PREPARATION OF ENZYME ELECTRODE FOR D-GLUCOSE DETERMINATION BY IMMOBILIZATION OF GLUCOSE OXIDASE ON COLLAGEN MEMBRANE

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Received January 15, 1990
Accepted February 28, 1990

An enzyme electrode for the determination of D-glucose was prepared by immobilization of glucose oxidase (EC 1.1.3.4.) on an activated collagen membrane using glutaraldehyde and the Ugi reaction resp. and by subsequent fixation of the membrane to an oxygen sensor of the Clark type. Two different procedures for the modification of the support, the composition of the reaction mixture and the immobilization time were examined. The electrode prepared was tested as regards the effect of pH and temperature on the magnitude of the response. The range of the linear dependence of the sensor response on substrate concentration ($1{\cdot}7 \cdot 10^{-5}$—$2{\cdot}0 \cdot 10^{-3}$), the apparent Michaelis constant of the immobilized enzyme ($K_{M(ap.)} = 4{\cdot}16 \cdot 10^{-3}$ mol dm^{-3}) and the stability of the biosensor as function of storage mode and number of assays performed with one electrode were determined. In view of the high stability and linear range of the concentration dependence the enzyme electrode is suitable for the determination of D-glucose in samples analyzed in agricultural and food laboratories.

The determination of D-glucose is of considerable importance in clinical biochemistry and also in different branches of food industry. A number of biosensors[1,2] have therefore been developed for this purpose. These sensors utilize the narrow substrate specificity of glucose oxidase[3] in combination with either amperometric[4] determination of oxygen or hydrogen peroxide, potentiometric detection[5] and recently also in combination with IFSET's (Ion-Sensitive Field Effect Transistor[6]), mediator transfer[7], optical sensors[8] or thermistors[9].

A number of papers[10–13] have been devoted to the immobilization of oxidoreductases on modified collagen membranes and to their use for the preparation of biosensors in combination with amperometric H_2O_2 detection. These procedures use enzymes immobilized by a technique involving esterification of carboxyl groups of collagen by methanol, subsequent hydrazide formation and its conversion into acyl azide by reaction with nitrous acid. The immobilization itself of the enzyme is effected by immersing the activated membrane into the enzyme solution.

As a continuation of our earlier work[14,15] on the preparation of biosensors based on the use of the Ugi reaction[16] for the immobilization of enzymes on partially

hydrolyzed nylon mesh we focused in this study our interest on the possibility of an analogous preparation of an enzyme electrode based on a collagen membrane.

EXPERIMENTAL

Immobilization of Glucose Oxidase on Collagen Membrane and Preparation of Enzyme Electrode

A solution (10 μl) of glucose oxidase (beta-D-glucose: O_2-1-oxidoreductase, EC 1.1.3.4, *Asp. niger*, Serva, F.R.G.), prepared by dissolving 50 mg of the preparation in 1·0 ml of 0·1M potassium phosphate buffer, pH 5·8, 2·5 μl of 2·5% glutaraldehyde solution (Koch–Light Laboratories, Great Britain) and, if necessary, 1·0 μl of cyclohexyl isocyanide (Fluka, Switzerland) were applied to a 0·195 cm^2 area of a collagen membrane (100 μm, Cutisin, Jilemnice, Czechoslovakia) wetted with the above buffer. After a 24- to 120-h immobilization period in a wet chamber at 4 °C the membrane was exhaustively washed with 0·1M potassium phosphate buffer in which it was also stored. When preparing the enzyme electrode the membrane together with the immobilized enzyme was fixed on the surface of an oxygen sensor of the Clark type (Chemoprojekt, Czechoslovakia) with a Pt cathode 0·2 mm in diameter.

D-Glucose Determination

The 0·1M potassium phosphate buffer (2·8 ml), pH 5·8, saturated by atmospheric oxygen, and 100 μl of ten times diluted suspension of catalase (H_2O_2 : H_2O oxidoreductase, EC 1.11.1.6, 1 g in 24·8 ml; 3 400 U mg^{-1}, Reanal, Hungary) were pipetted into a thermostated (at 30°C) and stirred reaction vessel (volume 3·0 ml) with the enzyme electrode. The reaction was triggered by the addition of 100 μl of D-glucose solution (containing an equilibrium mixture of both anomers). The rate of oxygen decrease beyond the enzyme membrane, proportional to D-glucose concentration in the reaction mixture, was assayed by a nanoamperometer equipped with a stabilized supply of direct polarization voltage [−650 ± 10 mV] and an element for signal derivatization (Chemoprojekt, Czechoslovakia). The simple signal and its differential were recorded in a TZ 4200 Recorder (Laboratorní přístroje, Prague, Czechoslovakia).

The dependence of the activity of the enzyme electrode on pH and temperature were measured by the method described above using an excess of D-glucose (100 μl of 1M D-glucose) at pH 2·0 to 8·0 in 0·1M potassium phosphate buffer (pH adjusted by either phosphoric acid or potassium hydroxide) and over the temperature range of 20 to 50°C, resp. After each pH or temperature change the system was allowed to stabilize for 10 min.

RESULTS AND DISCUSSION

Glucose oxidase was immobilized on the collagen membrane by covalent bonding using glutaraldehyde either in the absence or in the presence of cyclohexyl isocyanide, according to recorded data[17].

The effect of the quantity of cyclohexyl isocyanide and of glutaraldehyde applied was examined during the reaction (Table I) for 2 days at 4°C while the quantity of enzyme as well as the length of the immobilization period were kept constant. The maximal activity value was attained with 2·5 μl of 2·5% aqueous solution of glutaraldehyde in the absence of cyclohexyl isocyanide.

The optimal length of the immobilization period (Fig. 1) was determined with collagen membranes modified by different procedures. The aim of these modifications (hydrolyses and partial mechanical disintegration of collagen fibers, Table II) was to cleave a certain number of peptide bonds and to increase the binding capacity for enzyme immobilization.

In other experiments several collagen membranes were kept in distilled water with repeated freezing and thawing (5 times). The mechanical disintegration of the collagen fibers was due to slowly growing small ice crystals. The alterations were discontinued by exhaustive washing of the membranes with distilled water and 0·1M potassium phosphate buffer, pH 7·0. Simultaneously with the hydrolyzed membranes

TABLE I

Dependence of activity of glucose oxidase immobilized on collagen membrane on quantity of cyclohexyl isocyanide and glutaraldehyde used for immobilization at 4°C for 2 days (CHIK cyclohexyl isocyanide, GA glutaraldehyde)

CHIK μl	2·5% GA μl	Activity %
0	1·0	71·3
1·0	1·0	45·3
2·0	1·0	23·7
0	2·5	100·0
1·0	2·5	54·3
2·0	2·5	48·6
0	5·0	89·2
1·0	5·0	52·7
2·0	5·0	31·0

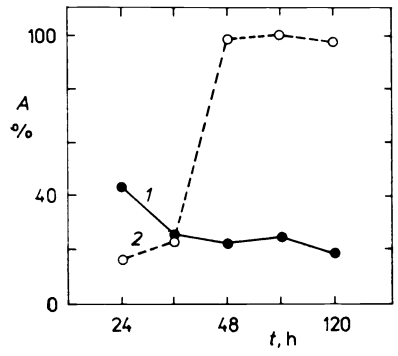

FIG. 1

Activity of glucose oxidase immobilized on collagen membrane as function of immobilization period. *1* Immobilization in presence of cyclohexyl isocyanide, *2* immobilization in absence of cyclohexyl isocyanide

collagen without any alterations except for wetting with the potassium phosphate buffer was also used for the immobilization. The latter was designated as "native".

TABLE II

Dependence of activity of immobilized glucose oxidase on mode of treatment of collagen membrane

Mode of treatment of collagen membrane	Glucose oxidase activity, %	
	immobilization in absence of CHIK	immobilization in presence of CHIK
Native	63	33
Repeated freezing	71	62
Urea[a]	100	52
Hydrochloric acid[b]	69	50
Lactic acid[c]	100	65
Lactic acid[d]	74	61
Pepsin[e]	28	28
Pepsin[f]	18	9

[a] 24-h treatment with 6M urea; [b] 24-h treatment with 5M hydrochloric acid; [c] 6-month treatment with 1M lactic acid at 4°C; [d] 6-month treatment with 5M lactic acid at 4°C; [e] 24-h treatment with 1% pepsin solution in 10 mM hydrochloric acid; [f] 24-h treatment with 5% pepsin solution in 10 mM hydrochloric acid.

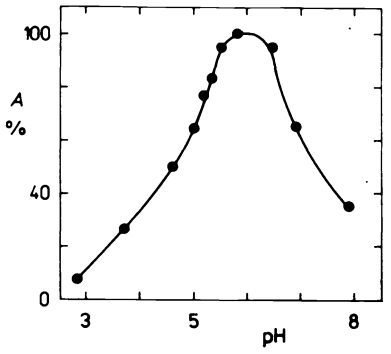

FIG. 2

Dependence of activity of glucose oxidase immobilized on collagen membrane on pH of reaction medium

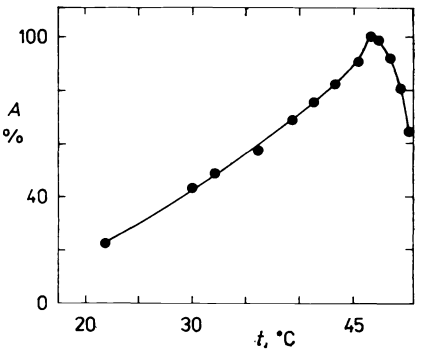

FIG. 3

Temperature dependence of activity of glucose oxidase immobilized on collagen membrane

The best results (Table II) were obtained after 96 h of immobilization of 10 µl of liquid glucose oxidase together with 2·5 µl of 2·5% glutaraldehyde solution in the absence of cyclohexyl cyanide using etiher the collagen membrane partially hydrolyzed by long-term treatment with 1·0M lactic acid or with the membrane which was placed for 24 h in 6M urea. Unlike in earlier experiments with the enzyme immobilized on nylon mesh[14,15] the activity of the enzyme always decreased markedly in all cases when the immobilization was carried out in the presence of cyclohexyl isocyanide. Activities of comparable magnitude were obtained with glucose oxidase immobilized on collagen disintegrated by repeated freezing and thawing, by treatment with 5·0M hydrochloric acid and with 5·0M lactic acid. These values were on the average by 10% higher than the activity of the enzyme immobilized on native collagen when the procedure was carried out in the absence of cyclohexyl isocyanide. Hydrolysis by 1% pepsin solution in 10 mM hydrochloric acid was not useful. The activity of immobilized glucose oxidase was by one half or even by two thirds lower than the activity of the enzyme immobilized on native collagen.

In all our subsequent experiments we used exclusively membranes after long-term treatment with 1M lactic acid or one-day treatment with 6M urea. The immobilization

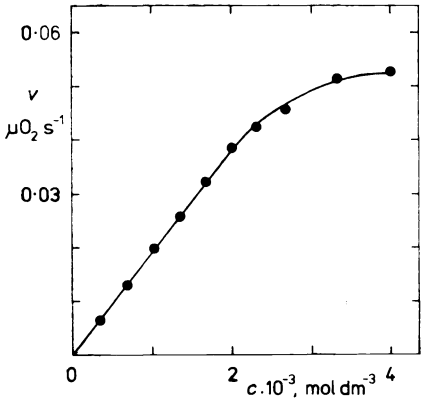

FIG. 4

Calibration curve for determination of D-glucose by biosensor based on glucose oxidase immobilized on collagen membrane. *v* Rate of oxygen decrease (µmol oxygen s^{-1}), *c* concentration of D-glucose in reaction vessel (mol dm^{-3})

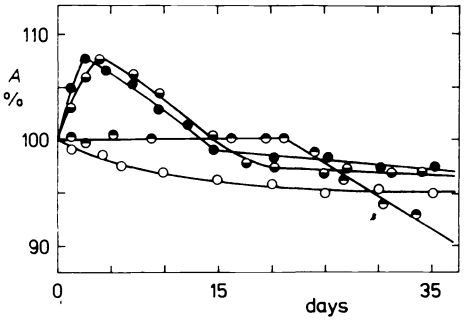

FIG. 5

Stability of enzyme electrodes as function of time. *A* Relative enzyme activity, glucose oxidase immobilized on collagen membrane, ● activated by urea in absence of cyclohexyl isocyanide, ○ activated by urea in presence of cyclohexyl isocyanide, ◒ activated by lactic acid in absence of cyclohexyl isocyanide, ◓ activated by lactic acid in presence of cyclohexyl iocyanide

was effected in a wet chamber for 96 h. Glucose oxidase immobilized on the collagen membrane was characterized by determination of the plot of activity versus pH of the reaction medium (Fig. 2). Compared to the pH-optimum of the free enzyme[18,19] (pH_{opt} = 6·5) a slight shift was observed with the immobilized conjugate toward the acid range (pH_{opt} = 5·8 to 6·3). The examination of the effect of temperature on the magnitude of the electrode response showed that the reaction rate was maximal at 46°C (Fig. 3). A rapid drop of the enzyme activity was observed above this value. In an effort to achieve the highest stability and with respect to our previous findings[20] we chose the temperature of 30°C for all D-glucose assays. The upper limit of the linear range of the calibration curve (pH 5·8; 30°C) was 60 mM D-glucose in injected sample, i.e. $2{\cdot}0 \cdot 10^{-3}$M D-glucose in the reaction vessel; the lower limit of reliably determinable concentration was $5{\cdot}0 \cdot 10^{-4}$M in injected sample, i.e. $1{\cdot}7 \cdot 10^{-5}$ glucose in the vessel (Fig. 4). The value of the apparent Michaelis constant $K_{M(app)}$, determined by the Gauss–Newton method of nonlinear regression from the calibration curves ($K_{M(app)} = 4{\cdot}15 \cdot 10^{-3}$ mol l^{-1}) was comparable to recorded data both for the free enzyme[18,19] ($K_{M(app)} = 33-110 \cdot 10^{-3}$ mol l^{-1}) and also for the enzyme immobilized on a membrane[21] ($K'_{M(app)} = 1{\cdot}6 \cdot 10^{-3}$ mol l^{-1}).

When examining the stability of the enzyme electrodes we performed 10 to 50 analyses daily. The electrode extension with the enzyme membrane was stored in 0·1M potassium phosphate buffer, pH 5·8 at 4°C. The value of the slope of the linear portion of the calibration curve was taken to represent 100% immediately after the immobilization. Thirty days later during which the stability of the enzyme electrodes was examined in the course of about 500 analyses (Fig. 5), the activity of the immobilized enzyme dropped by merely 3% of the original value. An initial increase of enzyme activity during the first 5 to 9 days after the immobilization was observed with these electrodes. The different modes of treatment of the collagen membranes were without effect on the character of these curves. No activity increase was observed when electrodes with glucose oxidase immobilized in the presence of cyclohexyl isocyanide were used (Fig. 5).

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Translated by V. Kostka.