Development of a glucose-6-phosphate biosensor based on coimmobilized p-hydroxybenzoate hydroxylase and glucose-6-phosphate dehydrogenase

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Abstract

This work reports the development of an amperometric glucose-6-phosphate biosensor by coimmobilizing p-hydroxybenzoate hydroxylase (HBH) and glucose-6-phosphate dehydrogenase (G6PDH) on a screen-printed electrode. The principle of the determination scheme is as follows: G6PDH catalyzes the specific dehydrogenation of glucose-6-phosphate by consuming NADP^+ . The product, NADPH, initiates the irreversible the hydroxylation of p-hydroxybenzoate by HBH in the presence of oxygen to produce 3, 4-dihydroxybenzoate, which results in a detectable signal due to its oxidation at the working electrode. The sensor shows a broad linear detection range between 2 μM and 1000 μM with a low detection limit of 1.2 μM . Also, it has a fast measuring time which can achieve 95% of the maximum current response in 20 s after the addition of a given concentration of glucose-6-phosphate with a short recovery time (2 min).

Keywords: Biosensor; Glucose-6-phosphate; p-Hydroxybenzoate hydroxylase; Glucose-6-phosphate dehydrogenase

1. Introduction

Glucose-6-phosphate plays an important role in the carbohydrate metabolism of all organisms from bacteria to plants and animals, and it can enter a number of catabolic pathways to yield energy ATP or reducing power NAD(P)H. In addition, the relative concentration of glucose-6-phosphate can be utilized to provide information for the activities of several enzymes due to being their substrate or product, such as glucose-6-phosphate dehydrogenase (G6PDH), phosphoglucomutase, hexokinase, phosphoglucose isomerase, etc., and also it is related to the regulations of some enzymes, such as glycogen synthase, protein kinase, etc. (Kirkman and Gaetani, 1986; Coevoet and Hervagault, 1997; Hogema et al., 1997; VillarPalasi and Guinovart, 1997; Sergeeva and Vreugdenhil, 2002; Bellaver et al., 2004).

Due to its importance, various methods have been developed for its determinations by the use of radioactive method, chromatographic method, or spectroscopic technique (Sapag-Hagar et al., 1973; Kirkman and Gaetani, 1986; Banhegyi et al., 1997; Miclet et al., 2001; Sekiguchi et al., 2005). However these methods are time consuming, labor-intensive, expensive, or need to be performed by skilled personnel.

The importance of enzyme-based biosensors has increased considerably in recent years thanks to the advantages of being highly sensitive, rapid, accurate, economical and easy-to-handle for specific measurement of target analyte in complex matrices such as blood, food products and environmental samples. Several types of biosensors have been developed for the measurements of glucose-6-phosphate based on combinations of G6PDH and different mediators or enzymes, including the combination of G6PDH and tetracyanoquinodimethane (Bassi et al., 1999), the combination of G6PDH and 3,3'-bis(benzo[a]phenoxazine-7-ium,5-amino-9-

(diethylamino))-1,4,N,N'-diamidobenzene (Skoog et al., 1991), the combination of G6PDH and poly-3, 4-dihydroxybenzaldehyde (Tzang et al., 2001), the combination of G6PDH and osmium (1,10-phenanthroline-5,6-dione)2-poly(4-vinylpyridine) (Iyer et al., 2003), the combination of acid phosphatase and glucose oxidase (Mazzei et al., 1996), and the combination of G6PDH, glutathione reductase, and polyethyleneimine ferrocene (Suye et al., 2005). However, these biosensors easily encounter mediator leakage or inhibition or are not sensitive enough. Recently, the coimmobilization of dehydrogenase with salicylate hydroxylase has been investigated for developing a series of biosensors that result in high performance characteristics based on the detection of co-substrate consumption or product generation through the two enzyme reactions (Gajovic et al., 1998; Mak et al., 2003; Kwan et al., 2005; Cui et al., 2006), which is also used to construct a glucose-6-phosphate biosensor (Cui et al., 2006).

In this work, we present the development of a new amperometric glucose-6-phosphate biosensor using the coimmobilization of G6PDH and another type of hydroxylase, *p*-hydroxybenzoate hydroxylase (HBH), which is the first report for coupling HBH and dehydrogenase on a screen-printed electrode to construct a biosensor. G6PDH catalyzes the specific dehydrogenation of glucose-6-phosphate by consuming NADP^+ to generate 6-phosphogluconate and NADPH Eq. (1). The product, NADPH, initiates the irreversible hydroxylation of *p*-hydroxybenzoate by HBH in the presence of oxygen to produce 3, 4-dihydroxybenzoate, H_2O and NADP^+ Eq. (2). During the measurement of glucose-6-phosphate, anodic current increased due to the oxidation of 3, 4-dihydroxybenzoate generated by G6PDH and HBH in the enzyme layer, and the increase of anodic current was proportional to the concentration of glucose-6-phosphate based on the equilibrium of the enzyme reactions:

2. Experimental

2.1. Apparatus

A potentiostat EP30 (Biometria, Göttingen, Germany) and a computer installed with the software FIABOLO (Biometria, Göttingen, Germany) were used. The screen-printed electrode (Bio Sensor Technologie GmbH, Berlin, Germany) with a two-electrode configuration was composed of a platinum (Pt) working electrode (diameter: 1 mm) and an Ag/AgCl reference/counter electrode. A stirred measuring cell (volume: 1 ml), connected to a syringe and installed with two connectors (cathode and anode) and an electrode-fitting site, was constructed for the experiments.

2.2. Chemicals

p-Hydroxybenzoate hydroxylase (HBH, EC 1.14.13.2, from *Pseudomonas* sp.) was purchased from Toyobo (Osaka, Japan). Sodium *p*-hydroxybenzoate, glucose-6-phosphate dehydrogenase (G6PDH, EC1.1.1.49, from *Leuconostoc mesenteroides*), β -nicotinamide adenine dinucleotide phosphate sodium salt (NADP⁺), glucose-6-phosphate sodium salt, glutaraldehyde, acetic acid, and 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIZMA[®] BASE, Tris) were all reagent grade purchased from Sigma–Aldrich (St. Louis, USA). Sodium acetate trihydrate, potassium dihydrogen phosphate (KH₂PO₄), dipotassium hydrogen phosphate (K₂HPO₄), and hydrochloric acid (HCl) were from Riedel-deHäen (Seelze, Germany). All solutions were

prepared in double distilled water.

2.3. Buffer

For the measurement of glucose-6-phosphate, except during the optimizations of NADP^+ , *p*-hydroxybenzoate and pH, Tris-HCl buffer (100 mM, pH 8.0) containing *p*-hydroxybenzoate (0.5 mM) and NADP^+ (0.1 mM) was used as the working buffer. For the study of NADP^+ loading, Tris-HCl buffer (100 mM, pH 8.0) containing *p*-hydroxybenzoate (0.5 mM) with various concentrations of NADP^+ (0–0.2 mM) was used. For the study of *p*-hydroxybenzoate loading, Tris-HCl buffer (100 mM, pH 8.0) containing NADP^+ (0.1 mM) with various concentrations of *p*-hydroxybenzoate (0–1.0 mM) were used. For the study of pH effects on biosensor performance, Tris-HCl buffer (100 mM) containing *p*-hydroxybenzoate (0.5 mM) and NADP^+ (0.1 mM) with various pH conditions (pH 7.0–9.5) was used. For the study of biosensor storage, acetate buffer (100 mM, pH 3.5–5.5), K-PBS buffer (100 mM, pH 6.0–7.5), and Tris-HCl buffer (100 mM, pH 8.0–8.5) were investigated to obtain an appropriate storage buffer.

2.4. Biosensor preparation

Enzyme solution containing $6 \text{ U } \mu\text{l}^{-1}$ HBH and $4 \text{ U } \mu\text{l}^{-1}$ G6PDH, was mixed with diluted glutaraldehyde (1% in H_2O) by a volume ratio of 1:1, and a $0.5 \mu\text{l}$ of this mixture was spread over the working Pt electrode of the screen-printed electrode, followed by drying and storing at 4°C overnight. Six kinds of enzyme electrodes, listed in Table 1, were prepared from mixtures of HBH and G6PDH for the optimization of enzyme loadings. The sensor was then screwed into the measuring cell which was filled with buffer solution, and rehydrated for around 30 min at room temperature (22°C) to allow the enzyme matrix swelling before usage.

2.5. Amperometric measurement

One milliliter of Tris-HCl buffer (100 mM, pH 8.0) containing 0.5 mM sodium *p*-hydroxybenzoate and 0.1 mM NADP⁺ was added into the measuring cell, which was performed by manually pipetting. Experiments were carried out at room temperature by applying the specific potential for this type of screen-printed electrode at 0.42 V (a recommended potential which was not optimized in this study) and magnetically stirring the solution at 300 rpm to obtain a uniform distribution of glucose-6-phosphate. After the achievement of a steady background current, measurement was started by adding various volumes (0.1–20 μ l) of standard glucose-6-phosphate solutions (20–200 mM) into the buffer solution contained in the measuring cell, and the current difference was recorded for plotting the calibration curve. After the measurement, a washing step was performed with the use of a syringe for sucking the buffer solution out of the measuring cell to remove glucose-6-phosphate in the solution, and thus to remove 3,4-dihydroxybenzoate on the electrode surface, followed by the addition of a blank buffer (containing no glucose-6-phosphate) to start a new measurement.

3. Results and discussion

3.1. Optimization of the enzyme loadings

Enzyme covered screen-printed electrodes with different loadings of HBH and G6PDH and their sensitivities were summarized in Table 1. The loadings of HBH were varied from 0.5 U to 1.5 U, and the loadings of G6PDH were varied from 0.2 U to 1.0 U. With a constant loading of HBH at 1.5 U, the sensitivity increased ($\sim$ 1.2 times) with doubling G6PDH loading from 0.5 U to 1.0 U. Similarly, with a constant G6PDH loading at 1.0 U, the signal response increased

slightly (~ 1.1 times) with a HBH loading varying from 1.0 U to 1.5 U. Maximum signal was found in the system of 1.5 U HBH and 1.0 U G6PDH, which was sufficient to obtain a maximum response and used as standard enzyme loadings for further experiments.

3.2. Optimization of the working buffer condition

To improve the biosensor performance, various loadings of cofactor NADP^+ , *p*-hydroxybenzoate and working buffer pH were investigated. The amounts of cofactor NADP^+ and *p*-hydroxybenzoate in the enzyme matrix should be sufficient in order to obtain a good linear range. The linear range of the biosensor ranging from 5 μM to 1000 μM was determined by using excess amounts of NADP^+ (5 mM) and *p*-hydroxybenzoate (5 mM) in the measurement of glucose-6-phosphate. For the optimization of NADP^+ , the biosensor response to 1000 μM of glucose-6-phosphate with various NADP^+ loadings was investigated. The response increased with increasing NADP^+ loading and became saturated at a loading of 0.1 mM. Similarly, the biosensor response to 1000 μM of glucose-6-phosphate with various loadings of *p*-hydroxybenzoate was investigated. The response increased with increasing loading of *p*-hydroxybenzoate and became saturated at a loading of 0.5 mM. For the optimization of the working buffer pH, the bienzyme sensor was studied in Tris-HCl buffer with pH values ranging from 7.0 to 9.5. The signal was obtained by measuring 500 μM of glucose-6-phosphate in the measuring cell. The maximum response was obtained between pH 8.0 and 9.0. Therefore, the optimized working buffer was standardized as Tris-HCl buffer (100 mM, pH 8.0) containing 0.5 mM *p*-hydroxybenzoate and 0.1 mM NADP^+ .

3.3. Characterization of the sensor performance

The sensor performance was characterized. Fig. 1 (A) shows the current–time curve of the bienzyme sensor obtained by adding various amounts of glucose-6-phosphate solutions. After the addition of glucose-6-phosphate, the anodic current increased due to the oxidation of 3, 4-dihydroxybenzoate generated by G6PDH and HBH in the enzyme layer. The increase of the anodic current was proportional to the concentration of glucose-6-phosphate. The sensor had a fast measuring time which can achieve 95% of the maximum current response in 20 s after the addition of a given concentration of glucose-6-phosphate with a short recovery time (2 min). The total measurement using the sensor took less than 3 min with high reproducibility. Fig. 1 (B) shows the calibration curve for glucose-6-phosphate with the bienzyme sensor. A linear relationship was observed between the current response and the concentration of glucose-6-phosphate from 2 μM to 1000 μM (slope: 510.4 nA mM^{-1} , $R^2 = 0.9955$, $n = 3$) with a current density of $6.5 \times 10^4 \text{ nA mM}^{-1} \text{ cm}^{-2}$, which was a quite sensitive detection range compared to those by other previously reported glucose-6-phosphate biosensors (Skoog et al., 1991; Mazzei et al., 1996; Bassi et al., 1999; Tzang et al., 2001; Iyer et al., 2003; Suye et al., 2005). For the detection limit, double distilled water (20 μl) was added as blank solution into the measuring cell in order to determine the blank signal. The detection limit of the sensor, calculated as mean blank signal plus three times the standard derivation of mean blank signal, was 1.2 μM of glucose-6-phosphate. Also, the method for this sensor development has a high reproducibility with a R.S.D. of 4.6% for five biosensors by testing their sensitivities.

3.4. Investigation of the sensor storage

The storage of the bienzyme sensor was investigated. Fig. 2(A) shows the pH effect of the storage buffer on enzyme stabilities, which was obtained by measuring 500 μM of glucose-6-

phosphate at room temperature in the working buffer after 50 h storage at 4 °C in various storage buffers with pH values ranging from 3.5 to 8.5. As shown in the figure, the stability was closely related to the buffer pH, and the maximum stability was obtained by storage in buffer with pH 5.5–6.0. Therefore, Tris–HCl buffer which maintains pH out of this range is not appropriate for storing the bienzyme sensor, and 100 mM K–PBS buffer at pH 6.0 was chosen as the storage buffer. Fig. 2(B) shows the storage stability of the bienzyme sensor over a period of 20 days. The sensor was stable and maintained around 100% of the maximum response during the first 5 days due to the freshly prepared enzymes and their high activities. Obvious reduction of the sensor stability was observed at day 7 and response continued to fall to 50% at day 11 and to 5% at day 20. The reduction of sensor performance was probably due to the different lifetimes of individual enzymes, which were reduced during storage of the biosensor in buffer.

4. Conclusions

We have demonstrated the development of a glucose-6-phosphate biosensor based on the coimmobilization of HBH and G6PDH on a screen-printed electrode. The sensor shows high performance characteristics with a sensitive detection range, a low detection limit and a short measuring time. Thus, this work provides a new analytical approach for the measurement of glucose-6-phosphate that is sensitive, rapid, economical and easy to handle. In addition, the sensor could also be used for the activity determination of several enzymes which involve glucose-6-phosphate as substrate or product in the enzymatic reaction. Moreover, the first time successful coupling of HBH and G6PDH on a screen-printed electrode to construct biosensor provides new opportunities for developments of other bienzyme and trienzyme biosensors utilizing NAD(P)H-dependent HBH and NAD(P)⁺-dependent dehydrogenases.

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Table

Table 1

Sensitivities of glucose-6-phosphate biosensors with different loadings of HBH and G6PDH

Electrode	HBH (U)	G6PDH (U)	Sensitivity (nA mM ⁻¹)
I	0.5	1.0	190.3
II	1.0	1.0	464.4
III	1.0	0.5	392.2
IV	1.5	0.2	168.1
V	1.5	0.5	415.9
VI	1.5	1.0	510.4

Figure legends

Fig. 1. Characterization of the sensor performance. (A) Current-time curve of the bienzyme sensor to (a) 250 μM , (b) 500 μM , and (c) 1000 μM of glucose-6-phosphate with (w) washing step. (B) Calibration curve for glucose-6-phosphate by the bienzyme sensor ($n=3$) (Sensor: 1.5 U HBH and 1.0 U G6PDH in 0.5 μl of enzyme matrix. Working buffer: 100 mM Tris-HCl buffer at pH 8.0, 0.1 mM NADP^+ , 0.5 mM p-hydroxybenzoate).

Fig. 2. Investigations of the bienzyme sensor storage. The sensors were stored in buffer solution at 4 $^{\circ}\text{C}$ after the measurement of 500 μM glucose-6-phosphate. (A) Determination of the optimum pH of storage buffer by measuring the sensor response after 50 hours' storage ($n=3$) (Storage buffer: 100 mM Tris-HCl buffer at pH 8.0–8.5, 100 K-PBS buffer at pH 6.0–7.5, 100 mM acetate buffer at pH 3.5–5.5). (B) Storage stability of the bienzyme sensor by using K-PBS (100 mM, pH 6.0) as the storage buffer ($n=3$). All analytical conditions were the same as in Fig. 1. The signal response (%) was calculated by normalizing the signal to the maximum signal obtained on the first day of measurement.

Fig. 1

Fig. 2