An Enzymatic Sensor for the Analysis of Glycerol in Beverages*

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A biosensor for the determination of glycerol based on bacterial membranes was developed. Bacterial membranes containing pyrroloquinoline quinone (PQQ) dependent glycerol dehydrogenase (GlycDH) were isolated from *Gluconobacter* sp. 33 and were immobilized on the carbon rod electrode. Phenazine methosulfate (PMS) was applied as redox mediator. The response of the biosensor is not sensitive to the oxygen concentration in the bulk. Maximal selectivity of the biosensor was observed at pH 8. The calibration graph of the constructed biosensor was linear up to 8—10 mmol dm^{-3} glycerol and possessed the correlation coefficient of 0.997. The selectivity of this biosensor to various compounds mostly found in the beverages was investigated. The concentration of glycerol in beverages was determined after biochemical elimination of glucose from the sample.

Glycerol is one of the most important compounds that need to be monitored in a sugar-fermentation plant in order to improve the quality control of the process [1]. Standard methods for the determination of free glycerol in the sample are based on the detection of the oxidation products of the glycerol. Formic acid produced by the oxidation reaction can be titrated with standard solution of sodium hydroxide [2]. Glycerol can also be determined spectrophotometrically with chromotropic acid after oxidation (with periodates) of glycerol to formaldehyde. Several enzymatic methods were proposed for the determination of glycerol. In the presence of glycerol kinase, glycerol can be converted to 1-glycerophosphate, which is oxidized by oxygen in the presence of glycerol phosphate oxidase, and dihydroxyacetone phosphate and hydrogen peroxide are produced under these reactions. Subsequently, the concentration of H_2O_2 can be evaluated by photometric methods using commercially available kits [3].

More attractive and cheaper analytical methods are based on biosensors. Over the last three decades, biosensors have become a popular tool in many fields of analytical chemistry due to their high sensitivity and selectivity. Immobilized glycerol oxidase was used for the electrochemical determination of glycerol (*via* amperometric determination of H_2O_2) [4]. Unfortunately, the working potential of electrochemical oxidation of H_2O_2 is high, and many compounds, including ascorbic acid, uric acid, free amino acids, *etc.* can be oxidized on the surface of the electrode and thereby can interfere with the determination of glycerol. Ferrocyanide was used as oxidizer in the enzymatic determination of glycerol. It allowed to reduce the potential of determination and thus to increase the selectivity of the analysis [5].

Analytical systems based on oxidases have some considerable drawbacks. The concentration of oxygen in the bulk is low (about 0.23 mmol dm^{-3}). It is lower than the concentration of analyte, and it is of the same range as K_m in regard to oxygen for this group of enzymes. It means that fluctuation of oxygen concentration in the bulk will directly influence response of the biosensor. All artificial acceptors which are used now, are still worse electron acceptors in comparison with oxygen (a natural electron acceptor of oxidases) and oxygen competition is significant drawback in mediator-based biosensors still now.

Recently some new analytical systems based on dehydrogenases and NAD/NADH couple were reported [6]. The determination of glycerol is achieved by sequence of the enzymatic reactions containing glycerol kinase, pyruvate kinase, and lactate dehydrogenase, or by enzymatic oxidation of glycerol with glycerol dehydrogenase in the presence of NAD. NAD/NADH conversion can be followed spectrophotometrically at

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340 nm [7], fluorometrically at 450 nm [8] or electrochemically on Pt electrodes at 0.7 V [9]. Due to the high overpotential and considerable dimerization, NAD/NADH cannot be detected properly by direct electrochemical methods on solid electrodes. Phenoxazine and phenothiazine mediators (Meldola blue, Toluidine blue) were used for the chemical oxidation of NADH. Electrochemical regeneration of reduced forms of such mediators can be performed at low potential on the carbon electrodes [10, 11]. Different carbon-based electrodes became a popular material in biosensors design due to their inert hydrophobic surface, excellent for the adsorption of enzymes. Moreover, in many cases the redox mediators can be easily reoxidized on the carbon surface. Irreversible adsorption of mediators on the surface of carbon electrode and high current density opens good possibilities for the creation of reagentless and sensitive enough biosensors, but soluble NAD/NADH couple makes them unsuitable for routine analysis of glycerol in foods.

PQQ-dependent dehydrogenases represent a new class of oxidoreductases with great potential in design of analytical systems [12]. The natural acceptors of electrons of PQQ dehydrogenases usually are cytochromes, quinones or ferrocyanide. The natural electron acceptors of PQQ-dependent enzymes can be replaced by the artificial electron acceptors such as ferrocene and its derivatives [6], or phenazine methosulfate [13], but not oxygen [14]. The PQQ-dependent glycerol dehydrogenase has been detected in certain bacteria [15]. PQQ-GlycDH was isolated from the membranes of mutant strain of *Gluconobacter* sp. 33. The purification of the enzyme was complicated and the yield was very low. The stability of the extracted enzyme was very low, too [16]. As an alternative biocatalytic material, immobilized whole cells have been used [17, 18]. The activity as well as the selectivity of such type of biosensors was low.

The goal of this work was to create a glycerol biosensor based on membranes of disrupted bacterial cells containing PQQ-GlycDH. A graphite electrode was selected as the suitable surface for enzyme immobilization. The application of bacterial membranes can be more promising because the enzyme will be left in the natural environment but the unwanted activity of intracellular enzymes will be avoided.

EXPERIMENTAL

A membrane fraction containing PQQ-dependent glycerol dehydrogenase was isolated from *Gluconobacter* sp. 33 as described below. Membranes of the alcohol dehydrogenase-deficient mutant *Gluconobacter* sp. 33 cells were used as a source of PQQ-dependent glycerol dehydrogenase. Cells were cultivated aerobically at 30 °C in a liquid medium, pH 5.5, of the following composition: yeast extract – 5 g dm^{-3}, D-mannitol – 10 g dm^{-3}, $(NH_4)HPO_4$ – 1 g dm^{-3}, $MgSO_4 \cdot 7H_2O$ – 2 g dm^{-3}. The cells harvested at the late exponential phase were washed with 0.9 % NaCl buffer, pH 8.0, and disrupted by ultrasonic treatment for 3 min. Unbroken cells were removed by centrifugation. The membranes exerting glycerol dehydrogenase activity were collected by high-speed centrifugation (125000 *g*, 3 h and suspended in 10 mmol dm^{-3} Tris-HCl buffer, pH 8.0).

Carbon rod ultra "F" electrodes (cat. No. 001281-10) of 3 mm diameter were purchased from Ultra Carbon Division (Bay City, USA). Glutaraldehyde (25 %) was purchased from Reanal (Budapest, Hungary). KCl, Na-acetate, KH_2PO_4, $Na_2B_2O_4$, KOH (anal. grade), and bovine serum albumin were obtained from Reakhim (Kiev, Ukraine). Glucose oxidase (EC 1.1.3.4) from *Aspergillus niger*, 200 units/mg, catalase (EC 1.11.1.6) from bovine liver, 1600 units/mg, glycerol dehydrogenase (EC 1.1.1.6) from *Cellulomonas* sp., 78 units/mg, phenazine methosulfate (PMS), dichlorophenol indophenol (DCPIP), $K_3[Fe(CN)_6]$ (ferrocyanide), trizma base, glycine, and glycerol were obtained from Sigma (St. Louis, USA). All solutions were prepared by using HPLC grade water purified in a "Purator-B" Glas Keramic (Berlin, Germany). Argon gas of 99.993 % purity (AS Elme Messer Gass, Tallin, Estonia) was used.

All amperometric measurements were performed with a PA-2 polarograph (Laboratorní přístroje, Prague, Czech Republic) together with an XY-recorder (Schlotheim, Germany). Three-electrode electrochemical cell, consisting of a working carbon electrode, a saturated Ag/AgCl reference electrode, and a Pt auxiliary electrode was used. The electrochemical cell was carefully protected from light. The biosensor for glycerol determination was assembled on the tip of graphite electrode. The bacterial membranes containing glycerol dehydrogenase were immobilized by using modified soft enzyme immobilization technology [19]. For this purpose, 3 mm^3 of membrane suspension in 0.05 mol dm^{-3} phosphate buffer solution, pH 7.0, were spread on the tip of the polished graphite electrode and dried. Then the electrode was exposed over a 3 % glutaraldehyde solution in hermetical vessel for 20 h at 4 °C. Afterwards, the electrode was washed with distilled water and kept wet in the refrigerator.

The anodic current of the prepared electrode was measured in 0.05 mol dm^{-3} buffer solution, pH 7.0, containing 0.1 mol dm^{-3} KCl, at 0.3 V *vs.* Ag/AgCl reference electrode. 1 mmol dm^{-3} phenazine methosulfate (PMS) was used as redox mediator. In some cases phosphate, borate, tris-malate, and glycine buffers were used. As a reference method of glycerol determination spectrophotometric NAD/NADH conversion by NAD-dependent glycerol dehydrogenase at 340 nm was used [12]. The elimination of glucose from tested wine was carried out by incubation of 10-fold diluted samples with 60 units of glucose oxidase and

200 units of catalase at 30 °C. Full glucose elimination was complete within 2 h. The absence of glucose in the samples was confirmed by a glucose analyzer EKSAN-G (Analita, Vilnius, Lithuania) [20].

RESULTS AND DISCUSSION

The action of the GlycDH biosensor is based on an enzymatic recognition and oxidation of glycerol by GlycDH. The reduced form of PQQ transfers electrons to carbon electrode *via* the redox mediators. The increase of anodic current of the biosensor correlates with the glycerol concentration in tested solution. PMS was selected as the best redox mediator because in the presence of 1 mmol dm^{-3} of PMS, DCPIP and ferrocyanide the relationship between amperometrical signals was as 50:15:1.

An approximation of the experimental data by the apparent Michaelis—Menten equation leads to the values of the parameters $K_{m,app}$ = 10.4 mmol dm^{-3} and I_{max}= 5.6 μA (Fig. 1). The linearity of sensor response was observed up to the concentration of 8 mmol dm^{-3} (Fig. 1). This wide linear region of the steady-state currents *vs.* concentration of glycerol as well as the about 12 times higher value of $K_{m,app}$ in comparison with the K_m value (0.83 mmol dm^{-3}) reported for the purified glycerol dehydrogenase [16] indicated that the biosensor was operating in a diffusion-limited mode.

This biosensor was supposed to be oxygen-independent because oxygen-independent glycerol dehydrogenase was used as the biocatalyst. A comparison

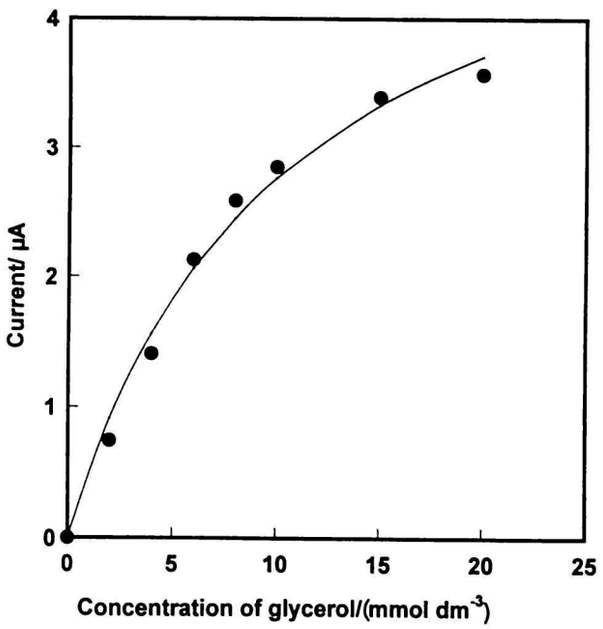

Fig. 1. Calibration graph of the glycerol biosensor in 0.05 mol dm^{-3} sodium acetate buffer (pH = 6).

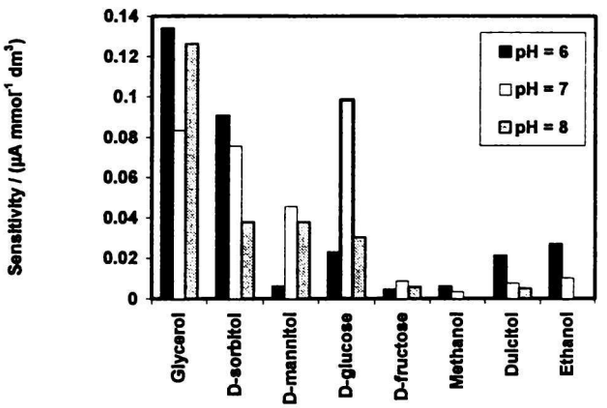

Fig. 2. Response of the *Gluconobacter* sp. membrane-based biosensor to various substrates. The sensitivity was measured in three buffer solutions: acetate, pH 6, phosphate, pH 7, and glycine, pH 8.

of biosensor signals obtained in deaerated (with argon) and air-saturated solutions, respectively, was performed in the interval of 1—10 mmol dm^{-3} of glycerol. Amperometric signals of the biosensor in the deaerated samples were only by 4 % higher in comparison with signals obtained in air-saturated solutions containing usual concentrations of oxygen. Probably reduced PMS was slowly oxidized by molecular oxygen. This side reaction had only small impact because electrochemical oxidation of reduced PMS on the electrode surface was much faster. This fact was in a good agreement with the results obtained by PMS-mediated alcohol biosensor where oxygen-independent quinohemoprotein alcohol dehydrogenase was immobilized on the surface of polypyrrole layer [21]. It means that our sensor can be used for the determination of glycerol in the probes with unstable oxygen concentration. This is especially important when glycerol concentrations are measured in wines. Bottled wines contain different concentration of oxygen, as well as concentration of oxygen is varying under the fermentation process.

The selectivity of the obtained biosensor to various mono- and polyhydroxylic compounds was investigated by comparing response (the steady-state current) of the biosensor to the different compounds 5 mmol dm^{-3} each (Fig. 2). Significant interferences from D-glucose, sorbitol, and mannitol were observed. Responses for fructose, methanol, and dulcitol could be neglected, because the concentrations of these compounds normally are significantly lower than that of glycerol in tested beverages. No response to ethanol was obtained at pH 8.0.

The sensitivity of the biosensor was higher for glucose and lower for sorbitol and mannitol in comparison to the sensitivity of a purified GlycDH measured by DCPIP-PMS assay in solution [16]. No responses of the biosensor to ethanol and methanol at pH 8 were observed. Although the used *Gluconobacter* sp. strain

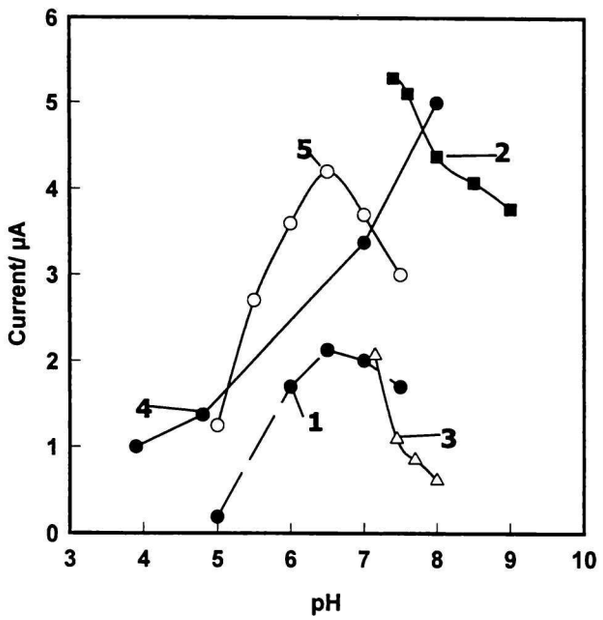

Fig. 3. Dependence of the response of the glycerol biosensor on pH in different buffer solutions. The glycerol concentration was 5 mmol dm^{-3} (*1.* phosphate buffer, *2.* borate buffer, *3.* tris-malate buffer, *4.* glycine buffer, *5.* acetate buffer).

was alcohol dehydrogenase-deficient, probably other alcohol-metabolizing enzymes in the membranes may act. The selectivity of the analysis may be increased by the optimization of the acidity of reaction media. At pH 8, the response to glycerol was significantly higher than response to other substances.

The dependence of the response of glycerol biosensor on pH was also affected by the nature of buffer solution. In a 0.05 mol dm^{-3} sodium acetate buffer, a sharp optimum was found at pH 6 (Fig. 3). In phosphate and tris-malate buffers the responses were lower, but with a similar pH optimum tendency. In glycine solutions, the sensitivity of the biosensor was higher at alkaline pH values (Fig. 3). For this reason, alkaline media (glycine buffer, pH 8.0) were selected for experiments with real samples.

The biosensor was applied for the detection of glycerol in four wine samples. The biosensor was inserted into 2.0 cm^3 volume reaction cell filled with a 0.05 mol dm^{-3} glycine buffer (pH = 8) containing 0.1 mol dm^{-3} KCl and 1 mmol dm^{-3} PMS. After adjusting the background current, 0.5 cm^3 of 10-fold diluted wine was added to the reaction mixture. The increase of anodic current was registered. For the elimination of the effect of possible inhibitors (that can be present in real samples) subsequent additions of glycerol standard solution to the sample were performed and sensitivity of biosensor was controlled. In order to eliminate interference of glucose, all tested samples were in advance incubated with glucose oxidase and catalase as described in Experimental. The results (Table 1) are in agreement with the results obtained by the spectrophotometric glycerol determination method and data presented in the literature for 2.8—15.3 g dm^{-3} of glycerol in dessert wines [22]. The values obtained by the biosensor were by 6—11 % higher than those obtained by the reference method. It can be due to the influence of some redox active compounds in the wine samples. In the future this influence can be reduced by application of some semipermeable layers on the working surface of the GlycDH modified electrode.

The storage stability of the biosensor (in a dry state, at 4 °C) was tested by periodic measurements of the response to glycerol at room temperature. Fig. 4 shows the decrease of the calibration slope during a 12-day period. The sensitivity of the biosensor during first two days after preparation decreased considerably (by approximately 25 %), probably due to the leakage of weakly immobilized or adsorbed biocatalyst from the electrode surface. After this storage period, the residual sensitivity was about 50 % and later decreased very slowly. The operational stability of the biosensor within 3—12 days after preparation was investigated in the real (wine) samples as a decrease of amperometric signals during continuous measurements with

Table 1. Detection of Glycerol in Wine Samples

Wines	Glycerol concentration determined by the biosensor		Glycerol concentration determined by the reference method	
	ρ/(g dm^{-3})	RSD*/%	ρ/(g dm^{-3})	RSD/%
Kadarka Royal (Peter F. Heering, Denmark)	17.6	5.2	15.1	2.1
Portwein semi-sweet (Puerto Pablo, Spain)	12.0	7.1	11.0	3.5
Dry White Chardonnay (Black Sea Gold, Bulgaria)	18.9	8.4	18.1	2.7
Tokaji aszu 3 puttonyos (Tokaj kereskedohaz rt., Satoraljaujhely Palackozouzem: Tolcsva, Hungary)	23.6	4.1	21.9	3.0

*RSD – relative standard deviation. The presented concentration values are the means of three measurements.

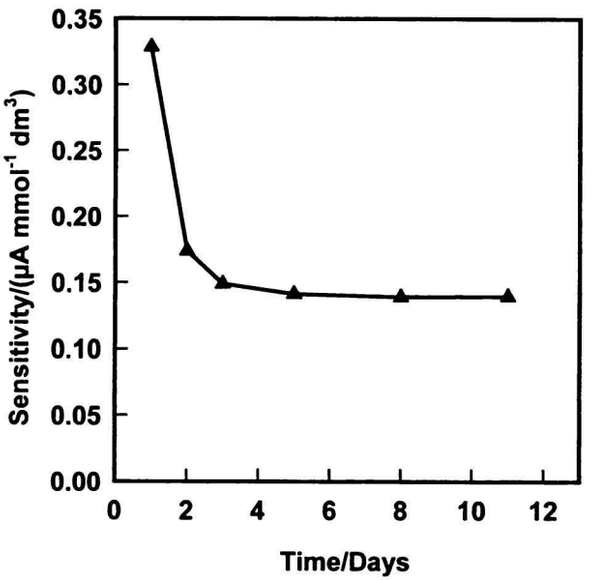

Fig. 4. Storage stability of the glycerol biosensor at 4 °C.

5 min period. Observed decrease was 1.5 % h^{-1}. A calibration of biosensor every 2—3 h is recommended.

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