AMPEROMETRIC ENZYME ELECTRODES FOR SUBSTRATES OF IMMOBILIZED PYRANOSE OXIDASE

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Two kinds of biosensors for the determination of pyranose oxidase substrates were developed, based on the detection of evolving hydrogen peroxide on a platinum or platinized graphite electrode at +650 or +400 mV, respectively. The membranes consisted of enzyme immobilized by covalent bonds on nylon net and were stable for 8 months of dry storage at 4 °C. In addition to D-glucose, low concentrations of D-xylose, D-galactose and L-sorbose can also be measured with the biosensor. The shift of the optimum pH of the immobilized enzyme to the alkaline region (8.0 – 9.5) is convenient for the borate buffer medium which extends the linear concentration region of the biosensor to 15 mmol l^{-1} for D-galactose, 30 mmol l^{-1} for D-xylose and 30 mmol l^{-1} for maltose. L-Sorbose provides no response up to a concentration of 10 mmol l^{-1} in 0.05 M borate and up to a concentration of 30 mmol l^{-1} in 0.2 M borate at pH 9.2. Interfering D-glucose was eliminated up to 2.5 mmol l^{-1} by means of an enzyme pre-membrane with immobilized hexokinase. The effect of ascorbate was eliminated, up to 75 mmol l^{-1}, by using a cellulose acetate electrostatic barrier. D-Galactose, however, decreases the sensor response to D-glucose.

Pyranose oxidase (PROD), also referred to as glucose-2-oxidase, was first purified to homogeneity from *Polyporus obtusus*[1,2], and its activity was subsequently found in mycelial cultures of 40 Basidiomyceta species[3]. The substrate specificity, pH optimum and thermal stability have been determined for PROD isolated from *Phanerochaete chrysosporium*[4,5]. This is one of the four enzyme types oxidizing D-glucose as the main substrate. In contrast to flavoprotein glucose-1-oxidase, NAD^+-dependent and quinoprotein dehydrogenase, PROD transforms D-glucose to D-glucosone[2,5]; hydrogen peroxide is formed as the second product from the consumed O_2. In addition to D-glucose the enzyme is also capable of oxidizing D-xylose, D-gluconolactone and L-sorbose, the low specificity, however, does not rule out analytical applications of the enzyme to the determination of total monosaccharide in sample. PROD has been applied as the recognition element in the flow determination of pyranoses in cellulose hydrolyzate[6], and a clinical test with soluble PROD simplifies the determination of 1,5-D-anhydroglucitol in blood plasma within the diagnosis of diabetes[7]. A glucose biosensor based on the

Clark oxygen sensor and PROD immobilized on an acetylcellulose membrane has also been developed[8].

The present paper deals with additional design principles of a single-enzyme electrode for the detection and determination of PROD substrates. The possibility of increasing the specificity of the biosensors by a suitable arrangement of the biorecognition layers and a suitable choice of the environment was also examined.

EXPERIMENTAL

Material. Pyranose oxidase EC (1.1.3.10.) with a specific activity of 23.3 U per mg was isolated from the mycelium of *Phanerochaete chrysosporium*[5]. Hexokinase (EC 2.7.1.1) with a specific activity of 140 U per mg (substrates: ATP, glucose) was supplied by Boehringer Mannheim (Germany). The nylon net (77 mesh cm^{-2}, 100 μm thickness) was a product of Bozzone (Appiano Gentile, Italy). Platinized graphite paper (PGP, platinum content 0.40 mg cm^{-2}) was obtained from E–Tek (Framingham, MA, U.S.A.). D-Glucose anomers were prepared according to Hudson and Dale[9]; the β-anomer was crystallized from pyridine, the α-anomer from acetic acid at room temperature. δ-D-Gluconolactone was synthesized by Spolek pro chemickou a hutni vyrobu (Prague, The Czech Republic). The other chemicals used were products of Lachema (Brno, The Czech Republic) supplied in the reagent grade purity. Lepox epoxy resin (Lachema Neratovice, The Czech Republic) and silver-containing conductive epoxy resin (Emerson and Cunning, Milan, Italy) were employed for manufacturing the electrodes. The homogeneity of the saccharides was tested by TLC on a Silufol foil. The standard glucose solution, 1 mol l^{-1}, was preserved with 0.02% sodium azide.

Immobilization of enzymes. *a*) Lysine was first bonded to the nylon net activated with dimethyl sulfate, and PROD was bonded to the lysine terminal amino group via glutaraldehyde as in ref.[10]. Membranes were stored in the dry state at 4 °C.

b) PROD was applied to the surface of PGP and allowed to dry at room temperature. Subsequently, the same amount of PROD was applied again and crosslinked with glutaraldehyde (total applied PROD activity was 80 U per cm^2). The top of the electrode was covered with a dialysis membrane and the electrode was stored dry at 4 °C.

c) Anti-interference hexokinase membrane was prepared by crosslinking hexokinase (70 U per cm^2) with bovine serum albumine and glutaraldehyde on a polyamide net[11].

An anti-interference acetylcellulose membrane (free from enzyme) was prepared following Mascini and coworkers[12].

Instrumentation. Amperometric electrodes of two kinds were employed:

a) A combined electrode manufactured by Krejci Engineering (Tisnov, The Czech Republic), consisting of a Pt-disk (0.6 mm in diameter) and a large-area Ag/AgCl reference electrode. A plastics jacket with a fixed enzyme membrane can be attached to the electrode system. The internal compartment of the jacket is filled with an electrolyte, viz. 3 M KCl for measuring O_2 at –650 mV, or 0.05 M phosphate at pH 7.3 for measuring H_2O_2 at +650 mV. For the oxygen measurement, the enzyme membrane rests on a Teflon membrane 15 μm thick. In all measurements the enzyme membrane was coated with a dialysis membrane 20 μm thick facing towards the working solution.

b) In the electrode of the other kind, the enzyme was immobilized on a PGP disk 5 mm in diameter, which was fixed with epoxy resin to a glass tube. The wire was fixed to the PGP by means of the conductive resin. The PGP electrode was polarized to a potential of +200 to +650 mV (vs the Ag/AgCl/3 M KCl electrode), with a simultaneous use of an auxiliary Pt electrode.

The electrodes were submerged in 3.5 ml of buffer accommodated in a thermostatted cell (30 °C) and magnetically stirred. The measurements were accomplished in the two-electrode (Pt electrode) or

three-electrode (PGP sensor) connection. An ADLC2 amperometric detector interfaced to a TZ 4200 recorder (both Laboratorni pristroje, Prague, The Czech Republic) and a digital voltmeter served as the potentiostat.

RESULTS AND DISCUSSION

Characteristics of the Biosensors

The basic properties of the two kinds of PROD electrode are compared in Table I. The assets of the PGP-based electrodes include a rapid and easy immobilization of the enzyme, rapid response, higher sensitivity, and the possibility of reducing the working potential to +400 mV; their use, however, is limited by a low storage stability (the response decreases by 65% in 7 days of dry storage at 4 °C). The enzyme electrodes with PROD covalently bonded on nylon, on the contrary, are highly stable: no measurable decrease in response was found in 8 months of dry storage at 4 °C, and the stability in 24 h of storage in phosphate (0.05 mol l^{-1}), pH 7.3, at 4 °C was 95%. The latter membranes were used in the subsequent measurements.

The attempt to prepare a functioning biosensor by fixing a thin layer of *Phanerochaete chrysosporium* mycelium to the electrode between two dialysis membranes or directly to the electrode surface by means of the nylon net and a dialysis membrane failed. The sensor responded to none of the PROD substrates tested, apparently due to the low specific activity of PROD in the mycelium[4]. The maximum activity of PROD in the mycelium has been reported to be 50 U per g of dry matter (0.05 M phosphate at pH 6.5, glucose as the substrate[5]), which, for the amount of wet mycelium used, corresponds to a PROD content which is at least 20-fold to 30-fold lower than in the immobilized enzyme membrane.

TABLE I
Chemometric characteristics of two kinds of PROD electrodes in the determination of D-glucose in 0.05 M phosphate at pH 7.3

Parameter	PGP (+400 mV)	Nylon (+600 mV)
Linearity, mmol l^{-1}	0 – 4.5	0 – 1.5
Sensitivity, μA l mol^{-1}	55.0	23.8
Response time[a], τ_{95}, s	25	80
Detection limit, μmol l^{-1} (signal/noise = 3)	3.0	3.0
Variation coefficient[a], %	2.5	3.2

[a] For a D-glucose concentration of 0.3 mmol l^{-1}.

As compared to the soluble PROD (refs [4,5]), which exhibits a wide pH optimum with the highest activity at about pH 7.5, the pH optimum of the immobilized PROD sensor is shifted to the alkaline region, both for D-glucose and D-xylose (Fig. 1). This could be made use of for measurements in alkaline borate buffer (see later). The electrode response only drops appreciably at a pH as high as 11.

We attempted to increase the electrode sensitivity by using trapping reagents for the PROD-catalyzed reaction product – glucosone. This can be converted with phenylhydrazine at room temperature to osazone, or with *o*-phenylenediamine in a strongly alkaline solution to 2-(tetrahydroxybutyl)quinoxaline[13]. Experiments with ethylenediamine, *o*-phenylenediamine and *trans*-1,2-diaminocyclohexane, however, failed, and the hydrazine derivatives used inhibited the enzyme reaction.

Biosensor Selectivity

Available data concerning the substrate specificity of PROD are often controversial[2,5,7,8]. There is consensus in that D-glucose, 6-deoxy-D-glucose, D-xylose, L-sorbose and δ-D-gluconolactone are good substrates. Our measurements confirmed that D-galactose, D-mannose, L-arabinose, maltose and myo-inositol[6,8] give a response as well (Table II), although the low sensitivity of the sensor limits its analytical applicability (except for D-galactose and maltose). For instance, the affinity of PROD for mannose is comparable with that of glucose-1-oxidase[14] (GOD).

D-Glucose is a preferred PROD substrate. As with GOD (ref.[8]), the β-D-anomer is a specific substrate for the enzyme from *Polyporus obtusus*, as was found after the apparent affinity for the α-anomer was corrected for mutarotation[2]. In agreement with the specificity of the enzyme from *Coriolus versicolor*[15], however, there was observed no

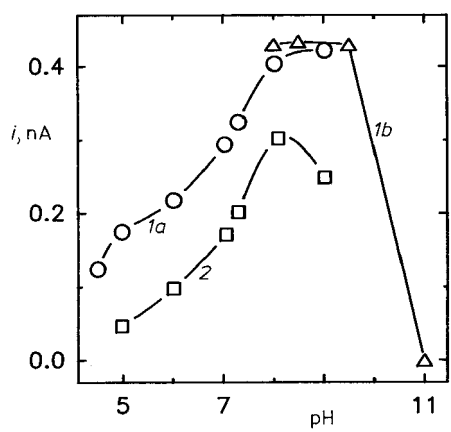

FIG. 1
Current vs pH plot for a Pt electrode with PROD covalently bonded on nylon net, for glucose (0.29 mmol l^{-1}) in 0.05 M phosphate (*1a*) and in 0.05 M diphosphate (*1b*) and for D-xylose (2.86 mmol l^{-1}) in 0.05 M phosphate (*2*); polarization potential +650 mV

significant difference in the response of the pO_2 biosensor with immobilized PROD (*Phanerochaete chrysosporium*) to the two anomers even in freshly prepared solutions.

Effect of Glucose and Galactose on the Detection of Other Saccharides

Calibration of the electrode with L-sorbose and D-xylose at various glucose concentrations in the solution revealed that up to a concentration of 1 mmol l^{-1}, D-glucose reduces the sensitivity of the sensor to those saccharides by 50% (while preserving the linearity range), and at 1.5 mmol l^{-1} the sensor fails to respond to additional amounts of the substrates. The effect of D-galactose is different. When the electrode response to D-glucose attains its steady state, addition of D-galactose reduces the current of anodic oxidation of peroxide. The dependence of that decrease on the concentration of D-galactose is linear up to a D-galactose concentration of 10 mmol l^{-1} (Fig. 2). Moreover, at D-glucose concentrations in excess of 5 mmol l^{-1} the decrease is independent of the activity of the enzyme immobilized on the electrode. Quantitation of galactose based on its inhibiting effect has a wider linearity range than the direct measurement of galactose with the PROD electrode (the sensitivity, expressed in terms of the calibration straight line slope, is about 40% lower).

Interferences

When quantitating saccharose, the D-glucose present was eliminated up to 2 mmol l^{-1} by means of a membrane with co-immobilized GOD and catalase[16]. A similar PROD system in combination with catalase is not efficient in our case due to the production of

TABLE II
Parameters of PROD electrode for various substrates using detection of H_2O_2 at +650 V. Enzyme covalently bonded on nylon, medium of 0.05 M phosphate at pH 7.3

Substrate	Linearity, mmol l^{-1}	Sensitivity, μA l mol^{-1}	Detection limit, μmol l^{-1}
D-Xylose	0 – 5.0	1.2	85
D-Galactose	0 – 5.0	0.8	56
L-Sorbose	0 – 25.0	1.2	85
Maltose	0 – 2.0	0.4	170
δ-D-Gluconolactone	0 – 18.0	0.08	280
L-Arabinose	0 – 15.0	0.04	280
D-Mannose	0 – 10.0	0.04	280
Myo-inositol	0 – 45.0	0.03	800

δ-gluconolactone, which is also a PROD substrate (Table II). An alternative approach consists in the use of a membrane with immobilized hexokinase (EC 2.7.1.1, henceforth HK) which, in the presence of ATP and $MgCl_2$, can eliminate glucose by phosphorylation to glucose-1-phosphate[11]. We examined the optimum procedure for immobilizing HK and the design of the anti-interference layer in front of the measuring electrode (Fig. 3). The results with HK bonded to the nylon net were unsatisfactory even if three anti-interference layers and a dialysis membrane were used, presumably due to an insufficient activity of the enzyme. HK immobilized by crosslinking with glutaraldehyde on a polyamide net, on the other hand, eliminated glucose up to a concentration of 2.5 mmol l^{-1}; similarly, D-xylose was eliminated up to 2 mmol l^{-1}. The anti-interference membrane with HK exhibited no measurable affinity to D-galactose or L-sorbose.

Since the results of analysis applying an external potential of +650 mV may be affected by the presence of additional electro-active substances in the biological sample, the elimination of ascorbic acid was also investigated. The simplest approach consists of the use of a membrane prepared from a solution of cellulose acetate, which con-

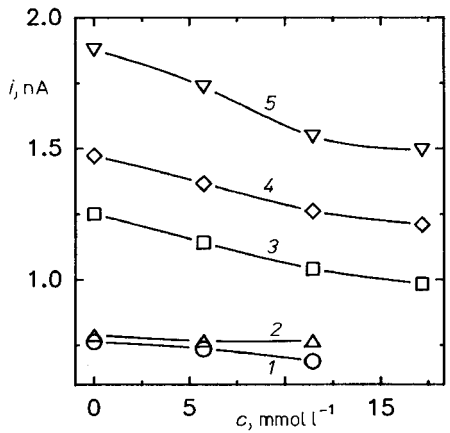

FIG. 2
Decrease in the PROD electrode current response to D-glucose due to the inhibiting effect of D-galactose. Medium of 0.05 M phosphate at pH 7.3, working potential +650 mV. Glucose concentration (mmol l^{-1}): *1* 1.43, *2* 2.86, *3* 8.55, *4* 14.3, *5* 28.5

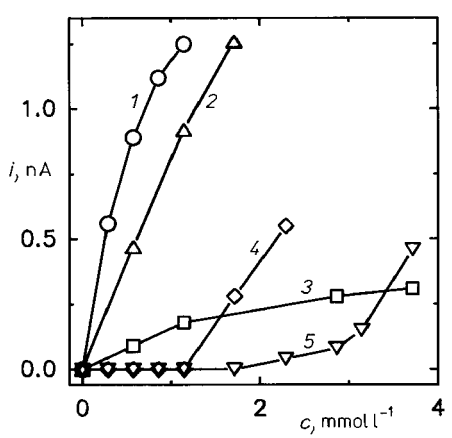

FIG. 3
Response of the PROD electrode to D-glucose using an anti-interference hexokinase membrane. Medium of 0.05 M phosphate of pH 7.3 and 0.02 M $MgCl_2$, working potential +650 mV. Arrangement: *1* without HK membrane, *2* HK covalently bonded on nylon, in a medium of ATP (15 mmol l^{-1}), *3* – *5* HK crosslinked with glutaraldehyde in the presence of ATP in a concentration of: *3* 0, *4* 5, *5* 15 mmol l^{-1}

stitutes an efficient electrostatic barrier to ascorbate. The enzyme electrode with such an anti-interference barrier was insensitive to ascorbate up to 75 mmol l^{-1}.

Effect of Borate Buffer on the Determination of Pyranoses

The electrode responses with the PROD electrode in phosphate buffer at pH 7.3 did not differ for the two D-glucose anomers. When measuring in 0.05 M borate at pH 9.2, the response to the β-anomer (which constitutes 63% of the equilibrium solution) had a two-stage character, after removing the dialysis membrane the peak shape was even similar to that with the membrane with GOD (ref.[17]). This effect was still more marked if a known amount of the solid anomer was added to the reaction vessel: there the increase in the steady-state current of oxidation of H_2O_2 was higher for the α-anomer. The effect of the basic borate buffer, however, appears to some extent for all of the substrates tested (Figs 4*a* and 4*b*): as compared to the phosphate medium at the same pH, the response of the sensor to an amount of analyte is lower; at the same time, the linearity span is wider. The borate anion generally forms with polyols complexes possessing the 1 : 1 stoichiometry (1 : 2 at high polyol concentrations). Complexes involving *cis*-hydroxyls at C1 and C2 or C4 and C6 are thermodynamically most favourable in the case of pyranoses[18,19]. The *cis*-1,2-diol configuration occurs in the α-anomers of D-glucose, D-galactose and D-xylose, whereas complexation to C4 and C6 can be expected in D-gluconolactone and maltose.

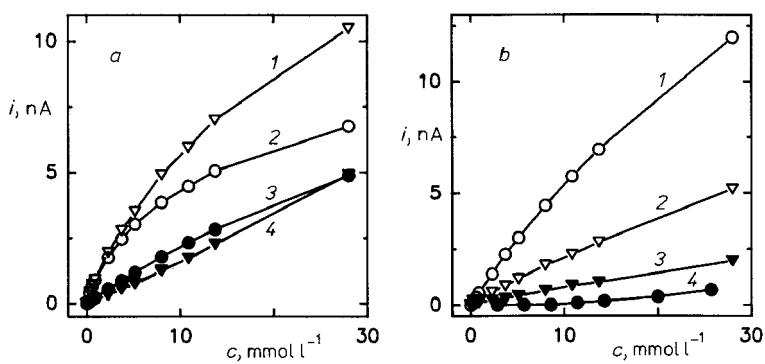

FIG. 4
Extension of the concentration region of the PROD biosensor at +650 mV using 0.05 M phosphate (○,▽) and 0.05 M borate (●,▼) buffer at pH 9.2. *a 1*, *4* D-Xylose, *2*, *3* D-galactose; *b 1*, *4* L-sorbose, *2*, *3* maltose

L-Sorbose probably behaves in a manner similar to that of D-fructose (a substantially higher fraction is bonded in borate complexes[19]). It is oxidized by the enzyme at C5, 5-keto-D-fructose being the reaction product[2]. In 0.05 M borate at pH 9.2 the electrode gave a response at L-sorbose concentrations above 10 mmol l^{-1}. The lower limit of detection of L-sorbose depends on pH and on the buffer concentration (10 to 35 mmol l^{-1}); the electrode does not saturate within the concentration region measured (up to 150 mmol l^{-1}), the calibration curves have concave shapes. The formation of highly stable complexes thus can account for the different results obtained in the measurement of L-sorbose (Fig. 5).

The electrode sensitivity decreases further in a more concentrated borate buffer and in buffer at a higher pH. Similar results were obtained with PROD electrodes sensitive to hydrogen peroxide or sensitive to O_2. The electrode phenomena occurring in borate buffer apparently include the effect of the diffusion barrier at the electrode. The character of the electrode response seems to be determined by the differences in the diffusion rates of the substrates and their borate complexes as well as in their oxidation rates.

CONCLUSIONS

The glucose biosensor based on amperometric detection of hydrogen peroxide produced by pyranose oxidase bonded on activated nylon net exhibits roughly the same sensitivity to D-glucose as a similar sensor containing glucose oxidase. Its lower selectivity, on the other hand, enables saccharides which are more difficult to quantitate, such as D-xylose, D-galactose and L-sorbose, to be determined as well. In such cases the interfering effect of glucose can be eliminated by means of an anti-interference membrane containing hexokinase in the presence of ATP in the reaction medium. For other potential substrates the biosensor is not sufficiently sensitive. The stability of the sensor

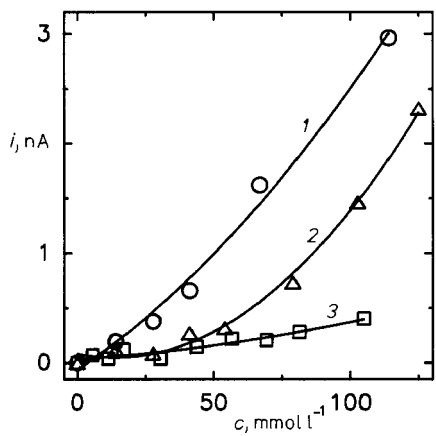

FIG. 5
Current vs concentration plot for L-sorbose, measured with the pO_2 cell with immobilized PROD (potential of −650 mV) in borate at pH 9.2. Borate concentration (mmol l^{-1}): *1* 50, *2* 100, *3* 200

also in basic borate allows the working concentration region for the saccharides to be widened substantially.

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