

Bioorganic Chemistry

low temperature Stabilization of glucose oxidase as a component of biological sensor

V.I. Grischenko, O.A. Nardid, K.D. Rozanova, M.I. Schetinsky, E.I. Naumenko, S.V. Dzyadevych

The Institute of Cryobiology and Cryomedicine, NAS of Ukraine

23, Pereyaslivska Str., Kharkiv, 61015, Ukraine

The Institute of Molecular Biology and Genetics, NAS of Ukraine

150, Zabolotny Str., Kyiv, 03143, Ukraine

The effect of freeze-thawing with various cooling rates on structural state of glucose oxidase using low temperatures has been studied for searching the ways of long-term preservation of biosensors. Such cryoprotectors as glycerol, 1,2-propane diol and DMSO were used for complete preservation of the enzyme properties in the process of low temperature preservation. The glycerol and 1,2-propane diol proved to be the most suitable for low temperature preservation of the glucose oxidase sensor that enabled to protect not only the protein part of biosensor but the biosensor protective surface as well.

Key words: *glucose oxidase, biosensor, low temperatures, cryoprotectants.*

Introduction. In recent years the problem of long-term low temperature preservation of biomacromolecules with enzyme activity gained both general biological and

applied meaning. To some degree it may be explained by the fact that enzymes are the main biological instrument of biosensors, the intensive production of which increases all the time. In comparison with other kinds of sensors, e.g. designed on the basis of biological tissues [1] and cells [2], enzyme biosensors

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have higher selectivity and sensitivity, which most often are the distinguishing indicators. Still the usage of enzymes is restricted by their comparatively low resistance at room temperature, thus the necessity of long-term maintenance of their functional properties during preservation is an important problem for many biosensors. Previously conducted researches allowed revealing some peculiarities of preserving biosensor detectors at temperatures, close to room temperature and household refrigerator [3, 4], but further increase of the biosensors preservation terms may be based only on the introduction of technologies of low temperature preservation using the temperatures of liquid nitrogen or close to it. Cryopreservation, in its turn, requires the study of the influence of freeze and subsequent thawing processes on the enzymes, the selection of conditions (the composition of the medium, speeds of freezing and thawing) for valuable restoration of structural-functional properties of proteins.

In our researches we used enzyme biosensors on the basis of glucose oxidase, which were first used several decades of years ago. They are still widely used both in medicine and other fields. But the problem of long-term preservation of such biosensors did not obtain its solution until the present time.

Taking the abovementioned into consideration, while performing this work we had the following purpose: to study the possibility of stabilization of glucose oxidase immobilized on the transducers' surface as a component of biosensors by low temperature. For this it was necessary to study the influence of free-thawing and cryoprotective substances on structural-functional properties of glucose oxidase in the solution; to estimate the possibility of using these substances for the preservation of the structure and enzyme activity of the isolated glucose oxidase at low temperatures.

Materials and methods. In the work we used glucose oxidase (-D-glucose: O₂-oxidoreductase KF 1.1.3.4) from *Aspergillus niger*, Faizyme company (South African Republic), purified additionally by the gel-chromatography method on the column with Sephadex G-200. Glycerol, 1, 2-propane diol and dimethylsulfoxide (DMSO) of chemical pure mark were used as cryoprotectants. The enzyme was dissolved in 0.05M potassium-phosphate buffer. Molecule conformation was defined using the absorption spectra and their first derivatives in UV and in visible region, as well as fluorescent spectra. The activity of glucose oxidase was defined at 22°C by the accumulation of hydrogen peroxide which at the action of peroxidase composes a

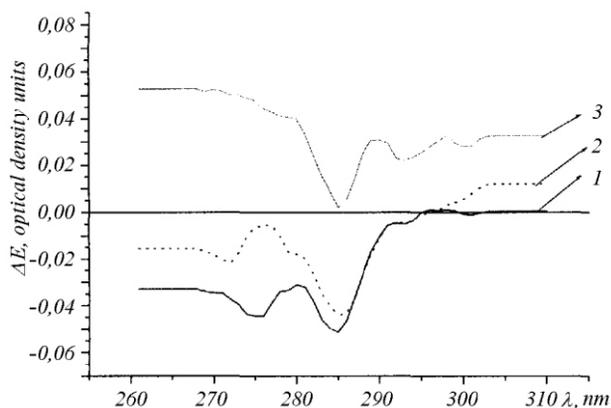


Figure 1. Differential absorption spectra of glucose oxidase after freeze-thawing: 1 – fast freezing; 2 – 5-times fast freezing; 3 – slow freezing

stained composition with phenol and 4-aminophenazone, measuring the degree of optic density increase at 540nm on the linear part of kinetic curve [5]. The absorption spectra were registered on the spectrophotometer Pye Unicam SP 8000 (Great Britain), fluorescent spectra – on the spectrophotometer Hitachi F-4010 (Japan).

In most cases freezing was conducted with the average speed of 100 degrees per minute, and for biosensors with the speed of 3000 degrees per minute it was slower, with the average speed of 5 degrees per minute. Thawing was performed in the water bath at the temperature of 22°C. The isolated enzyme was frozen-thawed in series of six samples; obtained results were worked out by accepted statistical methods.

Biosensors were made and tested at the Institute of Molecular Biology and Genetics, NAS of Ukraine. Immobilized glucose oxidase was analyzed in 5mM phosphate buffer, pH 7.4, at room temperature in the open volume at intensive stirring.

To estimate the influence of the degree of immobilized glucose oxidase response to the introduction of glucose depending on used regimes, we assembled a standard device [7]. Non-specific changes of the primary signal, connected with temperature variations, pH of solution and electrical induction were excluded due to the usage of differential mode of measurements in the work.

Results and discussion. Performed investigations showed that after freezing and subsequent thawing there are some changes which can be seen in the structure of absorption spectra of glucose oxidase in UV region. Fig.1 shows differential absorption spectra, the analysis of which proves, that the effect of low temperatures results in the availability increase of polar

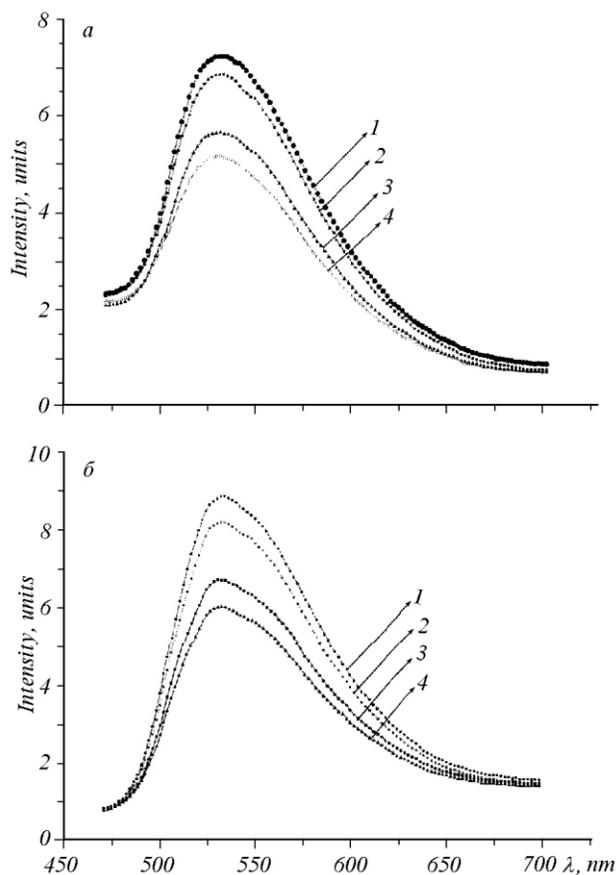


Figure 2. Fluorescent spectra of glucose oxidase after freeze-thawing: 1 – control; 2 - fast freezing; 3 – slow freezing; 4 – 5-times fast freezing

tyrosine sediments for the dissolvent, and these changes are more expressed after fast freezing. At slow freezing there are evident changes in the region of absorption of tryptophan sediments which testify to the increase of their availability for the dissolvent, and probably, partial aggregation of glucose oxidase molecules as the solution turbidity increases. Such assumption may be made on the basis of investigation results which show that the molecules aggregation of glucose oxidase occurs at the expense of hydrophobic interactions at molecule unfolding [8]. It is conditioned by the peculiarities of the structure of the mentioned enzyme: high content of tryptophan (10 sediments per monomer, seven of which are in the active centre and interact with flavine) and high negative charge on the molecule surface.

The intensity of flavine absorption changes also – it decreases in all the spectrum range. The latter testifies to the fact that freezing and subsequent thawing of

Table 1
The activity of glucose oxidase in 1 hour after freeze-thawing

Cryoprotectant	Cryoprotectant concentration, %	Glucose oxidase activity, % to the control
Without a cryoprotector	-	122±5
1, 2-propane diol	20	96±6
1, 2-propane diol	60	97±5
Glycerol	20	112±5

glucose oxidase result in the conformation change of polypeptide chains of glycoprotein which also covers the region of FAD location.

Fluorescent spectra of tryptophan ($\lambda_{\text{excitation}} = 295\text{nm}$) of glucose oxidase do not change much after freezing which proves that flavine does not segregate from the protein part of the molecule [9]. The intensity of flavine fluorescence ($\lambda_{\text{excitation}} = 370$ and 450nm) decreases considerably after freeze-thawing (Fig.2). It allows supposing the increase of availability of active enzyme centre for the dissolvent after both fast and slow freezing. Therefore, freeze-thawing results in conformation change of glucose oxidase which consists in protein globule losing as there is the increase of availability of protein chromophores for polar dissolvent. It is shown by the absorption and fluorescent spectra and changes in the active centre surrounding. Conformational rebuilding in a macromolecule are accompanied by the increase of enzyme activity of glucose oxidase in the first 24 hours after freeze-thawing (Table 1) and the decrease of activity at further preservation at the temperature of 4°C (Fig.3). In most cases enzyme immobilization occurs also at loosening of protein molecules [8], therefore the search of ways of functional activity protection of glucose oxidase in this case is a typical problem.

There are literature data concerning the fact that special buffer solutions, containing mostly zwitterions, were selected at the glucose oxidase stabilization at freeze-thawing [9]. At the same time it was shown that there is very limited usage of electrolytes and polyelectrolytes for enzymes stabilization in the biosensors composition, because they may influence the properties of other integral device elements [10].

There is wide usage of non-ionogenic organic additives in modern cryobiology for the preservation of structural-functional properties of proteins at the condition of low temperatures influence on them [11,

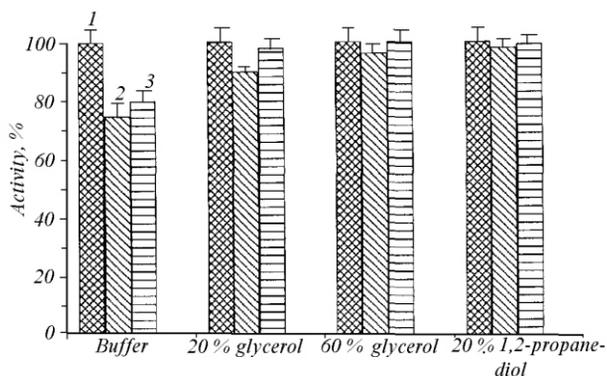


Figure 3. Glucose oxidase activity in 24 hours after freeze-thawing: 1 – control; 2 – fast freezing; 3 – slow freezing

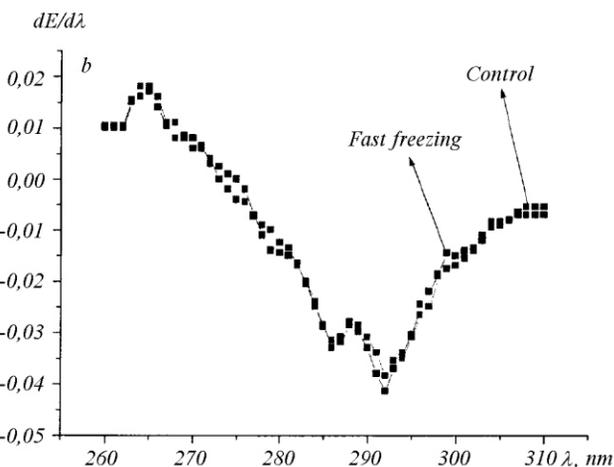
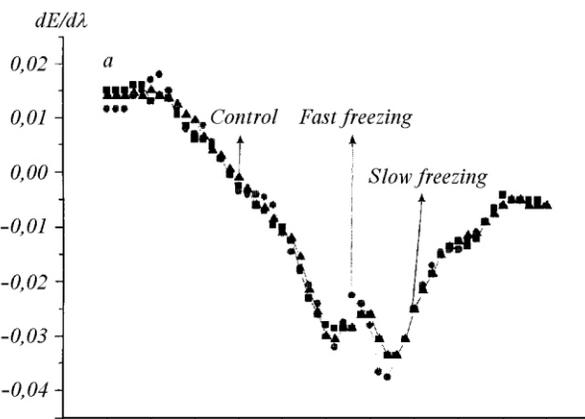


Figure 5. First derivatives of absorption spectra of glucose oxidase after freeze-thawing in 20% (a) and 60% (b) glycerol solutions

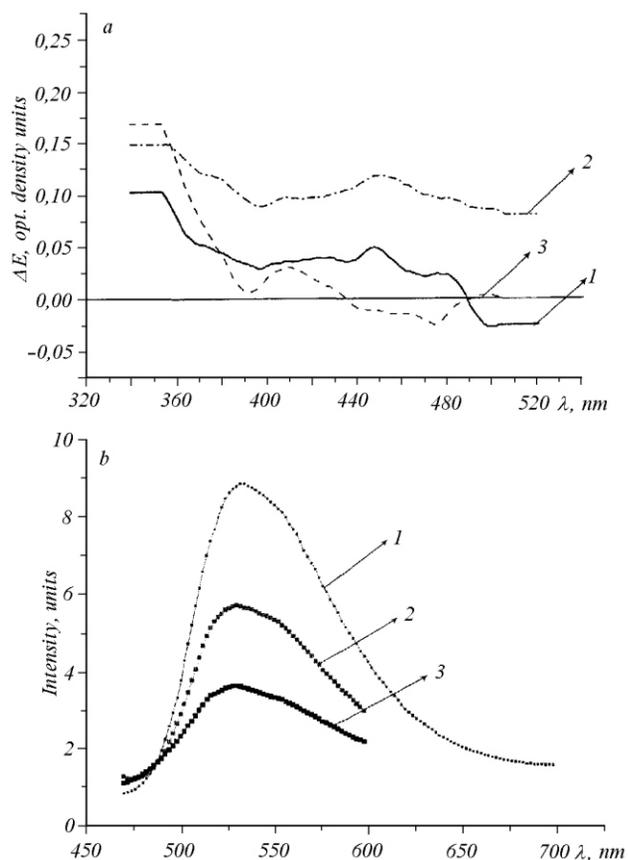


Figure 4. Differential absorption spectra (a: 1 – glycerol; 2 – 1, 2-propane diol; 3 - DMSO) and fluorescent spectra (b: 1 – control; 2 – 1, 2-propane diol; 3 - glycerol) of glucose oxidase in 60% solutions of cryoprotectants

12]. Therefore, for cryoprotection in our work we used glycerol, a well-known cryoprotectant, as well as 1, 2-propane diol and DMSO which recently proved to be suitable.

The investigation of differential spectra of glucose oxidase absorption in the visible region in 60% solutions of cryoprotectants showed that in the presence of glycerol and 1, 2-propane diol they have similar configuration which corresponds to the data of the literature [13] (Fig.4, a). Higher intensity of spectra in the solutions of 1, 2-propane diol is explained by bigger penetrability of its molecules into the region of active centre of glucose oxidase as a result of smaller molecule size. The absorption spectrum in the DMSO solution is considerably different which may testify to the significant change of glucose oxidase conformation. The study of enzyme activity after the exposition showed that glycerol and 1, 2-propane diol do not influence much the activity of glucose oxidase while the exposition in the DMSO solution results in its considerable decrease, therefore this substance was not used in the following experiments.

Table 2

The influence of the speed of freezing, cryoprotectants and conditions of preservation on the response degree of biosensors

Investigation example	Biosensor response, %
1. Control	100±3
2. +60% glycerol solution	91±5
3. +60% 1, 2-propane diol solution	95±5
4. Fast freezing without a cryoprotectant to -196°C	0
5. Slow freezing without a cryoprotectant to -196°C	23±4
6. Slow freezing with 60% glycerol solution to -196°C	68±7
7. Slow freezing with 60% 1, 2-propane diol solution to -196°C	46±7
8. Preservation in the course of 6 months in liquid nitrogen with 60% glycerol solution after slow freeze-thawing (1-30 additions)	63±7
9. Preservation in the course of 6 months at 4°C after first two additions	65±8
10. Preservation in the course of 6 months at 4°C after the following additions	0
11. Preservation in the course of 1 year in liquid nitrogen with 60% glycerol solution after slow freeze-thawing	65±8
12. Preservation in the course of 1 year at 4°C	0

Fluorescent spectra in the solutions of glycerol and 1, 2-propane diol ($\lambda_{excitation} = 450\text{nm}$) are characterized by less intensity (Fig.4, b) in comparison with buffer solution, and the effect of glycerol is more evident.

After slow freezing of the protein in the presence of 20% glycerol the absorption spectrum in UV region does not change practically (Fig.5). In case of fast freezing at this concentration there are some changes of the spectrum which are seen at freezing without cryoprotectants in comparison with the control.

Coming out of absorption spectra, at the usage of 60% glycerol it is possible to preserve protein conformation completely at all the investigated speeds of freezing: the structure of first derivative frozen

samples coincides with the structure of the control ones practically completely.

Glucose oxidase freezing in the presence of 20% 1, 2-propane diol allows preserving enzyme conformation at both used speeds of freezing.

As it was mentioned above, the study of enzyme activity of glucose oxidase in the solutions of investigated cryoprotectants showed (Fig.3) that before freezing the exposition in solutions of cryoprotectants does not influence the enzyme activity considerably. The biggest decrease of activity in comparison with the control was determined after freezing of glucose oxidase in 20% solution of glycerol. The activity decreased insignificantly after freezing in 60% solution of glycerol, minimum deviations (in the range of experiment error) were determined after freezing in 20% solution of 1, 2-propane diol. In all the investigated variants the highest activity decrease was seen after fast freezing.

There is a question of the way in which such conditions of freezing will influence the bioselective element of the biosensors i.e. glucose oxidase in the immobilized state.

For the experiments we selected biosensors on the basis of thin-film conductometric electrodes, produced in the Institute of Chemo- and Biosensorics (Germany) which proved to be suitable in practice.

The degree of biosensor response with glucose oxidase to the introduction of 1mM of glucose solution in potassium-phosphate buffer was accepted as 100%. As it is seen from Table 2, fast freezing without the usage of cryoprotectants stops its work completely. At slow freezing without cryoprotective substances the response degree amounted to about 23%. To provide the protection of the biosensor at freezing and subsequent thawing we used 60% solutions of glycerol and 1, 2-propane diol. The usage of high cryoprotectants concentrations is explained by the intention of obtaining the matrix, which gives in to scouring at freezing. As it is seen from the data of Table 2 the incubation of biosensors in the solutions of cryoprotectants with the subsequent three-times washing by the buffer in the course of 30 minutes worsens the response degree of the biosensor insignificantly.

The freezing of biosensors in the solutions of cryoprotectants improves their preservation. Thus, in case of using 60% solution of 1, 2-propane diol and slow freezing the response degree is 2-fold bigger, and while using 60% solution of glycerol it is 3-fold bigger than at freezing without cryoprotectants. We believe

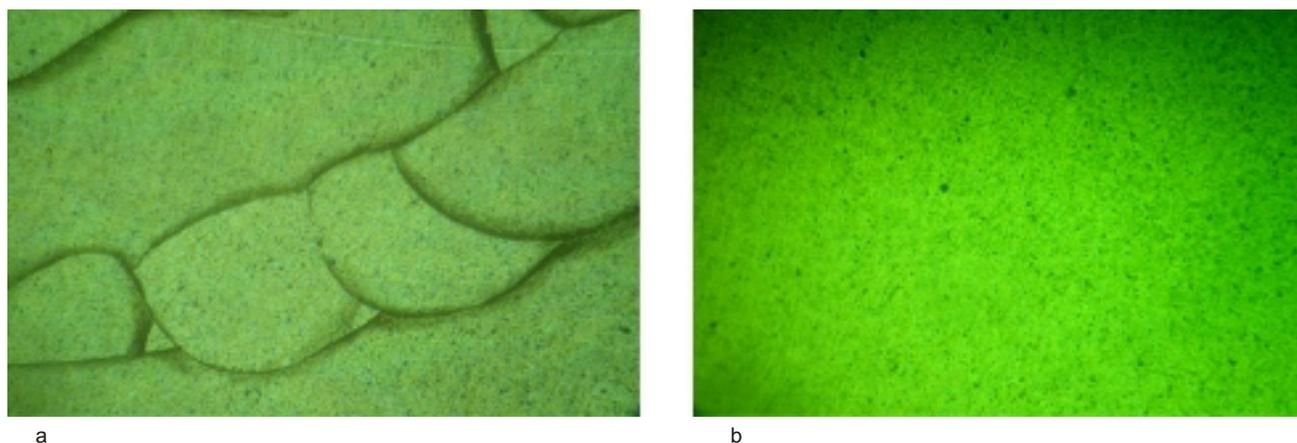


Figure 6. The condition of a protective layer of transducer after the action of fast freeze-thawing on it without previous applying 20% glycerol solution (a) and at its presence (b)

that glycerol is more appropriate as it is sometimes used while producing biosensors to improve glucose oxidase adhesion to the surface of a physical transformer.

Besides, as it results from the experiments, performed before, glycerol prevents the destruction of the protective layer of biosensor surface at freezing (Fig. 6). Therefore, slow freezing with 60% solution of glycerol was selected for the preservation of investigated biosensors in liquid nitrogen. The experiment was performed in such a way that after slow freezing with 60% solution of glycerol to -196°C , the biosensors, which were made and tested, were immersed into liquid nitrogen of low temperature depository. After preserving in the course of 6 months and 1 year the biosensors were thawed slowly on the water bath at 20°C and washed three times in 10mM phosphate buffer in the course of 30 minutes. Then we measured the responses of corresponding biosensors to the addition of 1mM solution of glucose which is a test to the biological activity of glucose oxidase.

According to the results, shown in Table 2 (lines 8, 10), response degrees coincide in the range of experiment error (63 ± 7 and 65 ± 8 correspondingly). Then the biosensors on the basis of glucose oxidase were preserved in the course of 6 months and 1 year at the temperature of liquid nitrogen and at 4°C for the comparison (Table 2, lines 8-12). As it is shown in our investigations, after first two additions of 1mM of glucose solution the response degrees of biosensors (Table 2, lines 8, 9 coincide in the range of experiment error (63 ± 7 and 60 ± 8 correspondingly)), but as a result of the following additions (Table 2, line 10) the response was absent completely, while biosensors, which were preserved at the temperature of liquid nitrogen,

remained in the working condition even after 30 and more additions (Table 2, line 8). In such conditions the biosensors, which were preserved in the course of 1 year at the temperature of 4°C by the prototype (Table 2, line 12) stop functioning completely (the response is absent), while the response degree of biosensors, which were kept in the course of 1 year at the temperature of liquid nitrogen (Table 2, line 11) corresponds to the response of biosensors which were kept in the course of 6 months (Table 2, line 8).

Then, to compare freezing with 60% solutions of glycerol (Table 2, line 6) and 1, 2-propane diol (Table 2, line 7) the biosensors were immersed into separate plastic containers, after that they were slowly ($1-5^{\circ}\text{C}$ per minute) frozen to -196°C . According to the results, given in Table 2, the usage of 60% glycerol solution was more effective for further preserving of biosensors on the basis of glucose oxidase. Therefore, presented data allow making the following conclusions:

- freezing and subsequent thawing of isolated glucose oxidase result in the change of its conformation, which is accompanied by the decrease of enzyme activity which is more expressed at high speeds of freezing;
- the presence of glycerol or 1, 2-propane diol in the freezing medium allows preventing changes, caused by the action of low temperatures;
- the investigation results, obtained on the isolated glucose oxidase allowed determining optimum composition of the medium and freezing regime, at which there is no loss of properties of glucose, immobilized on the physical basis, in the composition of a working biosensor at its preservation in the course of 1 year at the temperature of liquid nitrogen with subsequent slow thawing.

В. И. Грищенко, О. А. Нардид, К. Д. Розанова, М. И. Щетинский,
Е. И. Науменко, С. В. Дзядевич

Низкотемпературная стабилизация глюкозооксидазы в составе биологического сенсора

Резюме

Для поиска путей длительного хранения биосенсоров при помощи низких температур изучено влияние замораживания — оттаивания с разными скоростями охлаждения на структурное состояние глюкозооксидазы. Чтобы избежать утраты свойств фермента в процессе низкотемпературного хранения использованы следующие криопротекторы: глицерин, 1, 2-пропандиол и ДМСО. Глицерин и 1, 2-пропандиол оказались более пригодными для низкотемпературного хранения глюкозооксидазного сенсора, что дало возможность не только уберечь белковую часть биосенсора, но и не нарушить защитного слоя поверхности биосенсора.

Ключевые слова: глюкозооксидаза, биосенсор, низкие температуры, криопротекторы.

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