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Novel conductometric biosensor based on three-enzyme system for selective determination of heavy metal ions

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ABSTRACT

A differential pair of planar thin-film interdigitated electrodes, deposited on a ceramic pad, was used as a conductometric transducer. The three-enzyme system (invertase, mutarotase, glucose oxidase), immobilized on the transducer surface, was used as a bioselective element. The ratio between enzymes in the membrane was found experimentally considering the highest biosensor sensitivity to substrate (sucrose) and heavy metal ions. Optimal concentration of sucrose for inhibitory analysis was 1.25 mM and incubation time in the investigated solution amounted to 10–20 min. The developed biosensor demonstrated the best sensitivity toward ions Hg^{2+} and Ag^{+}. A principal possibility of the biosensor reactivation either by EDTA solution after inhibition with silver ions or by cysteine solution after inhibition with mercury ions was shown.

1. Introduction

Nowadays, ecological monitoring of environment becomes vital social challenge [1] associated with the development of chemical industry and intense usage of numerous chemical agents in agriculture and other fields of human activities. These harmful compounds and products of their degradation pollute air and soil and, consequently, drinking water and foodstuff, that results in numerous human diseases [2]. Pollution of the environment in large settlements, especially in industrious areas, is characterized by the presence of numerous heavy metal compounds in soil. Their sources can be technological processes in metallurgy and chemical industry as well as daily human activities. For example, silver ions come to surface water from groundwater, mine and mill wastewater, etc. In agricultural areas, the main source of pollution with heavy metal ions is farming industry. Heavy metals and their compounds in environment are characterized by relatively high stability, solubility in atmosphere precipitations, ability to be absorbed by soil and plants. Their accumulation in organisms of humans and animals results in harmful effects of wide spectrum and variety [3]. Considering the above mentioned, permanent effective control over the presence of these compounds in the environment and foodstuff appears to be extremely important for nature protection and improvement of life quality [4]. Conventional methods of high-precision analysis of heavy metals, i.e. gas and liquid chromatography, spectrophotometry, numerous chemical and physical techniques, require high-skilled personnel, complex and expensive equipment [5]. Besides, complicated sample pretreatment is necessary that makes the procedure more time- and costs-consuming. That is why, the development of easy-to-use, accurate, selective, fast and cheap method of analysis of heavy metal concentration in the samples of ambient environment and foodstuff appeared at present to be such actual problem.

Biosensors are an alternative for determination of toxic substances since they offer a promising approach to solve the above mentioned problems. At present, there are some variants of electrochemical biosensors for measurement of heavy metal ions [6–18]. Generally, they are based on enzyme urease [6–10]. The disadvantage of these biosensors is that they are not selective to a specific heavy metal, i.e. they can be applied only for determination of total toxicity of a sample. Other enzymes of horseradish peroxidase [11], alcohol oxidase [12], glucose oxidase (GOD) [13] are used as a bioselective element more rarely than urease. The GOD-based biosensors are supposed to be most sensitive to silver ions though there are the data on their application for determination of ions of other heavy metals, mercury in particular [14].

Amperometric biosensors based on a three-enzyme system (invertase, mutarotase and glucose oxidase) can also be used for heavy metal analysis [15,16]. In the first prototype, mutarotase and glucose

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oxidase were immobilized on the transducer surface while invertase was inserted into the reactor together with the sample; this complicated the procedure and raised the analysis price [15]. The second variant was more efficient as all three enzymes were immobilized concurrently, though mutarotase consumption was too large [16].

A new approach to the development of silver sensitive biosensors on the basis of square wave voltammetry with metallothionein as a bioselective element enables measurement of this toxin in very low concentrations (0.5 nM) [17]. Metallothionein-based biosensors are known as electrochemical detector for HPLC analyzing of silver ions [18]. Despite numerous reports on the development of laboratory prototypes of the biosensors, their application in food production and environmental protection is still limited. No commercial biosensor device is known so far to function for determination of heavy metals ions. Therefore the development and analysis of novel biosensoric approach are now of great interest and prospect.

Conductometric methods being quite simple, easy-to-use and accurate, suggest a solution of a number of essential scientific and technological tasks [19]. It was a reason that this work was aimed at the development of conductometric biosensor based on a three-enzyme system (glucose oxidase, mutarotase and invertase) for measurement of concentration of heavy metal ions in aqueous solutions.

2. Materials and methods

2.1. Materials

The enzymes used in the work were as follows: glucose oxidase (GOD) from *Penicillium vitale* (EC 1.1.3.4.) with activity of 130 U/mg, production of "Diagnosticum" (Lviv, Ukraine); invertase from baker yeast (EC 3.2.1.26.) with activity of 355 U/mg, production of Sigma-Aldrich Chemie (Germany); mutarotase (EC 5.1.3.3.) with activity of 100 U/mg, production of "Bioenzyme Laboratories Ltd." (Great Britain). Bovine serum albumin (BSA) (fraction V), 50% aqueous solution of glutaraldehyde (GA), cysteine and ethylenediaminetetra acetate (EDTA) was purchased from Sigma-Aldrich Chemie (Germany). As the substrates, sucrose and glucose of domestic production were used, as the inhibitors — aqueous solutions of heavy metals nitrates of domestic production. The compounds for buffer preparation and other inorganic compounds used were of domestic production and of analytical grade.

2.2. Sensor construction and general view

We used conductometric transducers manufactured in the V. Lashkaryov Institute of Semiconductor Physics of National Academy of Sciences of Ukraine (NASU) (Kyiv, Ukraine) in accordance with our recommendations and sketches. They consist of two identical pairs of gold interdigitated electrodes obtained by gold sputtering onto ceramized plate of $5*40\ mm^2$ area. The sensitive surface of each pair was about $1.0*1.5\ mm^2$, the distance between neighbor digits, as well as the width of each digit was 20 μm.

2.3. Preparation of bioselective elements

Working bioselective membranes based on the three-enzyme system were formed as follows. A mixture consisting of 10% invertase, 10% mutarotase, 10% glucose oxidase (GOD) and 10% BSA was prepared by dissolving the enzymes in 40 mM phosphate buffer, pH 6.5, containing 20% glycerol. The mixture for reference membrane was prepared by the same procedure using only BSA, the final protein concentration — 40%. Prior to deposition on the transducer surfaces, the prepared solutions (for both reference and working membranes) were mixed with 2.5% aqueous solution of glutaraldehyde at the ratio of 1:1. Immediately after that, the surfaces of working zones of conductometric transducers were entirely covered with the solutions obtained. The volume of each membrane was about 0.1 μl. Then the membranes were dried in open air at room temperature for 2 h. Before starting the experiments, the membranes were dipped into 5 mM phosphate buffer, pH 6.5, to washout excess of unbound GA.

2.4. Setup scheme and measurement principle

The flow diagram of measuring unit is shown in Fig. 1. The interdigitated conductometric electrodes (a differential pair) located in a cell with the tested solution, are supplied with alternating voltage (100 kHz frequency, 10 mV amplitude) from the low-frequency signal generator G3-118 (Ukraine). A differential mode is used to increase sensor sensitivity and minimize the noise of nonspecific effects. The sensor electrodes are coupled with load resistance $R = 1\ k\Omega$. The signal from electrodes through the differential amplifier Unipan-233-6 (Poland) enters the selective nanovoltmeter Unipan-233-6, and then is registered by a recording device.

2.5. Measurement procedure

Measurements were carried out at room temperature in the 5 mM phosphate buffer solution, pH 6.5, intensively stirred in an open cell (2 ml). The required substrate concentration in the cell was attained by inserting aliquots of the substrate stock solutions into the working buffer. The enzymes were inhibited by means of 20 min biosensor exposure to solutions of the metal salts of concentrations 0.1 to 100 μM. At least three series of experiments were performed. Non-specific changes in the output signal associated with fluctuations of temperature, medium pH, and electrical noise, were compensated by using differential mode of measurement.

3. Results and discussion

3.1. Basic principle of inhibitory analysis by three-enzyme biosensor

Operation of a bioselective element of the developed conductometric biosensor is based on a cascade of enzymatic reactions:

$$\text{Sucrose} + H_2O \xrightarrow{\text{Invertase}} \beta\text{–D–fructose} + \alpha\text{–D–glucose} \quad (1)$$

$$\alpha\text{–D–glucose} \xrightarrow{\text{Mutarotase}} \beta\text{–D–glucose} \quad (2)$$

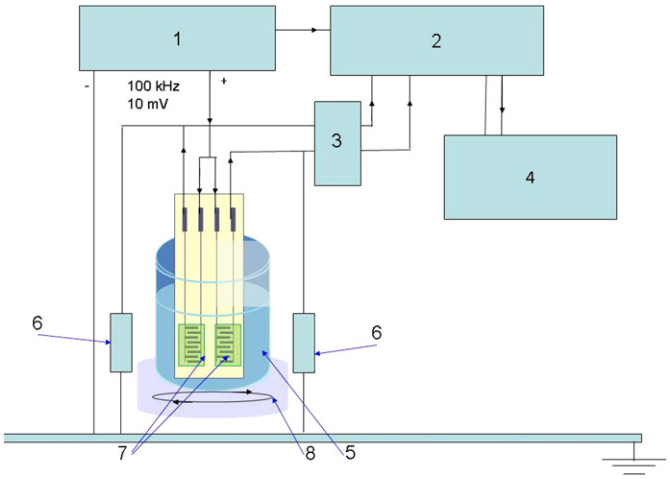

Fig. 1. Diagram of measuring setup (1 — generator, 2 — phase-sensitive nanovoltmeter, 3 — differential amplifier, 4 — recording device, 5 — electrochemical cell (2 ml), 6 — loading resistances (1 kΩ), 7 — electrodes with enzyme and reference membranes, 8 — magnetic stirrer).

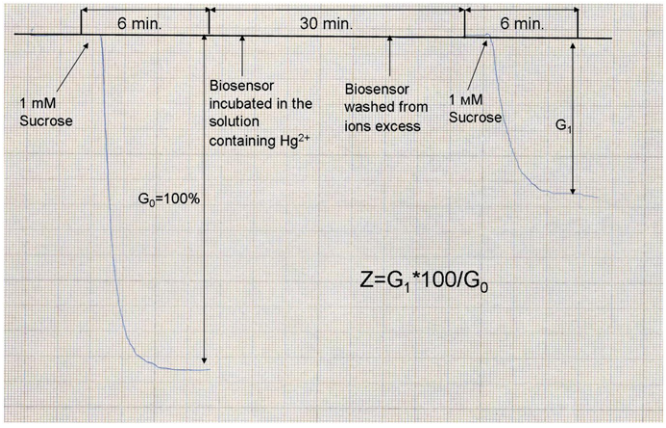

Fig. 2. Scheme of inhibitory determination of heavy metal ions (Hg^{2+} as an example) by conductometric enzyme biosensor.

$$\beta-D-\text{glucose} + O_2 \xrightarrow{GOD} D-\text{gluconolacton} + H_2O_2 \quad (3)$$

$$D-\text{gluconic acid} + H_2O \leftrightarrows \text{acid residue} + H^+. \quad (4)$$

Invertase, mutarotase and glucose oxidase disintegrate sucrose gradually to hydrogen peroxide and D-gluconolacton. The latter, in its turn, is hydrolyzed spontaneously to gluconic acid which dissociates for acid residue and proton, thus, the solution conductivity changes that can be registered by the conductometric transducer [19].

At inhibition, an interaction between ions of heavy metals and sulfhydric groups of enzymes [6,20] results in decrease of enzyme activity and corresponding reduction of number of the protons generated at enzymatic disintegration of sucrose.

The interaction in question can be reversible. Heavy metal ions segregate from reaction mixture in the presence of strong chelating agents, e.g. EDTA. For example, at reactivation of the enzyme system from silver ions, EDTA can be used since it interacts with four Ag^+ ions separating them from the inactivated enzyme [20].

As an example of typical inhibitor analysis, the diagram of Hg^{2+} determination is presented in Fig. 2. In the first place, the biosensor signal (G_0 — conductivity) to insertion of 1 mM sucrose into the cell was obtained and taken as 100%. Then the biosensor was incubated for 30 min in the solution containing Hg^{2+}, the concentration of which is to be measured, and washed from ions excess. Next, the biosensor signal (G_1 — conductivity) to insertion of the similar sample was again measured. Residual activity (Z) is determined by the formula $Z = G_1 * 100/G_0$. If the biosensor activity dropped insufficiently ($\leq 10\%$) at 100 μM inhibitor concentration, no further experiments with this metal were performed.

3.2. Optimization of biosensor enzyme membrane for inhibitory analysis

Working characteristics of conductometric biosensors depend on composition of the bioselective membrane and the method of enzyme immobilization. For example, the biosensor response to sucrose depends not only on the activity of enzymes and diffusion processes in the bioselective membrane, but also on the enzymes ratio in it. Also, difference between the biosensor responses to glucose and sucrose can be a ground for analysis, which enzyme is in excess and which — in deficiency regarding sensor sensitivity to the substrate. Invertase was shown to have the highest sensitivity to heavy metal ions among the tested enzymes [15,16]. That is why enzyme ratio in the bioselective membrane should be optimized in order to attain the highest sensitivity toward substrates and to avoid the invertase excess relative to other enzymes.

Optimal mutarotase concentration in the enzyme membrane was evaluated by following procedure. GOD and mutarotase mixtures were prepared separately in 40 mM phosphate buffers, pH 6.5, containing 20% glycerol. Mutarotase concentration was varied while GOD concentration was constant, 10%. Each enzyme solution was mixed with aqueous solution of glutaraldehyde in the ratio of 1:1, so concentration of GOD in the working membrane was 5% whereas that of mutarotase changed. 5% GOD was taken in experiments, since this concentration has been earlier declared as optimal for glucose determination [21], i.e. at this value an optimal proportion between sensitivity and dynamic range of the biosensor operation has been obtained. Prior to the measurements, an excessive amount of invertase was inserted into the working cell, so that the final mutarotase concentration was 100-fold higher than theoretically required and thus did not limit the reaction. Next, responses to 1 mM glucose and 1 mM sucrose were measured depending on mutarotase concentration, the response to 1 mM glucose was taken as 100%. As seen in Fig. 3(a), at 4% and higher mutarotase concentrations, the responses to glucose and sucrose become equal, hence, 5% mutarotase concentration in the membrane was taken in further experiments.

The next stage in optimization of enzyme composition of the membrane was determination of optimal invertase concentration. The prepared mixtures contained invertase of different concentrations along with 10% mutarotase and 10% GOD, i.e. after mixing with GA water solution (1:1) the concentrations of both mutarotase and GOD in the membrane were 5%. As seen from Fig. 3(b), at an increase of invertase concentration in the membrane to 5% and higher, the difference between the values of biosensor responses to glucose and sucrose is the least which, correspondingly, testifies to maximum membrane saturation with invertase. Since it is required that invertase should be

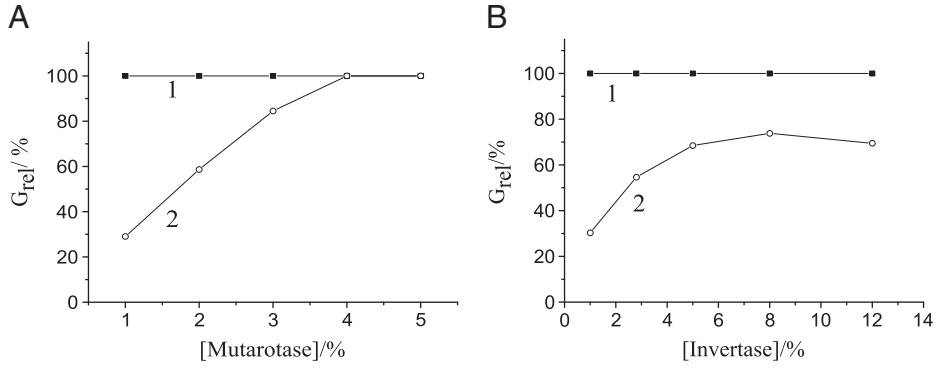

Fig. 3. Dependence of biosensor responses (relative conductivity G_{rel}) to 1 mM glucose (1) and 1 mM sucrose (2) on mutarotase (A) and invertase (B) concentrations in enzyme membrane. $G_{rel} = G_s \cdot 100\%/G_g$, where G_s — biosensor response (change of conductivity) to sucrose, G_g — biosensor response (change of conductivity) to glucose (response to 1 mM glucose is taken as 100%). Measurements in 5 mM phosphate buffer, pH 6.5.

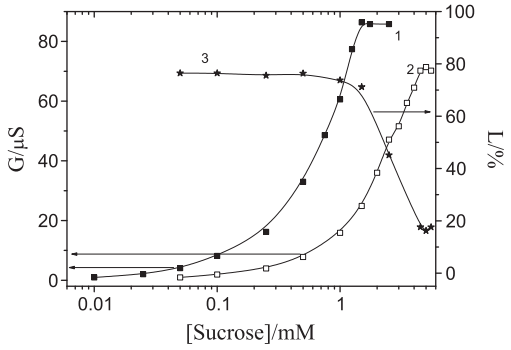

Fig. 4. Dependences of conductometric biosensor responses (G — change of conductivity) before (1) and after (2) inhibition with 50 μM Hg^{2+} solution, and level of inhibition (L) of three-enzyme system (3) on different sucrose concentration. $L=(G_0-G_1)\cdot 100\%/G_0$, where G_0 — biosensor response before and G_1 — *biosensor response* after inhibition. Measurements in 5 mM phosphate buffer, pH 6.5, inhibition time 30 min. (The x-axis is a logarithmic scale of sucrose concentration).

completely involved in sucrose conversion in the bioselective membrane, the invertase concentration of 5% was finally used.

So, optimal enzymes concentrations in the bioselective membrane were found to be 5% for each of the enzymes tested, i.e. invertase, mutarotase and GOD.

3.3. Investigation of optimal conditions for biosensor operation

The concentration of substrate sucrose should be optimized to provide the best sensitivity of the developed biosensor to heavy metal ions. Theoretically, optimal value of substrate concentration is in the region of enzyme saturation with the substrate where every enzyme molecule is maximally involved in the process of substrate transformation into the final product, which results in changes of conductivity and generation of maximum response. To determine optimal substrate concentration in the inhibitory analysis, dependence of biosensor response on the sucrose concentration before and after inhibition with 50 μM Hg^{2+} solution was studied. As seen from Fig. 4, at the sucrose concentration up to 1.0 mM the classic dependence of response and independence of inhibition level on the substrate concentration are revealed. Further increase of substrate concentration from 1.0 to 1.25 mM results in higher response values up to saturation (at different substrate concentrations before and after inhibition), but the inhibition level drops. So, in further experiments we used sucrose of concentration of 1.25 mM, which provided maximum biosensor response at still sufficiently high inhibition level, i.e. sensitivity to heavy metal ions.

Another important characteristic at inhibitory analysis is duration of biosensor incubation in the solution tested. Dependence of residual activity of the bioselective element based on three-enzyme system on inhibition time was studied at four values of Hg^{2+} concentration (10, 25, 50 and 100 μM) (Fig. 5). As seen, the curves obtained are essentially different. Besides, to attain the highest biosensor sensitivity to the toxicant, duration of incubation is to be increased and, consequently, the whole analysis will last longer. Thus, optimal inhibition time depends on mercury concentration.

3.4. Biosensor sensitivity to different toxicants

Considering the data obtained, in subsequent experiments 5 mM phosphate working buffer, pH 6.5, and sucrose of concentration of 1.25 mM were used, inhibition time — 20 min. The curves of dependence of residual activity of the three-enzyme biosensor system on ion concentration of various heavy metals (Fig. 6) demonstrate that Ag^{+} and Hg^{2+} influence the enzyme activity considerably, while the ions of other metals at their concentrations below 100 μM have a slight effect on the activity of enzyme system. Analyzing the results obtained, we concluded that the three-enzyme system based on glucose oxidase, invertase and mutarotase can be effectively used for highly sensitive determination of ions of mercury and silver, with detection limits ($3\times S/N$) of 25 nM and 100 nM respectively.

The developed conductometric biosensor based on the three-enzyme system was next investigated regarding its selectivity toward different groups of toxicants (formaldehyde, pesticides and herbicides) which potentially can be present in real environmental samples. The biosensor inhibition with 100 μM mercuric nitrate was taken as 100%. As seen, the presence of formaldehyde, pesticides and herbicides of 1 mM concentration in the solution had no effect on the biosensor response. This testifies to high selectivity of the biosensor developed.

3.5. Enzymatic membrane reactivation

In our case inhibition of the bioselective element with heavy metal ions is irreversible, therefore the biosensor can be used for measurement only once which makes its application ineffective. To provide repeated usage of the biosensor we studied a possibility of its reactivation.

Since EDTA and cysteine are known as reactivators of enzyme systems of biosensors for heavy metal determination [22], we used the solutions of 10 mM cysteine and 5 mM EDTA with neutral pH to evaluate a possibility of reactivation of the developed biosensor. For better understanding of inhibition of a specific enzyme with corresponding heavy metal ions and, thus, of processes of reactivation of the entire three-

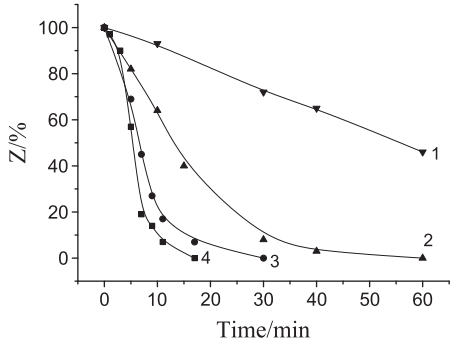

Fig. 5. Dependence of residual activity (Z) of biosensor three-enzyme system after inhibition on time of biosensor incubation in 10 μM (1), 25 μM (2), 50 μM (3) and 100 μM (4) Hg^{2+} solutions. $Z=G_0\cdot 100\%/G_1$, where G_0 — biosensor responses before and G_1 — biosensor responses after inhibition. Measurement in 5 mM phosphate buffer, pH 6.5, substrate concentration — 1.25 mM sucrose.

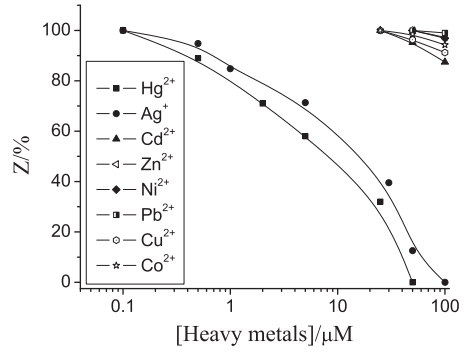

Fig. 6. Dependence of residual activity (Z) of biosensor three-enzyme system on concentration of ions of different metals. $Z=G_0\cdot 100\%/G_1$, where G_0 — biosensor responses before enzyme inhibition and G_1 — biosensor responses after enzyme inhibition. Measurements in 5 mM phosphate buffer, pH 6.5, sucrose concentration — 1.25 mM, inhibition time — 20 min. (The x-axis is a logarithmic scale of concentration of heavy metals).

Table 1
Reactivation of three-enzyme biosensor after inhibition with silver ions.

Residual activity of bioselective element, %			
After inhibition		After reactivation in 5 mM EDTA for 45 min.	
Response to sucrose	Response to glucose	Response to sucrose	Response to glucose
80	100	100	100
70	100	100	100
64	68	95	100
63	85	90	100
40	64	85	100
3	12	3	65
2	12	7	62

enzyme system, the biosensor responses to both glucose and sucrose were measured over time of investigation. The response to glucose can be considered as correspondent to the glucose oxidase activity in three-enzyme system while the response to sucrose — proportional to the total enzyme activity of the bioselective element. Therefore, at first we measured responses to addition of glucose and sucrose into the cell, then the biosensor was inhibited with the solutions of mercury or silver ions during 10–20 min depending on their concentration (the developed biosensor was highly sensitive just to these metals). Next, the responses to glucose and sucrose of the same concentrations were measured once more. If the responses decreased significantly, reactivation was repeated. The data obtained are presented in Tables 1 and 2. As seen, inhibition with mercury ions resulted in reduction of the response to sucrose while the response to glucose almost did not change which testifies to inactivation of invertase and, possibly, mutarotase. With silver ions as an inhibition agent, the responses to both sucrose and glucose decreased which evidenced that glucose oxidase was more inactivated.

After inhibition, the biosensors were placed for 45 min into intensively stirred EDTA or cysteine solution. It was revealed that EDTA did not reactivate the enzymes inhibited with mercury ions while the enzymes inhibited with silver ions were reactivated efficiently. The effect of cysteine was just opposite, i.e. weak reactivation of the enzymes inhibited with silver, and better — with mercury ions. The data on reactivation of three-enzyme biosensor inhibited with silver and mercury ions are presented in Tables 1 and 2, correspondingly. The results obtained at reactivation with different complexons can be also used to identify which heavy metal (either mercury or silver) is present in the sample analyzed.

4. Conclusions

For the first time a conductometric biosensor with three-enzyme system (invertase, mutarotase and glucose oxidase) was developed for inhibitory determination of some heavy metal ions. This method is based on the effect of inhibition of the cascade of enzymatic reactions resulting in changes of the medium conductivity. The composition of bioselective membrane and measurement conditions were optimized. The main working characteristics of the biosensor for inhibitory analysis of toxicants in water solutions were determined. Sensitivity of three-enzyme biosensor toward different heavy metal ions was investigated. The biosensor was shown to have the highest sensitivity toward Ag^{+} and Hg^{+2}, it can be used for selective analysis of these toxic substances as well as for determination of the assay total toxicity. Principal possibility of biosensor reactivation with EDTA and cysteine solutions was shown. In future the developed biosensor can be used for express evaluation of the presence of heavy metal ions in water samples.

Table 2
Reactivation of three-enzyme biosensor after inhibition with mercury ions.

Residual activity of bioselective element, %			
After inhibition		After reactivation in 10 mM cysteine for 45 min	
Response to sucrose	Response to glucose	Response to sucrose	Response to glucose
81	92	100	100
73	100	100	100
40	95	100	100
0	92	83	100
0	93	84	98

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