CHEMIJA. 2014. Vol. 25. No. 2. P. 115–118


# Prussian Blue as an alternative to catalase: a bioelectrocatalytic system for production of dihydroxyacetone phosphate

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The bioreactor for the production of dihydroxyacetone phosphate (DHAP) was developed by immobilization of α-glycerophosphate oxidase (GPO) on the Prussian Blue-(PB) modified carbon fibre (CF) electrode. DHAP reached 32.0 mM with a yield of 64 ± 5% from 50 mM L-α-glycerophosphate (GP), while the maximum concentration of hydrogen peroxide did not exceed 2 mM. A conjugation of two catalytic activities, the enzymatic oxidation of GP to DHAP by GPO and the electrochemical elimination of hydrogen peroxide with PB, allowed exclusion of catalase from a biocatalytic process. The PB-modified electrodes showed a great potential for the development of biocatalytic procedures based on hydrogen peroxide-generating oxidases. These electrodes could be used in the designing of improved biocatalysts.

**Key words:** biocatalysis, bioreactor, dihydroxyacetone phosphate, α-glycerophosphate oxidase, Prussian Blue

## INTRODUCTION

Dihydroxyacetone phosphate-dependent aldolases are known to catalyze the condensation of a number of aldehydes with dihydroxyacetone phosphate (DHAP) to give monosaccharides and other chiral compounds with related structures [1]. Therefore, DHAP is an essential building block for many syntheses. Notwithstanding the commercial availability, DHAP is usually too expensive to be purchased for large-scale synthesis. Nevertheless, while mass production of this compound requires efficient and reliable syntheses, many procedures of enzymatic or chemical production of DHAP have been described [2–4]. DHAP can be prepared by oxidation of L-α-glycerophosphate (GP), mediated by a FAD containing glycerophosphate oxidase (GPO) (E.C.1.1.3.21) [5, 6]. GPOs have been purified from a number of microorganisms: *Streptococcus* sp. [7, 8], *Enterococcus* sp. [9], and *Aerococcus viridians* [10]. The main drawback of the GPO application is a formation of hydrogen

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peroxide, which is detrimental to the activity of the enzyme [4]. The application of various oxidases in bioconversion tackles with the problem of the elimination of hydrogen peroxide. Although for the removal of hydrogen peroxide a number of systems using various catalysts, including bimetallic alloys, mixed-metal oxides or catalase exist, it is not feasible to integrate them into bioreactors to operate in continuous cycles [11–13].

An efficient electrochemical method for the determination of hydrogen peroxide, based on the reduction of hydrogen peroxide on the Prussian Blue (PB)-modified electrode, has been described previously [14, 15]. Moreover, it has been shown that GPO from *Enterococcus* sp. RL1 can use PB as a mediator both under aerobic and anoxic conditions [16].

In this paper we show that the PB-modified electrodes can be applied as a catalase substitution for the electrochemical conversion of GP into DHAP.

## EXPERIMENTAL

### General

All reagents were of analytical grade and were used as received without further purification. Carbon cloth C-BX 200 made of graphite fibres (diameter 6–9 μm) was obtained from Toray Ind. (Saint-Gobain BTI Ltd., UK) and cut into pieces of 4 × 5 cm prior to further modification.

### Purification of the recombinant GPO from *Enterococcus* sp. RL1

*Escherichia coli* BL21 (DE3)/pET21d-*gpo* cells used for GPO expression were grown in the Brain Heart Infusion medium (Oxoid, UK) containing ampicillin (50 mg $l^{-1}$) and induced with isopropyl β-D-1-thiogalactopyranoside (0.1 mM). The GPO was purified on the HiTrap IMAC FF (GE, USA) column. For the elution of bound proteins, a linear gradient of imidazole (0–0.5 M) was used. The fractions containing GPO activity were pooled and dialyzed against 50 mM phosphate buffer solution (PBS), pH 7.2. The stock solution of GPO had an activity of 470 U $ml^{-1}$ (16.6 mg protein $ml^{-1}$) in 50 mM PBS, pH 7.2.

### Enzyme assay procedures

GPO activity was assayed using the peroxidase/*o*-dianisidine method by measuring $H_2O_2$ formation. The reaction mixture contained 50 mM PBS, pH 7.2, 0.3 mM *o*-dianisidine, 5 U of horseradish peroxidase type I, 25 mM of GP, and an appropriate amount of the enzyme solution in a total volume of 1.0 ml. The reaction was started by adding the GPO, and an increase in the absorbance at 430 nm was recorded. One unit of GPO activity was defined as an amount of the enzyme that catalyzed the formation of 1 μmol of $H_2O_2$ per minute under the conditions of the assay.

Protein concentration was measured routinely by the method of Lowry, using bovine serum albumin as a standard.

### Electrode pre-treatment and modification

Carbon fibre (CF) cloth was sonicated in the presence of non-ionic detergent for 10 min, rinsed in water and dried at room temperature. The CF cloth was then sealed into a plastic jacket with Cu wire contact to fabricate an electrode with working area of 20 $cm^2$. The mixture of 50 mM $K_3[Fe(CN)_6]$ and 50 mM $FeCl_3$ dissolved in 100 mM HCl solution was used to modify the CF cloth. CF electrode was immediately immersed in the mixture and incubated for 10 min at room temperature. The PB directly precipitated on the electrode surface. The PB modified CF (CF/PB) electrode was then washed with 100 mM HCl and dried at 80 °C for 90 min.

### Reactor construction

CF/PB/GPO cloth (4 × 5 cm) for the reactor was prepared by covalent immobilization of GPO on CF/PB. In total, 200 μl of GPO stock solution was mixed with 200 μl of 5% (w/v) gelatine solution in PBS, pH 7.0, and 5 μL of 5% (v/v) glutaraldehyde. The mixture was deposited on the CF/PB surface immediately. A working electrode in reactor I was made of CF/PB (Fig. 1a), whilst in reactor II it was made of CF/PB/GPO (Fig. 1b). The working electrode was placed into the cell made of a plastic tube (7 cm in length, 2.5 cm in diameter). The stainless steel tube of 4 cm in length and 0.5 cm in diameter, connected to the outlet of the cell, was used as an auxiliary electrode. The cell was additionally equipped with an Ag/AgCl reference electrode.

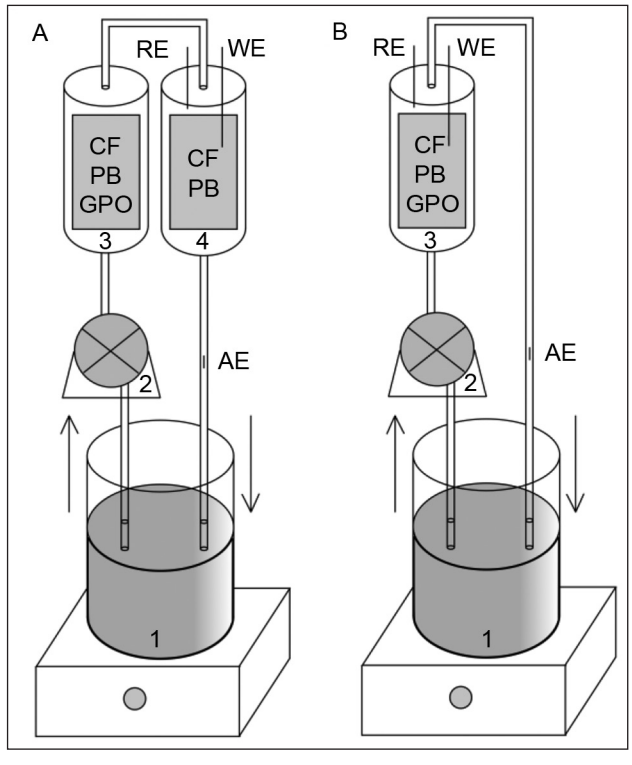


**Fig. 1.** The semi-continuously operating reactor for DHAP production. A – reactor I, B – reactor II. RE – reference electrode, WE – working electrode, AE – auxiliary electrode. *1* – substrate solution, *2* – peristaltic pump, *3* – CF/PB/GPO cloth (A) or electrode (B), *4* – CF/PB electrode

The system was filled with 30 ml of 5 or 50 mM GP solution prepared in 50 mM PBS, pH 7.2. The substrate was pumped at a flow rate of 50 ml $min^{-1}$. The samples were taken periodically to determine the concentrations of GP, DHAP and $H_2O_2$.

**Other analyses**

Concentration of $H_2O_2$ was measured using a three-electrode cell (1 ml) equipped with a magnetic stirrer, using a working Pt electrode (diameter 1 mm) at 0.6 V vs. Ag/AgCl and Ti plate as an auxiliary electrode. The linear range of the assay was 0.01–10 mM of $H_2O_2$.

DHAP was analysed using glycerol-3-phosphate dehydrogenase. The standard reaction mixture contained 50 mM PBS, pH 7.2, 0.2 mM NADH, 2.8 U glycerol-3-phosphate dehydrogenase, and the sample from the reactor in a total volume of 1 ml. The assay was started by the addition of the sample and conducted at 25 °C. The change in absorbance at 340 nm resulting from NADH oxidation ($\varepsilon_{340} = 6\,220\ M^{-1}\ cm^{-1}$) was recorded.

After the removal of $H_2O_2$ from the samples with an excess of $MnO_2$, GP was determined spectrophotometrically by a GPO-peroxidase coupled assay. The standard reaction mixture contained 50 mM PBS, pH 7.2, 0.3 mM *o*-dianisidine, 5 U of horseradish peroxidase type I, 2.3 U of GPO, and the sample in a total volume of 1 ml. The assay was started by the addition of GPO and conducted at 25 °C. The absorbance at 420 nm was recorded. The GP standards from 0 to 0.25 mM of GP were analysed in parallel.

Electrochemical measurements were performed using a potentiostat Voltalab PGZ 402 (Radiometer).

## RESULTS AND DISCUSSION

**Bioreactor for DHAP production**

Although a number of methods for DHAP synthesis have been already described, the efficient biocatalytic system, which could be successfully applied to the biotechnological production of DHAP, is yet to be developed. The aim of the work presented here was the creation of the GPO bioreactor containing an integrated electrochemical module that removes $H_2O_2$. For this, the reactor I was constructed. It consisted of two parts: one (CF/PB/GPO) with immobilized enzyme and the other (CF/PB) – as a working electrode for decomposition of $H_2O_2$ (Fig. 1a). After the reactor I was filled with 5 mM GP solution, production of DHAP and $H_2O_2$ was measured periodically. It is noteworthy that during the first 60 min of the experiment the working electrode was turned off. As shown in Fig. 2a, during the first stage of bioconversion the amount of $H_2O_2$ produced was equal to that of synthesized DHAP. When the CF/PB electrode was turned on, $H_2O_2$ concentration stopped increasing while DHAP was further produced, indicating that the CF/PB electrode eliminates $H_2O_2$.

When the working electrode was turned on at the beginning of the experiment (Fig. 2b), the rate of DHAP production was approximately 0.05 mM $min^{-1}$, and GP was almost completely (>98%) converted to DHAP in 2 hours. Within the first 30 min of the process the maximum $H_2O_2$ concentration (1.2 ± 0.3 mM) was observed, and it was stable throughout the bioconversion.

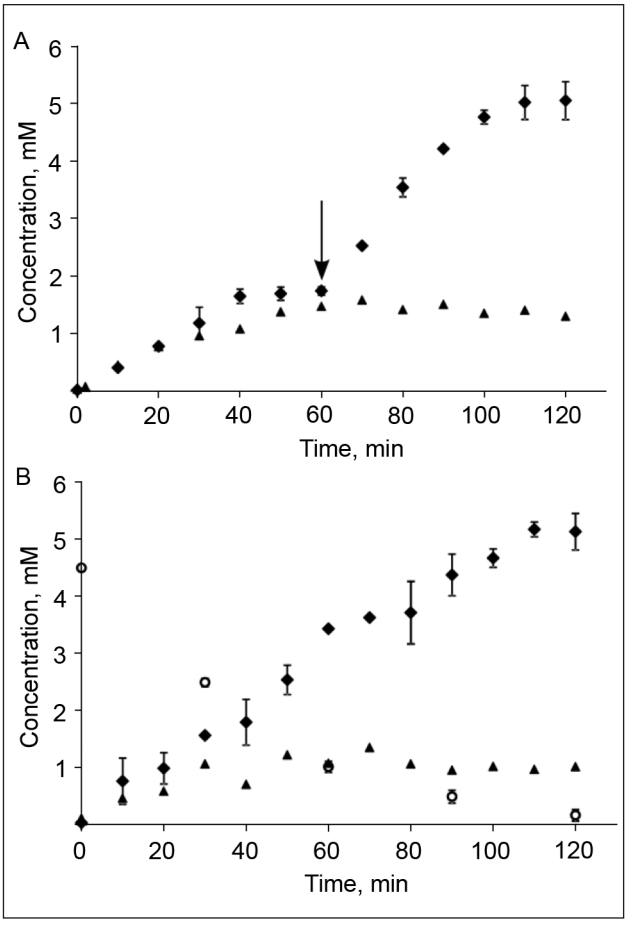


**Fig. 2.** Electrochemical conversion of GP into DHAP in the reactor I. CF/PB electrode potential +500 mV; 30 ml of 5 mM GP stock solution was pumped through the column at the rate of 50 ml $min^{-1}$. A – DHAP and $H_2O_2$ production in the absence of the active CF/PB electrode during the first 60 min of the process; the arrow indicates the moment when the CF/PB electrode was turned on. B – CF/PB electrode turned on throughout the conversion. Closed diamond – DHAP; open circle – GP; closed triangle – $H_2O_2$. The average of triplicate experiments is presented

**Optimization of the DHAP bioreactor**

Substrate concentration is a very important parameter in the economy of bioconversion process, since the higher the concentration of substrate the lower the cost of the product separation. Therefore, the efficiency of the reactor I at higher initial concentration of substrate was examined. The system was filled with 50 mM GP solution. Such increase in the substrate concentration accelerated threefold the rate of DHAP production.

To simplify the system, the reactor II consisting of CF/PB/GPO as a working electrode for decomposition of $H_2O_2$ was constructed (Fig. 1B). No operational differences

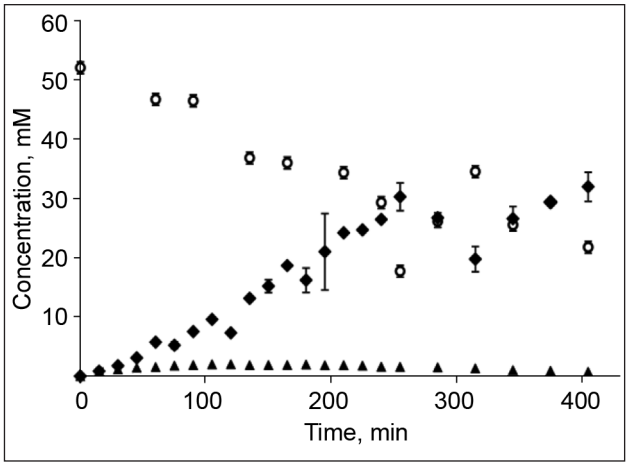


**Fig. 3.** Electrochemical conversion of GP into DHAP in the reactor II. CF/PB/GPO electrode potential +500 mV; 30 ml of 50 mM GP stock solution was pumped through the column at the rate of 50 ml $min^{-1}$. Closed diamond – DHAP; open circle – GP; closed triangle – $H_2O_2$. The average of triplicate experiments is presented

including the rate of DHAP production and the yield of the process between the reactor I and the reactor II were observed. The amount of DHAP produced was equivalent to the amount of GP consumed throughout the conversion (Fig. 3). Results from Fig. 3 demonstrate that 64 ± 5% of GP was consumed for the production of DHAP. Moreover, the concentration of $H_2O_2$ did not increase above 2 mM. Thus, it can be concluded that the catalase-independent DHAP production system presented here is both high-speed and highly efficient.

## CONCLUSIONS

Although many procedures for DHAP production by GPO [2–6] have been already described, this is the first report that demonstrates the application of the PB-modified CF electrode for the elimination of $H_2O_2$. Therefore, such electrode with the immobilized GPO can be used as an efficient electrochemical reactor for DHAP production. Moreover, this work opens a possibility to apply the PB-modified electrodes for optimization of other biocatalytic processes where $H_2O_2$-producing oxidases are used.

## ACKNOWLEDGEMENTS

The work was supported by the European Social Foundation and the Government of the Republic of Lithuania, Project No. VP1-3.1-ŠMM-08-K-01-001. We thank Dr. Laura Kalinienė for critical reading of the manuscript.

Received 5 February 2014
Accepted 26 February 2014

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## BERLYNO MĖLIS – KATALAZĖS ALTERNATYVA: BIOELEKTROKATALIZINĖ SISTEMA DIHIDROKSIACETONO FOSFATO SINTEZEI

*S a n t r a u k a*

Dihidroksiacetono fosfato sintezei skirtas bioreaktorius buvo sukonstruotas α-glicerofosfato oksidazę imobilizavus ant anglies pluošto elektrodo, modifikuoto Berlyno mėliu. Dihidroksiacetono fosfato sintezės iš L-α-glicerofosfato išeiga buvo 64 ± 5 % ir koncentracija tirpale siekė 32,0 mM, o vandenilio peroksido koncentracija visos biokonversijos metu neviršijo 2 mM. Sujungus du katalizinius aktyvumus, fermentinę glicerofosfato oksidaciją iki dihidroksiacetono fosfato naudojant α-glicerofosfato oksidazę ir elektrocheminį vandenilio peroksido pašalinimą Berlyno mėliu, biokataliziniame procese katalazė tapo nebereikalinga.