oxidase[16], no increase in sensitivity of the sensor to this amine was observed. The reproducibility of the determination is comparable with the oxygen method, using recorders of the same scale. The root-mean-square deviations for additions of 0·18 and 0·3 μmol of spermidine were 28·5 ± 1·05 mm (= 34·2 ± 1·26 . 10^{-9} A) and 43·6 ± 1·32 mm (= 52·3 ± ± 1·58 . 10^{-9} A), *i.e.* 3·7% and 3·0%, respectively.

From the practical point of view we investigated the stability and operation lifetime of the biocatalytic layers kept separately from the electrochemical sensor used (Tab. I). Experiments have shown that the enzyme layers can be stored dry or in a buffer at a temperature up to 20°C. In continuous measurement by the oxygen method the electrode response slowly increased, exceeding the two-fold of its original value in 65 days. This is, with cross-linked enzyme layers, rather a common phenomenon, ascribed to the formation of diffusion canals. The change in properties of the membrane is easily eliminated by periodic calibration of the sensor. Membranes stored in a dry state at 4°C for 8 months were still of use, the ratio of their sensitivities to spermidine and spermine being constant. Judging by the number of additions of a pure polyamine, hundreds of analyses can be carried out with one freshly prepared layer.

The aldehydes n-butanal, benzaldehyde and anisaldehyde, which stimulate animal polyamine oxidase[17], do not influence the electrode response to a polyamine, whether they were used alone or in the presence of 5 mmol l^{-1} dithiothreitol. The sensor is not sensitive to histamine or to any of the six tested aliphatic α,ω-diamines, with 2 to 6 and 10 methylene groups in the chain. Nor did their addition diminish the responses to spermine and spermidine. Of other substances that might be possibly expected to have an adverse effect on the function of the sensor we tried urea and ammonium ions, with a view to using the sensor for analysis of urine. It has turned out that urea in the physiological and higher concentrations (up to 2 mol l^{-1}) has no effect. The ammonium ions, by contrast, even at a concentration of only 0·1 mol l^{-1}, suppressed the sensitivity of the sensor by about 20%. After transferring the sensor to a solution free of ammonium ions it recovered its original properties. Analyses of 50 μl samples of human urine have shown that the sensor method as described is little sensitive for detection of the polyamines in non-hydrolyzed urine, since they are present mainly in the conjugated forms.

Acknowledgement is due to Miss J. Haubrová for careful technical assistance.

REFERENCES

1. Tabor C. W., Tabor H.: Annu. Rev. Biochem. *45*, 285 (1976).
2. Campbell R. A., Morris D. R., Bartos D., Davis G. D., Bartoa F.: *Polyamine Research* 1 and 2. Raven Press, New York 1979.

3. Janne J., Poso H., Raina A.: Biochim. Biophys. Acta *473*, 241 (1978).
4. Russel D. H.: Clin. Chem. *23*, 22 (1977).
5. Lipton A., Sheehan L. M., Kessler G. F.: Cancer *35*, 464 (1975).
6. Marton L. J., Lee P. L. Y.: Clin. Chem. *21*, 1721 (1975).
7. Newton N. C., Ohno K., Abdel-Monem M. M.: J. Chromatogr. *124*, 277 (1976).
8. Adler H., Margoes M., Snyder L. R., Spitzer C.: J. Chromatogr. *143*, 125 (1977).
9. Perini F., Sadow J. B., Hixson C. V.: Anal. Biochem. *94*, 431 (1979).
10. Toul Z., Macholán L.: This Journal *40*, 2208 (1975).
11. Suzuki Y., Hirasawa E.: Phytochemistry *12*, 2863 (1973).
12. Hirasawa E., Suzuki Y.: Phytochemistry *14*, 99 (1975).
13. Smith T. A., Bickley D. A.: Phytochemistry *13*, 2437 (1974).
14. Posádka P., Macholán L.: This Journal *44*, 3395 (1979).
15. Suzuki Y., Yanagisawa H.: Plant Cell Physiol. *21*, 1085 (1980).
16. Smith T. A.: Phytochemistry *16*, 1647 (1977).
17. Hölttä E.: Biochemistry *16*, 91 (1977).

Translated by J. Salák.