A BIOSPECIFIC MEMBRANE SENSOR FOR THE DETERMINATION OF SUCROSE

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The paper describes a rapid method for the biospecific determination of sucrose using an oxygen electrode of the Clark type or a platinum disc anode, the measuring part of which is coated by a thin film of invertase, mutarotase and glucose oxidase co-crosslinked by glutardialdehyde together with serum albumin. After injecting the sample into the reaction vessel the current corresponding to the decrease of the oxygen content or to the formation of hydrogen peroxide in the enzyme reaction layer is registered. The steady state electrode response is proportional to the concentration of sucrose within the range of 0·03 to 1·5 mmol . l^{-1} and is attained during 1—2 minutes with a reproducibility of 3—4%.

Recently much attention has been devoted to the determination of biologically important substances using biospecific sensors, in which the enzyme reaction is transformed into an electric signal. No reagents are needed and a rapid analysis of nontransparent mixtures is possible. In the analysis of sugars various modifications of the glucose electrode[1-3] are highly appreciated, a galactose electrode is known[3,4] and so is the bienzyme sensor for maltose[5], which makes use of coupled reactions catalyzed by glucoamylase and glucose oxidase.

In this paper the construction and the properties of an amperometric membrane sensor is described, which detects sucrose in the following way. In its catalytic layer three subsequent enzyme reactions take place: first the disaccharide is cleaved by invertase (EC 3.2.1.26) to a mixture of α-glucose and β-fructose, then the α-glucose is transformed by mutarotase (EC 5.1.3.3) to its β-anomer, which is the true substrate for glucose oxidase (EC 1.1.3.4), the last enzyme in the whole sequence:

$$\text{sucrose} + H_2O \xrightarrow{\text{invertase}} \alpha\text{-glucose} + \beta\text{-fructose} \qquad (A)$$

$$\alpha\text{-glucose} \xrightarrow{\text{mutarotase}} \beta\text{-glucose} \qquad (B)$$

$$\beta\text{-glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2 \qquad (C)$$

Behind the enzyme film either the decrease of the oxygen content by the Clark oxygen cell at −0·7 V reduction potential is measured amperometrically or the hydrogen peroxide formed is detected using the platinum disc anode at a constant H_2O_2 oxidation potential +0·6 V. Because of its rapidity and sensitivity the new sensor method can be successfully applied for following the kinetics of formation and utilization of sucrose.

EXPERIMENTAL

Material and Methods

Glucose oxidase (Grade II, specific activity 733 nkat/mg) was obtained from Boehringer (Mannheim, FRG). Invertase (β-D-fructofuranosidase) was isolated from brewery yeast[6] and as a lyophilized preparation it had an activity 13.1 μkat/mg. Mutarotase (aldose-1-epimerase) was purified from pig kidney cortex according to Lapedese and Chase[7] with some modifications[8]. Enzyme of specific activity 160 nkat/mg of protein was purified from NH_4^+ ions by dialysis and kept in 50 μl aliquots at −16 C.

The activity of glucose oxidase was tested from the initial velocity of oxygen consumption measured at 30°C with a Clark oxygen cell in 0·1 mol . l^{-1} potassium phosphate buffer of pH 5·6 containing 0·1 mol . l^{-1} glucose (reaction *C*). The solution of glucose was preserved by 0·02% sodium azide and used 24 h after the establishment of the mutarotation equilibrium. The activity of mutarotase was measured polarographically[9] at 30°C from the velocity of α-glucose oxidation before and after the addition of the enzyme in the presence of an excess of glucose oxidase (reaction *B* and *C*). Reaction medium of pH 7 contained 20 mmol . l^{-1} of EDTA. The invertase activity was determined at 30°C either by a conventional colorimetric method[10], or using an enzyme electrode (in the catalytic layer of which the invertase was omitted, see further) in 0·1 mol . l^{-1} sodium phosphate buffer, pH 6·0, containing 0·2 mol . l^{-1} sucrose. The colorimetric procedure gave results about 23% lower obviously owing to the inhibition of glucose oxidase by dianisidine[1].

Preparation of Enzyme Layers and the Method of Measurement

The enzyme layer for the determination of sucrose was prepared on a polyamide network (silon, 25 mesh/mm^2) by crosslinking 6 μl of invertase (394 nkat), 6 μl of mutarotase (7·6 nkat), 3 μl of glucose oxidase (0·24 mg, 176 nkat) and 4 μl of 10% bovine serum albumin using 2 μl of 2% glutardialdehyde. The aldehyde was added as the last component, the mixture was spread on both sides of a circular area with a diameter of 7 mm and dried for 3 hours at 4°C. Enzyme membranes were kept in a dry state at 4°C.

The basic sensor used for the determination of sucrose by the oxygen method was the Clark cell (Au–Ag/AgCl, 2M-KCl, polypropylene membrane) connected to an oximeter VD Czechoslovak Academy of Sciences Mod. 66 with a compensating recorder e KBT 1 EN (Meßgerätewerk, Magdeburg, GDR). A gold cathode of a diameter of 7 mm was polarized to a constant potential of −0·7 V. For the peroxide method a platinum disc anode of a diameter of 3 mm was used, which was polarized to +0·6 V *vs* S.C.E and connected to an universal polarograph OH-105 (Radelkis, Hungary). In both cases the recorder scale was 250 mm with a speed drive of 1 cm per min. On the measuring part of the sensor electrode a wet enzyme membrane was stretched and fixed by a rubber 0 ring. The enzyme layer of the Pt anode in the direction to the solution was covered by cellophane (25 μm) in order to limit the stirring effect. The glass reaction vessel equipped with a water jacket and a magnetic stirrer contained 3 ml of 0·1 mol . l^{-1} phosphate buffer

with 0·027% of sodium azide. The sample or the standard of sucrose was injected by a Hamilton repeating dispenser. The voltametric limiting current was measured and its steady state value, taken here for the response of the enzyme sensor, is expressed in microamperes in the case of the peroxide method and in mm of the recorder scale or in kPa (after recalculating to pO_2) in the case of the oxygen method.

RESULTS

Preliminary results with a membrane containing immobilized invertase and glucose oxidase only showed that for sucrose the response of the sensor is slow and cannot be well read (Fig. 1) owing to low spontaneous mutarotation of α-glucose. Accelerators of mutarotation *i.e.* phosphate ions or histidine did not have a substantial effect on the response. Only after incorporating mutarotase into the reaction layer, an increased sensitivity and a rapid response with a well developed plateau corresponding to the steady state current value was obtained (Fig. 1).

The composition of the three-enzyme layer was optimalized so that the effect of the variable amount of individual enzymes was appreciated according to the slope of the calibration curve measured at the pH corresponding to the average pH of the pH optima of respective enzymes (Fig. 2). Constant amounts of 2 µl of 2% of

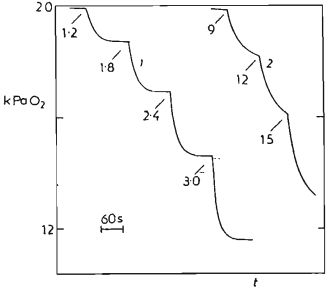

FIG. 1

Electrode responses for subsequent additions of various amounts of sucrose (in µmols) into the buffer. *1* sensor with a three-enzyme layer, 2 mutarotase was omitted. *x* axis — time, *y* axis — the decrease of pO_2 behind the membrane in kPa.

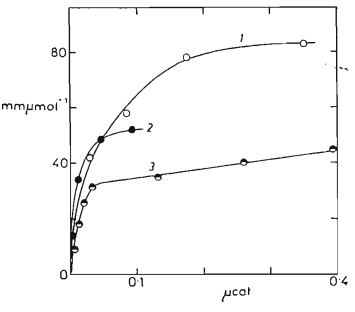

FIG. 2

Optimalization of the enzyme composition of the sensor reaction layer, *x* axis — enzyme activity in µkat, *y* axis — slope of the calibration curve for the respective substrate. *1* glucose oxidase at pH 5·6, *2* mutarotase (multiplied by a factor of 10) at pH 6·3, *3* invertase at pH 6·0.

glutaraldehyde and 0·4 mg of bovine serum albumin were always used to form a compact membrane. In the case of the membrane with glucose oxidase only, 180 nkat was a sufficient amount to reach the optimal steady state response for an equilibrium mixture of α- and β-glucose. Two-enzyme membrane containing in addition to the above mentioned amount of glucose oxidase a variable amount of mutarotase needed an amount of 8 nkat of the latter enzyme (determined, however, using a subsaturation concentration of α-glucose[9]). In the third series of measurements with a complete three-enzyme membrane electrode containing a variable amount of invertase an optimum amount of 400 nkat of the enzyme was found using sucrose as a substrate.

The optimalized membrane contained a total of 0·72 mg of the protein, which corresponds to a weight ratio of 18 : 1 with the respect to the amount of glutaraldehyde used. The amount of glutaraldehyde is sufficient for the reticulation of 1 mg of the protein[11].

The response of the enzyme sensor depends on the pH of the reaction medium. In 0·1 mol . l^{-1} potassium phosphate buffer the pH optimum is at pH 6·5 (Fig. 3). Compared with the arithmetic mean value of the pH optima of the three components of the enzyme membrane it is shifted by 0·5 pH unit towards the neutral region. Obviously, the acidification of the microenvironment of the anchored enzymes by the resulting gluconic acid must be compensated by a higher pH of the buffer.

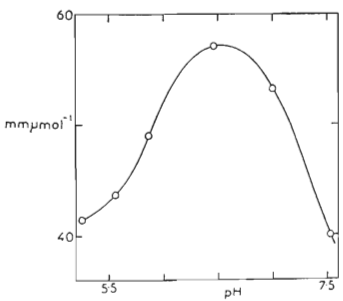

FIG. 3

pH Profile of the sucrose sensor in 0·1 mmol . . l^{-1} potassium phosphate buffer at 30°C. *x* axis — final pH of the reaction medium, *y* axis — slope of the calibration curve.

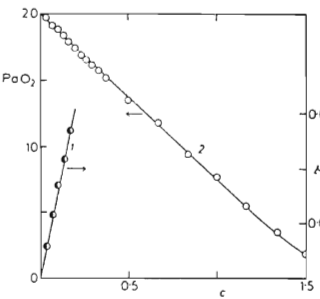

FIG. 4

Calibration curve for sucrose measured by the hydrogen peroxide method (*1*) and by the oxygen method (*2*). *x* axis — concentration of sucrose in mmol . l^{-1}, *y* axis — steady state electrode response in μA (H_2O_2 measurement) and in pO_2, respectively.

The dependence of the steady state sensor response on the variable sucrose concentration in potassium phosphate buffer, pH 6·5, was studied with respect to the linearity of the calibration curve. Using the oxygen method a linear dependence in a concentration range 0·03 to 1·5 mmol . l^{-1} (Fig. 4) was found. The lower limit is given by the sensitivity of the respective type of the Clark cell and the upper limit corresponds to the diffusion current of oxygen decreased virtually to the residual current value of the oxygen cell. The correlation coefficient in a series of 15 calibration curves had an average value of 0·998 and the variation coefficient for repeated addi-

TABLE I

Stability of the three-enzyme sensor layer. Steady state response of the sucrose sensor was measured by the oxygen method at 30°C and pH 6·5 and is expressed by the slope of the calibration curve (ΔpO_2 in kPa/μmol of sucrose).

Period of storage days	Membrane in a buffer at				Dry membrane at			
	20°C		4°C		20°C		4°C	
	slope	rel. %	slope	rel. %	slope	rel. %	slope	rel. %
1	4·7	100	4·8	100	4·0	100	5·3	100
15	4·6	98	4·6	96	3·7	92	4·9	92
22	3·8	81	4·5	94	3·7	92	4·6	87
36	3·1	66	4·3	90	3·6	90	4·7	89
50	3·0	64	3·7	77	3·6	90	4·6	87

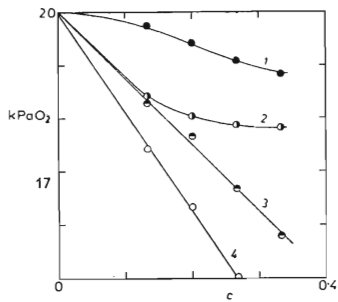

FIG. 5

Sensitivity of the sucrose sensor for various saccharides. *x* axis — saccharide concentration in mmol . l^{-1}, *y* axis — steady state electrode response in pO_2. *1* rafinose, *2* maltose, *3* sucrose, *4* glucose, 0·1 mol . l^{-1} potassium phosphate buffer, pH 6·5, 30°C.

tions of the sucrose starting with 0·6 μmol was ±4%. Using the peroxide method linearity was obtained up to the concentration of 0·33 mmol . l^{-1} only (Fig. 4) with a variation coefficient for a series of sucrose samples ±3%.

The saccharide-type substances as lactose, L-sorbose, D-fructose, D-galactose, D-ribose, D-arabinose, D-xylose, D-manitol, volemitol and glucosamine gave no response. None of these substances decreased the response of the sensor for sucrose. Compared with sucrose, only glucose (145%), rafinose (35%) and maltose (Fig. 5) gave positive responses. The latter sugar displayed a non-linear concentration dependence due to the inhibition of glucose oxidase. The inhibition is reversible, in a new medium without maltose the sensor recovers its original sensitivity to sucrose. The interference of maltose is probably connected with the presence of traces of glucoamylase in the enzymes used. On the other hand the sensitivity of the sensor to rafinose is due to inherent properties of the yeast invertase. However, this trisaccharide usually does not occur in any substantial amount in biological material. The effect of glucose present in the sample in addition to sucrose can be largely eliminated if the total response is corrected for the value measured under the same conditions by a sensor in the active layer of which invertase was omitted.

The operational durability of the three-enzyme electrode was followed in the course of one week, during which the sensor was in use a total of 16 hours and 270 additions of 0·15 mol . l^{-1} of a sucrose standard were measured. The sensor was kept overnight in a buffer, pH 6·5, at 4°C. After this period the slope of the calibration curve was 2% lower.

The long-term stability was controlled by a periodical measurement of the response with the oxygen method using four optimalized layers kept separatedly from the electrochemical sensor at two temperatures in a 0·1 mmol . l^{-1} phosphate buffer, pH 6·5, and in a dry state (after the measurement of the response the layer was desiccated again). From the results shown in Table I it follows that it is suitable to keep the layer dry. After 50 days the slope of the calibration curve was only about 10% lower and the time neccessary for attaining a steady response was moderately prolonged (90% of its value within 1 min). The membranes kept in the buffer desintegrated in the course of time possibly owing to bacterial degradation.

REFERENCES

1. Clark L. C. jr: Methods Enzymol. *56*, 448 (1979).
2. Thévenot D. R., Sternberg R., Coulet P. R., Laurent J., Gautheron D. C.: Anal. Chem. *51*, 96 (1979).
3. Baum G., Weetal H. H.: Methods Enzymol. *56*, 479 (1979).
4. Johnson J. M.: *Thesis*. Wright State University, Dayton, Ohio 1976.
5. Coulet P. R., Bertrand C.: Anal. Lett. *12* (B6) 581 (1979).
6. Myrbäck K., Schilling W.: Enzymologia *29*, 306 (1965).

7. Lapedes S. L., Chase A. M.: Biochem. Biophys. Res. Commun. *31*, 967 (1968).
8. Okuda J., Miwa I.: Methods Biochem. Anal. *21*, 155 (1973).
9. Miwa I.: Anal. Biochem. *45*, 441 (1972).
10. Messer M., Dahlquist A.: Anal. Biochem. *14*, 376 (1966).
11. Posádka P., Macholán L.: This Journal *44*, 3395 (1979).

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