PREPARATION AND UTILIZATION OF A BIOSENSOR BASED ON GALACTOSE OXIDASE

Eva VRBOVÁ, Jitka PECKOVÁ and Miroslav MAREK

Department of Biochemistry and Microbiology, Prague Institute of Chemical Technology, 166 28 Prague 6

Received February 6, 1992
Accepted March 18, 1992

An enzyme electrode for D-galactose determination was prepared by fixation of a carrier with immobilized galactose oxidase (E.C. 1.1.3.9) or coimmobilized galactose oxidase and catalase (E.C. 1.11.1.6) to a Clark-type oxygen sensor. The enzymes were immobilized either on a partially hydrolyzed nylon mesh or on a native collagen membrane using the Ugi reaction with cyclohexyl isocyanide and glutaraldehyde. The biosensors were characterized by the specific activity of the immobilized galactose oxidase, the apparent Michaelis constant $K_{M(app.)}$, and the stability expressed by time and a number of the performed analyses. The substrate specificity of the biosensor and the effect of pH and temperature of the reaction mixture on the response magnitude were also tested. The prepared biosensor was used for the determination of D-galactose content in samples of blood plasma and serum of patients with suspected galactosemia.

In recent years many applications of glucose oxidase for analytical purposes have been described. This enzyme may be used in combination with a Clark-type oxygen sensor in a biosensor for D-galactose determination, similarly as glucose oxidase is used for determination of D-glucose. Contrary to D-glucose, under catalysis with galactose oxidase (D-galactose : O_2-6-oxidoreductase) D-galactose is oxidized on the sixth carbon atom to give galactosedialdose[1]. Since the oxidation of D-galactose does not require any free hemiacetal hydroxyl group, the use of galactose oxidase makes it possible to directly determine galactosides and oligosaccharides (including not reducing ones) which contain D-galactose in their molecule[2]. Galactose oxidase is substantially less specific than glucose oxidase[3,4]. It is also active in oxidation of e.g. D-talose, L-altrose, 2-deoxy-L-*lyxo*-hexose (2-deoxy-D-galactose), 2-amino-2-deoxy-D-galactose, etc., an important condition being the orientation of the hydroxyl group on the fourth carbon atom (C-4). On the contrary, D-glucose, which differs from D-galactose in configuration on C-4, does not undergo oxidation with galactose oxidase; this may be of considerable importance in view of occurence of the individual saccharides in samples of the native material.

For the preparation of biosensors, galactose oxidase was immobilized on the surface of an acetylcellulose membrane[5], a nylon mesh[6], and a collagen membrane[7,8]. The im-

mobilization procedures were time-consuming and the stability of these biosensors was low.

Our previous studies were devoted to preparation of biosensors for determining D-glucose[9,10], vitamin C (ref.[11]) and alcohols[12] by immobilization of the corresponding enzymes on a nylon net or collagen membrane using the Ugi reaction. As a continuation, we have now investigated the application of this method to the preparation of a biosensor based on galactose oxidase.

EXPERIMENTAL

Immobilization of Enzymes on Nylon Mesh[10] and Preparation of Biosensor

A nylon mesh was partially hydrolyzed with 100 μl of 25% HCl at 20 °C for 1 s, washed thoroughly with distilled water and dried. On the hydrolyzed area (0.2 cm^2) there were applied 20 μl of a solution of galactose oxidase (D-galactose : O_2-6-oxidoreductase, E.C. 1.1.3.9, 12.5 U mg^{-1}, lyophilized preparation from *Dactylium dendroides*, Sigma, U.S.A.) prepared by dissolution of a powdered sample (1.6 mg) in 0.1M potassium phosphate buffer, pH 7.0 (100 μl), 2.5% solution of glutaraldehyde (5 μl; Koch–Light Laboratories, Great Britain), and cyclohexyl isocyanide (1 μl; Fluka, Switzerland) and, if needed, also 5 μl of a suspension of catalase (H_2O_2 : H_2O_2–oxidoreductase, E.C. 1.11.1.6, 2 000 U mg^{-1}, 1 g = 24.8 ml; Reanal, Hungary). After mixing, the enzyme (or enzyme mixture) was incubated in a moist chamber at 4 °C for 42 – 168 h. Then the mesh with the bound enzymes was washed with the above-mentioned buffer, fastened in a holder and fixed on the surface of a Clark-type oxygen sensor (SOPS 31, Chemoprojekt, Czechoslovakia) with a 0.2 mm diameter platinum cathode.

Immobilization of Enzymes on Collagen Membrane[13] and Preparation of Biosensor

The same amount of galactose oxidase or its mixture with catalase as in the above experiment was immobilized on a 100 μm thick collagen membrane (area 0.2 cm^2; Cutizin, Czechoslovakia) either untreated or activated with 1M lactic acid for 6 months or with 6M urea for 24 h) that had been thoroughly washed with distilled water and partially dried using the same procedure as described for the immobilization on the nylon mesh. From the collagen membrane a small disc was cut which was fixed on a Clark-type oxygen sensor by means of a holder.

Determination of Saccharides

Potassium phosphate buffer, pH 7.0, (1.4 ml of a 0.1M solution) was added into a thermostatted (30 °C) reaction vessel with immersed biosensor. In case of the measurement with free catalase, the reaction vessel contained 1 300 μl of potassium phosphate buffer and 100 μl of a ten times diluted suspension of catalase. Under stirring and saturation by air oxygen, a sample (100 μl) of the corresponding saccharide was added. The enzyme electrode signal was measured by a nanoamperometer with a stabilized direct polarization voltage source (−650 ± 10 mV) and a signal derivation element. The simple signal (current drop vs time) as well as its differential, were registered using a TZ 4200 recorder (Laboratorní přístroje, Czechoslovakia). From the height of the differential record or the initial slope of the simple curve the calibration curve was constructed (for standard solutions) which then served for determination of the corresponding saccharides in the analyzed samples.

Determination of Immobilized Galactose Oxidase Activity

The activity of the immobilized enzyme was measured after the fixation of the carrier with galactose oxidase or its mixture with catalase on the oxygen sensor according to the above-described method (0.1M potassium phosphate buffer, pH 7.0, 30 °C) in an excess of D-galactose (30 mmol l^{-1}).

Characterization of Enzyme Electrodes

The determination of optimum pH was carried out in an aerated 0.1M potassium phosphate buffer (1.4 ml) at 30 °C in the pH range 2.5 to 8.0 (the limit pH values were adjusted with 1M phosphoric acid and 1M potassium hydroxide), with a standard D-galactose concentration (6.67 mmol l^{-1}).

The effect of temperature was studied in the region 20 – 50 °C at pH 7.0, using the above-described method. The D-galactose concentration amounted to 6.67 mmol l^{-1}. After each change of pH or temperature, the system was allowed to stabilize for 10 min.

RESULTS AND DISCUSSION

The amounts of compounds used in the immobilization resulted from an optimization study[14] on the ratio of the enzymes, glutaraldehyde and cyclohexyl isocyanide. Of the immobilization procedures tested, only immobilizations on a nylon mesh and untreated collagen membrane were successful. Activation of the collagen membrane either with lactic acid or urea proved to be disadvantageous.

The activity of the immobilized galactose oxidase depended on the immobilization time: we found 64 h at 4 °C (Fig. 1) to be the optimal time.

During the measurements we observed that in the absence of catalase the immobilized enzyme was inhibited by the arising H_2O_2. The presence of catalase prolonged the stability of the biosensor, shortened duration of the analysis (because of faster stabilization of the current baseline) and, above all, enabled a more accurate determination without frequent verification of the response to standard solutions. Fixation of a carrier with coimmobilized galactose oxidase and catalase to the oxygen sensor afforded a very sensitive and stable biosensor.

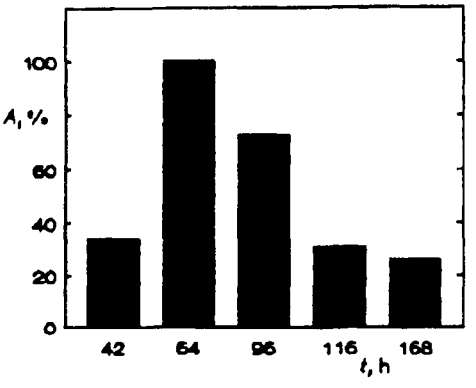

FIG. 1
Dependence of activity of nylon mesh-immobilized galactose oxidase on duration of immobilization

The specific activity of the immobilized enzyme on a nylon mesh, determined in the presence of free catalase, amounted to 5.50 U mg^{-1}, in the presence of coimmobilized catalase 9.30 U mg^{-1}, the activity of enzyme immobilized on the native collagen membrane was 4.70 U mg^{-1} (with free catalase). The response depended linearly on the substrate concentration in the region 10^{-4}M to 8 . 10^{-2}M – 0.1M of D-galactose in the injected sample (i.e. 6.67 . 10^{-6}M and 5.3 . 10^{-3}M to 6.67 . 10^{-3}M of D-galactose in the reaction vessel, respectively), the lower limit of the interval being related to determinations in the absence of catalase (Fig. 2).

From the plot of activity of immobilized galactose oxidase vs pH (Fig. 3) we found the pH optimum to be about 7.0 (immobilization on the nylon mesh) or 7.1 – 7.4 (immobilization on the collagen membrane). These values practically agree with the literature data[15] stating for free galactose oxidase (from *Dactylium dendroides*) the value pH 7.0 – 7.3.

The measured values of temperature dependence of galactose oxidase activity (Fig. 4) show that in the case of immobilization on the nylon mesh maximum activity was achieved at 41.5 °C whereas with the collagen membrane the maximum was at 45 °C.

From the calibration curves (Fig. 2) (i.e. the dependence of oxygen decrease rate in the reaction mixture on substrate (D-galactose) concentration) we determined the apparent Michaelis constant $K_{M(app.)}$ for galactose oxidase immobilized on the nylon mesh (1.93 . 10^{-2} mol dm^{-3}) and on the collagen membrane (1.97 . 10^{-2} mol dm^{-3}), using the Gauss–Newton iteration method of nonlinear regression. These values are

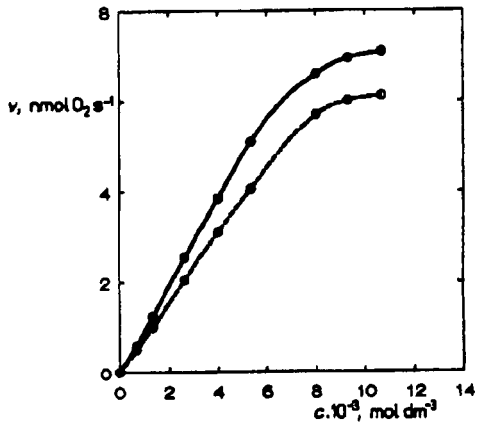

FIG. 2
Calibration curves for determination of D-galactose. v Rate of oxygen decrease in the reaction mixture (nmol oxygen s^{-1}), c D-galactose concentration in the reaction mixture (nmol dm^{-3}), ● immobilization on nylon mesh, ○ immobilization on collagen membrane

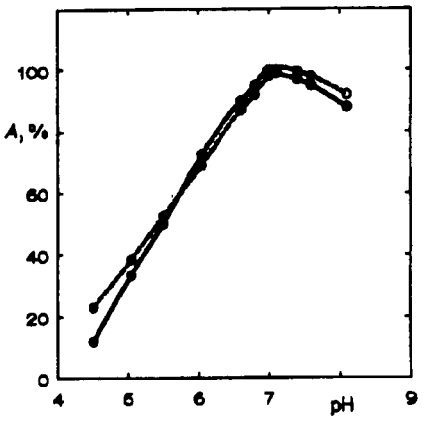

FIG. 3
Dependence of galactose oxidase activity on pH of the reaction medium. ● Immobilization on nylon mesh, ○ immobilization on collagen membrane

almost identical with the value of K_M stated[14] for the free enzyme ($2.0 . 10^{-2}$ mol dm^{-3}). For the coimmobilized system galactose oxidase–catalase on the nylon mesh the apparent $K_{M(app.)}$ was $2.73 . 10^{-2}$ mol dm^{-3}.

As concerns stability, we found that the immobilized galactose oxidase retains its activity for a long time (even after 30 months after immobilization on the nylon mesh, the enzyme was highly active) but only if it was stored in 0.1M potassium phosphate buffer, pH 7.0, at 4 °C, when it was not used for measurements. During measurement the enzyme was deactivated with the arising hydrogen peroxide and the biosensor stability was thus influenced by the total number of analyses. In 13 days during which about 200 analyses were carried out the activity of immobilized galactose oxidase dropped to 25% of its original value. With coimmobilized galactose oxidase and catalase no inactivation was observed because the arising hydrogen peroxide was decomposed with the catalase. In such case, the enzyme activity dropped to 25% only after 30 days and after 500 analyses. The parameters of the galactose biosensor are given in Table I.

We also paid attention to the question of substrate specificity of the prepared biosensor. The specificity was studied using the following compounds: D-galactose, D-xylose, L-lyxose, xylitol, dulcit, lactose, raffinose, 2-deoxy-D-lyxo-hexose, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, methyl 3,4-O-isopropylidene-β-D-galactopyranoside, methyl 6-deoxy-3,4-O-isopropylidene-α-D-galactopyranoside and methyl 2,3-di-O-(*p*-toluenesulfonyl)-α-D-galactopyranoside. Table II lists the relative rates of oxidation and apparent Michaelis constants $K_{M(app.)}$ (Fig. 5) for the most common D-galactose-containing oligosaccharides and basic galactopyranosides. For comparison, Table II lists also published data on relative rates of oxidation of substrates with galactose oxidase from various microorganisms because the substrate specificity of the enzyme depends on its source. The values measured by us agree well with those for free extracellular galactose oxidase from the same microorganism[4].

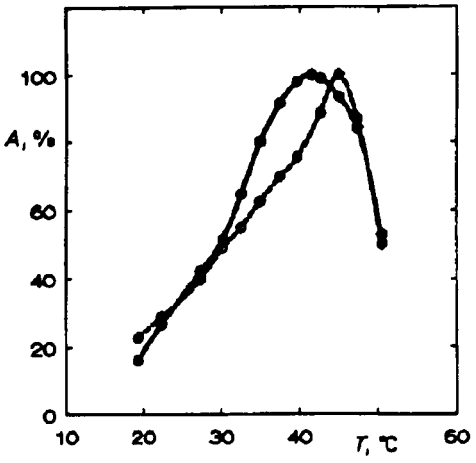

FIG. 4
Temperature dependence of galactose oxidase activity. ● Immobilization on nylon mesh, ○ immobilization on collagen membrane

However, this relatively broad substrate specificity of the enzyme[2-4], and thus also of the prepared biosensor, has no adverse effect on the determination of D-galactose in blood plasma[5] or in blood serum[16]. The biosensor with coimmobilized galactose oxidase and catalase on the nylon mesh was used for the determination of D-galactose

TABLE I
Characterization of the galactose biosensor and comparison with published values (enzyme from *Dactylium dendroides*; E values found in this study, P enzyme from *Polyporus circinatus*)

Experimental data	E	E	Ref.[6]	Refs[7,8]	P (ref.[5])
Carrier	nylon mesh	collagen membrane	nylon mesh	collagen membrane	acetylcellulose membrane
Detection	O_2	O_2	H_2O_2	H_2O_2	H_2O_2
Activity nmol min^{-1} cm^{-2}	15 260	13 670	200	15 – 20	
Linear response region, mmol l^{-1}	0.0067 – 6.67	0.0067 – 6.67	0.2 – 2.8	$5 . 10^{-4}$ – 0.6	0 – 28
Stability days	30	30	90		8 – 10
Number of analyses	500	500			

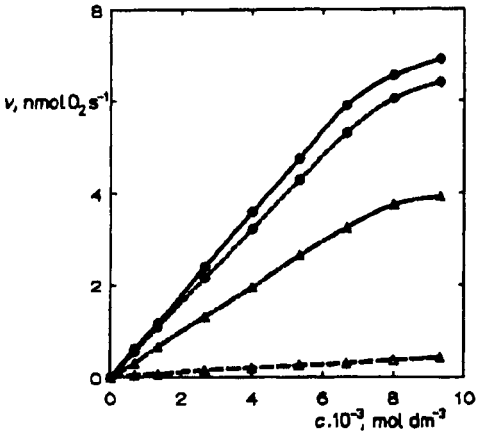

FIG. 5
Substrate specificity of galactose oxidase immobilized on nylon mesh. *v* Rate of oxygen decrease in the reaction mixture (nmol oxygen s^{-1}), *c* substrate concentration in the reaction mixture (mol dm^{-3}), ● D-galactose, ○ raffinose, ▲ 2-deoxy-D-*lyxo*-hexose, Δ lactose

TABLE II
Substrate specificity of galactose oxidase (E values found in this study; D1 *Dactylium dendroides*, intracellular enzyme[4]; D2 *Dactylium dendroides*, extracellular enzyme[4]; P enzyme from *Polyporus circinatus*[2])

Substrate	$K_{M(app.)}$ mol dm^{-3}	Relative oxidation rate				
	E	E	D1	D2	ref.[3]	P
D-Galactose	$1.93 . 10^{-2}$	100	100	100	100	100
2-Deoxy-D-*lyxo*-hexose	$1.94 . 10^{-2}$	58	52	65		32
Lactose	$2.25 . 10^{-2}$	6	11	13		2
Raffinose	$1.93 . 10^{-2}$	95	83	96	145	180
Methyl-α-D-galactopyranoside[a]	$2.45 . 10^{-2}$	106	170	106	118	125
Methyl-β-D-galactopyranoside[a]	$2.07 . 10^{-2}$	134				340
Methyl-2,3-di-O-(*p*-toluenesulfonyl)-α-D-galactopyranoside[a]	$4.23 . 10^{-2}$	40				

[a] Determined in the presence of catalase.

TABLE III
Determination of galactose content in blood plasma samples

Sample	Galactose, enzyme electrode, mmol dm^{-3}	Activity of galactokinase[a] μcat h^{-1} g^{-1} of haemoglobine
1	0.65	8.61
2	0.39	12.79
3	0.50	13.10
4	0.43	14.42
Healty donor	0.20	19.3 ± 2.4

[a] The values were measured by Dr Kulichová, Faculty Hospital II, Prague.

content in samples of blood plasma and serum of patients with a suspected metabolic disorder called galactosemia*. This disease is usually caused by a low galactokinase activity. The measured values (Table III) correlated well with the found activities of galactokinase[18].

REFERENCES

1. Pearson D., Tubbs P.: Biochem. J. *105*, 953 (1967).
2. Avigad G., Amaral D., Asensino C., Horecker B.: J. Biol. Chem. *237*, 2736 (1962).
3. Schlegel R. A., Gerbeck C. M., Montgomery R.: Carbohydr. Res. *7*, 193 (1968).
4. Mendonca M., Zancan G.: Arch. Biochem. Biophys. *252(2)*, 507 (1987).
5. Taylor P. J., Kmetec E., Johnson J. M.: Anal. Chem. *49*, 789 (1977).
6. Mascini M., Iannello M., Palleschi G.: Anal. Chim. Acta *146*, 135 (1983).
7. Bertrand C., Coulet P. R., Gautheron D. C.: Anal. Chim. Acta *126*, 23 (1981).
8. Blum L. J., Bertrand C., Coulet P. R.: Anal. Lett. *16*, 525 (1983).
9. Valentová O., Marek M., Albrechtová I., Albrecht J., Káš J.: J. Sci. Food Agric. *34*, 748 (1983).
10. Vrbová E., Marek M.: Anal. Chim. Acta *239*, 263 (1990).
11. Macháčková M., Šimánková J., Vrbová E., Marek M.: Sborník ÚVTIZ – Potravinářské vědy *9*, 173 (1991).
12. Vrbová E., Horáková I., Marek M.: Anal. Chim. Acta, in press.
13. Vrbová E., Marek M.: Collect. Czech. Chem. Commun. *55*, 2568 (1990).
14. Vrbová E.: Unpublished results.
15. Cooper J., Smith W., Bacila M., Medina H.: J. Biol. Chem. *234*, 445 (1959).
16. Buffone J., Johnson J. M., Lewis S. A., Sparks J. W.: Clin. Chem. *26*, 339 (1980).
17. Anderson E. D., Kalchar H. M., Kurahashi K., Isselbacher K. J.: J. Lab. Clin. Med. *50*, 469 (1957).
18. Misuma H., Kawakami M., Ninomiya H., Shohmori T.: Clin. Chem. Acta *111*, 27 (1981).

Translated by M. Tichý.

* Maximum normal D-galactose level in blood is 0.2 mmol l^{-1}; levels exceeding this value may indicate galactosemia[17,18].