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Yeast-modified electrodes as amperometric biosensors for glycerophosphate

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Several strains of baker's and wine yeast *Saccharomyces cerevisiae* and yeast *Kluyveromyces lactis* were screened for possible sources of the enzyme L-glycerophosphate oxidase in whole cell amperometric biosensors for glycerophosphate. The sensitivity of a yeast-modified carbon paste electrode to glycerophosphate depended on the yeast sample, the mediator and the mode of electrode preparation. Potassium hexacyanoferrate (III) was found to be the most suitable mediator in terms of electrode sensitivity and stability. The best biosensor was based on commercially available baker's yeast. The detection limit of such electrodes was in the range 33 to 48 μM of D,L-glycerophosphate. The response was linear in the concentration range from 0 to 2.4 mM.

Key words: bosensor, carbon paste, yeasts, glycerophosphate

INTRODUCTION

Biosensors have been most commonly constructed by using purified enzymes with high specific activities. However, purified enzymes are expensive and often not very stable. Therefore, whole bacterial and yeast cells were proposed as enzyme sources for biosensor construction [1–3]. Long and costly enzyme purification is eliminated; besides, enzymes remain in their natural environment that has an advantageous effect on their stability.

Our former research of feasibility to use several samples of baker's and wine yeast *Saccharomyces cerevisiae* as the sources of the enzyme flavocytochrome b_2 was devoted to the development of amperometric yeast-based biosensors for lactic acid [4–7]. The fact that dry baker's yeasts could serve as a source of the enzyme glycerophosphate oxidase (GPO) [8] has motivated this investigation of yeast-modified electrodes as biosensors for glycerophosphate.

GPO is a flavoenzyme that catalyses the oxidation of L-α-glycerophosphate to dihydroxyacetone phosphate according to the reaction

$$\text{L-α-glycerophosphate} + O_2 \xrightarrow{\text{GPO}} \text{dihydroxyacetone phosphate} + H_2O_2. \quad (1)$$

GPO has been isolated from various bacteria [9–15]. The enzymes differed in their optimal pH values, Michaelis constants, and did not show high stability. For analytical purposes, GPO has been used for developing biosensors for determination of glycerophosphate (an intermediate metabolite in the anabolic and catabolic pathways of lipids) [16–19]. The combination of GPO with other enzymes has also been employed for the determination of various substances such as triglycerides [20, 21], creatine kinase [16, 17], glycerol [18, 19] or adenosine triphosphate [20].

Several strains of baker's and wine yeast *Saccharomyces cerevisiae* and yeast *Kluyveromyces lactis* were screened for possible sources of the GPO in whole cell amperometric biosensors for glycerophosphate. Yeast-modified electrodes were prepared either by incorporating the yeast cells into the bulk carbon paste or by placing on the top of the carbon paste electrode surface. The research was carried out by using artificial mediators instead of oxygen according to the scheme:

$$\text{L-α-glycerophosphate} + \text{mediator}_{(ox)} \xrightarrow{\text{GPO}} \text{dihydroxyacetone phosphate} + \text{mediator}_{(red)} \quad (2)$$

$$\text{mediator}_{(red)} \rightarrow \text{mediator}_{(ox)} + e^-, \quad (3)$$

where Eqs. 2 and 3 are, respectively, chemical and electrochemical reactions. The latter reaction was used for amperometric measurements.

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EXPERIMENTAL

Two samples of instant dry baker's yeast (Fermipan, Holland and "dr. Oetker", Germany) and pressed baker's yeast *Saccharomyces cerevisiae* (Lallemand, Poland, with a shelf life not less than 2 weeks as specified by the producer) were obtained from a local market. Further in the text, these samples are nominated, respectively, BY1 (Fermipan), BY2 ("dr. Oetker") and BY3.

Two strains of wine yeast *Saccharomyces cerevisiae*: wine strain type K2 killer Rom K-100 HM/HM wt [kill-K2] and α'1 MATα leu2[kil-0] and two strains of yeast *Kluyveromyces lactis*: MD2/1(MATα argA lysA ura3 K^- $pKD1^-$) and MW270-7B(MATa leu2 ura3 K^+ $pKD1^-$) [27] from the collection of the Institute of Botany (Vilnius, Lithuania) were grown on the YEPD medium (1% yeast extract, 2% peptone, 2% glucose and 2.5% agar) until stationary phases. The plates were kept for 3 days at 30 °C. Afterwards the samples of yeasts were stored in the fridge (+4 °C). These samples are nominated, respectively, WY1, WY2, KL1 and KL2.

BY3, WY1, WY2, KL1 and KL2 were dried at room temperature for 24 hours and at elevated temperatures (30, 50 or 90 °C) for 2 hours.

Sodium-α-glycerophosphate (or D,L-glycerophosphate) was from Shuchardt (Munchen).

$K_3[Fe(CN)_6]$ and 2,6-dichorophenolindophenol sodium salt hydrate (DCPIP) were from Fluka. 1,2-naphthoquinone-4-sulfonic acid sodium salt (NQS) was from Merck. Phosphate buffer was prepared from 0.1 M KH_2PO_4 and contained additionally 0.1 M KCl (both from Fluka). The values of pH were adjusted with KOH or HCl.

Solutions were deoxygenated by purging nitrogen for 20 min before and during the measurements.

Plain carbon paste was prepared by mixing 200 mg of graphite powder (Merck) with 100 μL of paraffin oil (Fluka). The paste was packed into an electrode body consisting of a plastic tube (diameter 2.9 mm) and a copper wire serving as an electrode contact. The yeast suspensions were prepared from, respectively, 80 or 40 mg of intact or dried yeast, in 1 mL of phosphate buffer at pH 7.3 and were kept at room temperature under stirring at least for 30 min before use. A layer of the yeast on the surface of a plain carbon paste electrode was formed by dipping the electrode into the suspension of yeasts. These electrodes are denoted as CP/Y further in the text. The CP/Y was allowed to dry at room temperature for 30 to 40 min and then was covered with a dialysis membrane (Aldrich-Sigma).

Bulk yeast modified electrodes (CPY_{bulk}) were prepared by mixing of 80 mg of intact yeast with 120 mg of graphite and 100 μL of paraffin oil. The paste was packed into an electrode body consisting of a plastic tube (diameter 2.9 mm) and a copper wire serving as an electrode contact. After smoothing the surface with white paper, the electrode was covered with a dialysis membrane.

Electrochemical experiments were carried out with a BAS-Epsilon Bioanalytical system (West Lafayette, USA) and a three-electrode cell arranged with a magnetic stirrer. Platinum wire and Ag/AgCl, 3M NaCl were, respectively, counter- and reference electrodes. A modified carbon paste electrode served as a working electrode. Measurements were performed in a thoroughly stirred solution (15 mL) containing mediators $K_3[Fe(CN)_6]$, DCPIP or NQS. The operational potentials were somewhat higher than the values of the formal potentials determined by cyclic voltammetry [7] and were 0.3 V, 0.1 V and 0.2 V for, respectively, $K_3[Fe(CN)_6]$, DCPIP and NQS. After the steady states of the background currents were achieved, aliquots of D,L-glycerophosphate were added and electrode responses were measured.

RESULTS AND DISCUSSION

Electrode modification with a layer of yeasts was performed manually by dipping a top of the electrode into a yeast suspension; therefore, it was hard to expect very reproducible responses. To compare the results, three CP/Y electrodes were prepared from the same suspension during the same day. Three bulk yeast-modified electrodes (CPY_{bulk}) were prepared from the same yeast-modified paste during 2 days. Yeast-modified electrode responses to 0.6 mM glycerophosphate in 0.5 mM mediator solutions at pH 7.3 are summarized in Tables 1 and 2.

Table 1. **CP/Y electrode responses to 0.6 mM D,L-glycerophosphate in 0.5 mM mediator solutions at pH 7.3. Operating potentials: 0.3 V for $K_3[Fe(CN)_6]$, 0.1 V for DCPIP, 0.2 V for NQS**

Yeast sample	Drying conditions	Current responses with		
		$K_3[Fe(CN)_6]$, nA	DCPIP, nA	NQS, nA
BY1		82 ± 7	17 ± 11	32 ± 11
BY2		78 ± 3	32 ± 11	40 ± 9
BY3	20 °C, 24 h	95 ± 11	34 ± 14	32 ± 17
BY3	50 °C, 3 h	100 ± 18	42 ± 12	29 ± 4
BY3	90 °C, 2 h	–	–	–
WY1	20 °C, 24 h	46 ± 16	17 ± 6	11 ± 5
WY1	50 °C, 2 h	49 ± 13	11 ± 7	9 ± 4
WY1	90 °C, 2 h	–	–	–
WY2	20 °C, 24 h	18 ± 6	9 ± 6	11 ± 7
WY2	40 °C, 3 h	17 ± 11	–	9 ± 6
KL1	20 °C, 24 h	44 ± 19	27 ± 6	17 ± 6
KL1	40 °C, 3 h	32 ± 6	17 ± 6	27 ± 9
KL2	20 °C, 24 h	26 ± 7	11 ± 4	17 ± 6
KL2	50 °C, 2 h	32 ± 6	6 ± 3	11 ± 6
BY3	intact	17 ± 11	17 ± 6	11 ± 5
WY1	intact	11 ± 6	9 ± 3	6 ± 3
WY2	intact	6 ± 3	6 ± 3	–
KL1	intact	6 ± 3	–	–
KL2	intact	–	–	–

Table 2. **CPY_{bulk} electrode responses to 0.6 mM D,L-glycerophosphate in 0.5 mM mediator solutions at pH 7.3. Operating potentials: 0.3 V for $K_3[Fe(CN)_6]$, 0.1 V for DCPIP, 0.2 V for NQS**

Yeast sample	Current responses with		
	$K_3[Fe(CN)_6]$, nA	DCPIP, nA	with NQS, nA
BY3	54 ± 18	17 ± 6	12 ± 4
WY1	17 ± 4	9 ± 3	17 ± 6
WY2	11 ± 6	9 ± 4	6 ± 3
KL1	17 ± 11	9 ± 6	9 ± 6
KL2	14 ± 4	6 ± 3	–

The responses of yeast-modified electrodes to glycerophosphate depended on the yeast sample, the mediator and the mode of electrode preparation. The highest electrode sensitivities to glycerophosphate were obtained with CP/Y containing commercially available dry baker's yeast (BY1 and BY2). The most effective mediator was $K_3[Fe(CN)_6]$. CP/Y with intact yeast cells showed the lowest responses to glycerophosphate as well as in the cases of CP/Y sensitivity to lactic acid [5–7] possibly due to restricted permeation of mediators and analytes through a cell wall and/or a membrane. A simple procedure of drying the yeasts at temperatures up to 50 °C before electrode modification has also caused the increase in electrode sensitivity. CP/Y containing yeast samples dried at 90 °C were insensitive to glycerophosphate, whereas the electrodes with these samples were still sensitive for L-lactic acid [7] indicating lower thermal stability of GPO compared to that of flavocytochrome b_2.

CP/Y electrodes were more convenient for the screening of the GPO activity in the yeast samples compared to CPY_{bulk} electrodes, as their sensitivities were higher (Table 1). Besides, the responses were more reproducible, the values of the background currents (0.005 to 0.15 μA) were lower, the noise levels were lower and the time to achieve a constant background current was shorter (about 20 min) compared to those of CPY_{bulk}.

The electrode responses with DCPIP and NQS as mediators were significantly lower compared to those obtained with $K_3[Fe(CN)_6]$ (Tables 1 and 2).

To test the operational stability of the electrodes, the measurements of the current responses to gradual addition of D,L-glycerophosphate up to 3.6 mM were successively repeated for three times. When oxygen was not removed from the solution, 90 to 80% and 74 to 65% of the initial sensitivities, during, respectively, the second and the third run were obtained for CP/Y (yeast samples BY1, BY2 and BY3, dried at room temperature or at 50 °C) with $K_3[Fe(CN)_6]$ as a mediator. The stability of the responses was improved when oxygen was removed from the solutions: there was no loss of sensitivity after running five consecutive calibration curves in the range 0.6 to 3.6 mM of D,L-glycerophosphate. The influence of oxygen in the solution on the stability of yeast-modified electrode responses could probably suggest an idea that some amount of hydrogen peroxide was formed in the presence of oxygen and has affected the stability of the enzyme, as it is known that oxidases deactivate in the presence of hydrogen peroxide [28]. In the cases when NQS or DCPIP were used as mediators, the removal of oxygen from the solutions practically did not influence the operational stability of the electrodes (yeast samples BY1, BY2, BY3, WY1): the electrodes lost 10 to 15% of the initial sensitivity with each subsequent run both in de- and non-deoxygenated solutions. Operational stability of CPY_{bulk} (yeast sample BY3) in non-deoxygenated solutions of $K_3[Fe(CN)_6]$ was relatively higher compared to that of CP/Y (sample BY3, dried at room temperature). After running three consecutive calibration curves in the range 0.6 to 3.6 mM of D,L-glycerophosphate, 92 to 78% of the initial sensitivity was obtained. The removal of oxygen has also increased the stability of CPY_{bulk} in the presence of $K_3[Fe(CN)_6]$, however, with DCPIP and NQS gradual decrease of current responses of CPY_{bulk} was still observed. Thus, further research of electrode properties and optimal operating conditions (such as mediator concentration, optimal pH region, thermal stability) was carried out with $K_3[Fe(CN)_6]$ and purging nitrogen both before and during the amperometric measurements.

The dependence of the responses of CP/Y (sample BY1) on the concentration of $K_3[Fe(CN)_6]$ is shown in Fig. 1 and was similar for all tested CP/Y. The electrode responses to D,L-glyceroposphate practically did not vary at $K_3[Fe(CN)_6]$ concentrations higher than 0.2 mM.

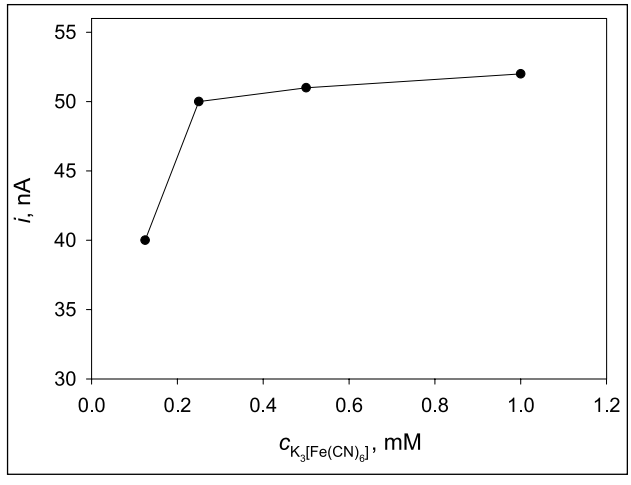

Fig. 1. Dependence of the current responses of CP/Y (sample BY1) on the concentration of $K_3[Fe(CN)_6]$ in de-oxygenated phosphate buffer at pH 7.3 containing 0.6 mM D,L-glycerophosphate, operating potential 0.3 V

The dependences of the responses of CP/Y to glycerophosphate on solution pH are given in Fig. 2. Compared with the optimal pH region around pH 8 obtained for L-glycerophosphate oxidase isolated form dried baker's yeast by measuring the consumption of oxygen [8], the optimal pH region for CP/Y with *Saccharomyces cerevisiae* (Fig. 2, solid and long-dashed lines for, respectively, BY3 and W1,

both dried at room temperature) was somewhat shifted to more acidic region of pH 7 to 8. The results with wine yeast W2 were not reproducible, thus, the optimal pH region was not determined. For CP/Y containing *Kluyveromyces lactis* (Fig. 2, dotted and short-dashed lines, for respectively, KL1 and KL2), the optimal pH was also shifted to more acidic region of pH 6.3 to 7.3.

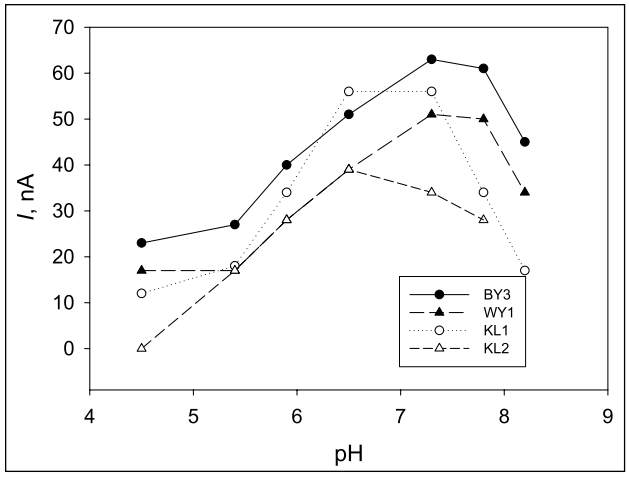

Fig. 2. Dependence of the current responses of CP/Y on solution pH in de-oxygenated phosphate buffer containing 0.6 mM D,L-glycerophosphate and 0.5 mM $K_3[Fe(CN)_6]$, operating potential 0.3 V

As the most sensitive to glycerophosphate, CP/Y with commercially available dry baker's yeast BY1, BY2 and dried BY3 samples were the most suitable for analytical purposes. The dependences of electrode responses on the concentration of D,L-glycerophosphate were similar for all these electrodes (Fig. 3a) with the linear range up to 2.4 mM with R^2 value not lower than 0.991. The apparent Michaelis constants K'_M and the maximum values of the currents (i_{max}) were obtained from Lineweaver–Burke plots (i^{-1} vs [D,L-glycerophosphate]$^{-1}$) and were, respectively, in the range 8.2 ± 3.2 mM to 13.4 ± 2.7 mM and 1.6 ± 0.4 μA. Detection limits calculated from the response ratio to noise 3:1 for these electrodes were in the range 33 to 48 μM of D,L-glycerophosphate.

Figure 3b shows the dependence of current responses of CPY_{bulk} (sample BY3) on the concentration of D,L-glycerophosphate. The R^2 value in the concentration up to 3.6 mM was not lower than 0.997. The K_M' and i_{max} values were, respectively, 20.0 ± 6.7 mM and 1.9 ± 0.5 μA. The detection limit obtained with the most sensitive electrode was about 110 μM of D,L-glycerophosphate.

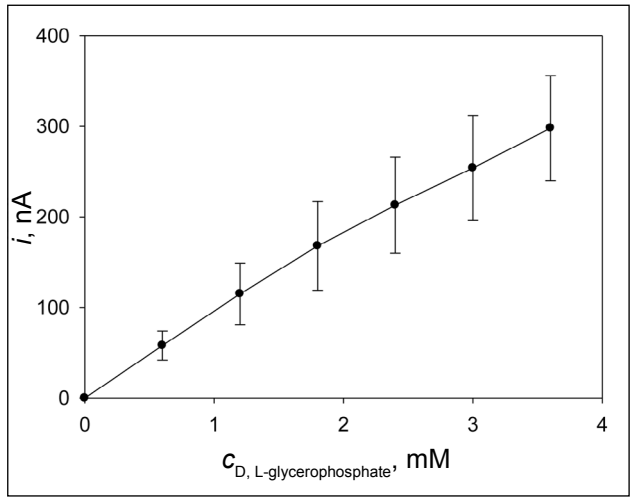

Fig. 3b. Dependence of the current responses of CPY_{bulk} (sample BY3) on the concentration of D,L-glycerophosphate in de-oxygenated phosphate buffer at pH 7.3 containing 0.5 mM $K_3[Fe(CN)_6]$, operating potential 0.3 V

The investigation of the dependence of CP/Y responses to glycerophosphate on the solution temperature (Fig. 4)

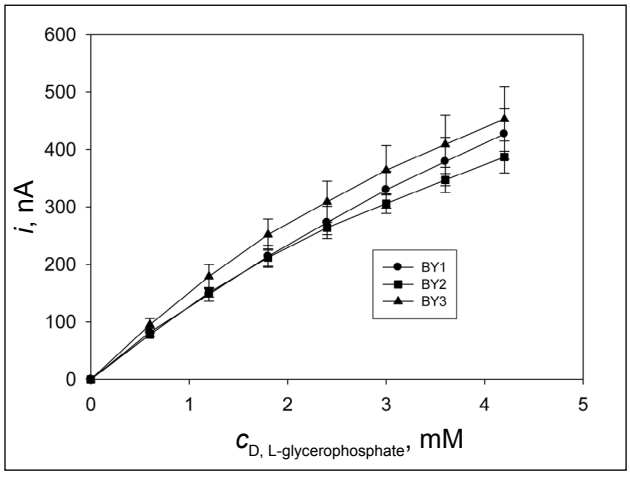

Fig. 3a. Dependence of current responses of CP/Y on the concentration of D,L-glycerophosphate in de-oxygenated phosphate buffer at pH 7.3 containing 0.5 mM $K_3[Fe(CN)_6]$, operating potential 0.3 V

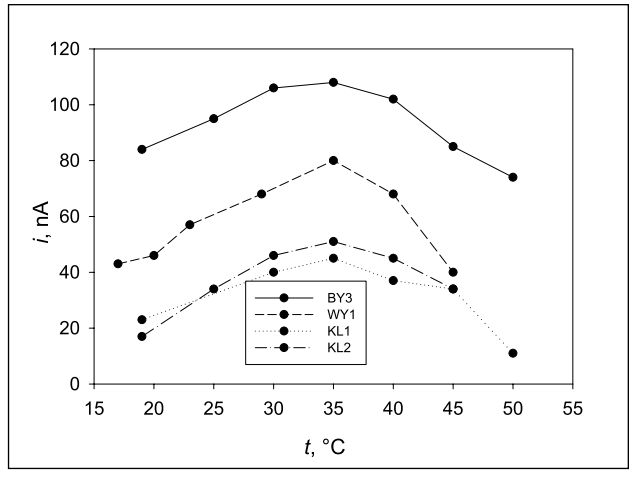

Fig. 4. Dependence of the current responses of CP/Y on the temperature in de-oxygenated phosphate buffer at pH 7.3 containing 0.6 mM D,L-glycerophosphate and 0.5 mM $K_3[Fe(CN)_6]$, operating potential 0.3 V

showed that the currents of all tested electrodes irreversibly decreased at temperatures above 35 °C. However, the results obtained with CP/Y containing BY3 dried at 50 °C (Table 1) showed that the electrodes still possessed relatively high sensitivity. This probably could suggest an idea that the amount of water is very important for the thermal stability of the enzyme.

CONCLUSIONS

Yeast-modified electrode responses to glycerophosphate showed that all tested samples of baker's and wine yeast *Saccharomyces cerevisiae* and yeast *Kluyveromyces lactis* could be possible sources of the enzyme L-glycerophosphate oxidase in whole cell amperometric biosensors.

The sensitivity of a yeast-modified carbon paste electrode to glycerophosphate depended on the yeast sample, the mediator and the mode of electrode preparation. The highest and the most stable current responses were obtained in deoxygenated solutions with commercially available baker's yeast placed on the top of the electrode surface and potassium hexacyanoferrate (III) as a mediator.

Compared to amperometric biosensors based on isolated L-glycerophosphate oxidase that could be used in the micromole range [16, 17], the sensitivities of electrodes containing a layer of commercially available baker's yeast were significantly lower and could be used to determine glycerophosphate in the millimole range.

The advantage of these yeast-based biosensors is that the electrodes can be easily prepared from cheap material. On the other hand, amperometric measurements of yeast-modified electrode responses to glycerophosphate could be used as a means for fast and simple screening of L-glycerophosphate oxidase activity in various yeast cells.

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MIELĖMIS MODIFIKUOTI ELEKTRODAI KAIP AMPEROMETRINIAI GLICEROFOSFATO BIOJUTIKLIAI

S a n t r a u k a

Konstruojant amperometrinius glicerofosfato biojutiklius, keletas kepimo ir vyno mielių *Saccharomyces cerevisiae* bei *Kluyveromyces lactis* pavyzdžių buvo panaudoti kaip galimi fermento L-glicerofosfato oksidazės šaltiniai. Biojutikliai buvo ruošiami modifikuojant anglies pastos elektrodus: uždedant mielių sluoksnį ant elektrodo paviršiaus arba sutrinant mieles kartu su grafitu. Amperometriniai matavimai buvo atliekami naudojant įvairius mediatorius. Modifikuoto elektrodo atsako į glicerofosfatą dydis priklausė nuo mielių rūšies, mediatoriaus ir elektrodo paruošimo būdo. Jautriausi (detekcijos ribos – 33–48 μM, tiesinė srovės priklausomybė nuo koncentracijos – iki 2,4 mM glicerofosfato) ir stabiliausi glicerofosfato biojutikliai buvo gauti naudojant kepimo mieles ir mediatorių kalio heksacianoferatą.