UDC 543.55:543.8

BIOSENSOR WITH L-PHENYLALANINE DEHYDROGENASE AND DIAPHORASE IMMOBILIZED ON NANOCOMPOSITE ELECTRODE FOR THE DETERMINATION OF L-PHENYLALANINE IN URINE

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Abstract

Bienzymatic amperometric biosensor consisting of L-phenylalanine dehydrogenase and diaphorase for the determination of L-phenylalanine has been developed. Enzymes were immobilized between chitosan layers onto the surface of planar nanocomposite electrodes based on multi-walled carbon nanotubes. Wide linear concentration range was obtained for 12–1250 μM of L-phenylalanine in phosphate buffer solution of pH 9.0 and in the presence of 2 mM NAD^+ and 2.5 mM ferricyanide mediator. The limit of detection was 6.4 μM, the sensitivity 22.3 nA μM^{-1} cm^{-2} and the response time within 60 s. The biosensor exhibited good storage (90% of initial sensitivity after 12 months) and operational stabilities (sensitivity above 90% after 5 days of use). It is proposed for the potential use in self-monitoring of phenylalanine in urine by phenylketonurics. A low signal was measured when tested for urine samples of healthy people, which indicated a low susceptibility for various interferences. Recovery values of about 100% were obtained after spiking urine samples with phenylalanine.

Keywords: biosensor, L-phenylalanine dehydrogenase, diaphorase, L-phenylalanine determination, nanocomposite, phenylketonuria, multi-walled carbon nanotubes, urine.

Introduction

Phenylketonuria (PKU) is a generic disorder resulting in mental retardation if not detected soon after birth [1, 2]. PKU is a panethnic disease with an estimated birth prevalence varying from 1:2,500 to 1:125,000 [3]. The symptoms of the disease are directly associated with deficiency of the liver enzyme phenylalanine hydroxylase that catalyses the conversion of phenylalanine into tyrosine [4]. When this major metabolic pathway is disrupted, excessive phenylalanine accumulates in the serum, and together with its metabolites is excreted in the urine [5]. An early detection of PKU combined with the introduction of a therapeutic, low phenylalanine diet lead to normal development of the affected child. Subsequently people must be monitored for their phenylalanine levels often and for their entire lives [1, 6–8]. The National Institutes of Health suggests that they should be tested one to two times per month and up to twice weekly during pregnancy.

There are several semi-quantitative [9] as well as quantitative methods for the phenylalanine determination in blood and urine. Some of them are listed by Pijanowska and Remiszewska [10] including spectrophotometry, fluorometry, gas chromatography, and electrophoresis. More recently, isotope dilution liquid chromatography/tandem mass spectrometry (MS/MS) and amino acid analysis by electrospray–MS/MS have

been developed as reference methods for the determination of phenylalanine level in the human serum [11, 12]. These methods are often time-consuming and require complex, tedious sample preparation by skilled technicians using highly sophisticated instrumentation. Although the determination of phenylalanine is typically performed in blood, the analysis using urine is preferred by adults and chemists since urine testing is non-invasive and urine has a higher concentration of phenylalanine [13, 14] than blood (the clinical range of phenylalanine in human urine is 20 to 60 mM for people with PKU compared to 0.6 to 3.8 mM in blood).

Therefore, improved methods that are simple, inexpensive, and offer rapid and quantitative determination of phenylalanine in human urine are needed. Biosensors have been shown to be progressive analytical tools capable to perform quick and selective detection of many types of important analytes [15]. Recently, several phenylalanine enzymatic tests and biosensors have been described such as phenylalanine dehydrogenase (PDH) based colorimetric [16] or potentiometric methods [10], PDH-based amperometric biosensors [17, 18], and optical biosensor employing phenylalanine ammonia lyase [19].

In this paper we present an easy-to-prepare phenylalanine amperometric biosensor utilizing bienzymatic composition consisting of PDH and diaphorase (DP) immobilized between chitosan layers onto nanocomposite electrode with a high sensitivity and a long-term stability. The proposed biosensor could be the first step in the development of a cheap and an easy-to-use system (sensor, kit, pocket-sized analyzer) for the highly required phenylalanine home self-monitoring.

1. Experimental

1.1. Materials. PDH (38 U mg^{-1} protein) was obtained from Unitika (Japan), DP preparatives (57 or 62.4 U mg^{-1} solid) and NAD^+ were purchased from Sorachim (Lausanne, Switzerland). Silica gel rubin, sodium phosphate tribasic dodecahydrate, potassium hexacyanoferrate(III), N-eicosane, L-phenylalanine, and chitosan from shrimp shells (85% deacetylated) were supplied by Sigma-Aldrich (St. Louis, USA). Potassium phosphate monobasic, potassium phosphate dibasic and tartaric acid were purchased from Riedel-de Haen (Seelze, Germany). Water deionized by a Millipore Milli-Q purification system was used. All chemicals used were of analytical grade. Multi-walled carbon nanotubes (MWCNT) (d = 60 to 100 nm, l = 5 to 15 μm, 95% purity) were obtained from NanoAmor (Houston, USA). Basic metal planar electrodes with diameter of 1.6 mm equipped with Ag/AgCl reference electrode (d = 2 mm, screen-printed) deposited on the planar glass-epoxy-laminate substrate were obtained from Biorealis (Bratislava, Slovakia).

1.2. Apparatus. Electrochemical studies were performed with electrochemical analyzers Autolab M101 (Methrom Autolab, Netherlands) and Omnilab from Biorealis (Bratislava, Slovakia).

1.3. Preparation of nanocomposites. The procedure developed by our research team and described previously for nanocomposite electrodes was used for the nanocomposite fabrication [20, 21]. Briefly, 100 mg of N-eicosane were melted at a temperature of 45 °C. After this, 10 mg of MWCNT were added and the mixture was

stirred vigorously with a spatula until the homogenous mixture was obtained. The suspension was subsequently transferred and spread on the surface of the metal electrode ($d \approx 1.6$ mm) equipped with an Ag/AgCl reference electrode. Finally, the layer of the nanocomposite was left to solidify and the surface was smoothed on a sheet of paper.

1.4. Preparation of biosensors. The electrodes were carefully cleaned with Milli-Q water. The immobilization of enzymes on the working electrodes surface was carried out by their sandwiching between the chitosan layers (1% w/w, aqueous solution). DP and PDH were dissolved in Milli-Q water before the deposition. The quantities of the enzymes on the electrodes were optimized from 0.5 to 4 U for DP and 0.1 to 1 U for PDH, respectively. Each layer was deposited after the previous one was dried. The prepared biosensors were stored at room temperature in a desiccator.

1.5. Amperometric measurements. Chronoamperometry was performed by applying selected constant potential (vs. Ag/AgCl) after inserting the biosensor in the volume of a test solution either 1 mL in a microtube or 10 mL in a beaker under stirring at laboratory temperature. The potential from +100 to +400 mV was tested to optimize the working potential value. The pH values of 0.4 M phosphate buffer measuring solution were optimized by test within pH 7.5 to 10. Similarly, the suitable concentrations of a coenzyme NAD^+ (from 0.02 to 5 mM) and an electrochemical mediator ferricyanide (from 0.5 to 10 mM) in the working media were also investigated.

After measurements, the biosensors were stored in 0.4 M phosphate buffer solution of pH 9.0 at laboratory temperature (up to 8 hours) or at 4 °C (for longer operational stability studies). The biosensors were kept dry in a desiccator at laboratory temperature for the storage stability studies.

2. Results and discussion

The illustration scheme of the phenylalanine determination principle is shown in Fig. 1. L-PDH catalyzes the specific dehydrogenation of L-phenylalanine accompanied with deamination in the presence of cofactor NAD^+, which is reduced to NADH. Subsequently, DP catalyzes the reduction of ferricyanide, which acts as a hydrogen acceptor from NADH.

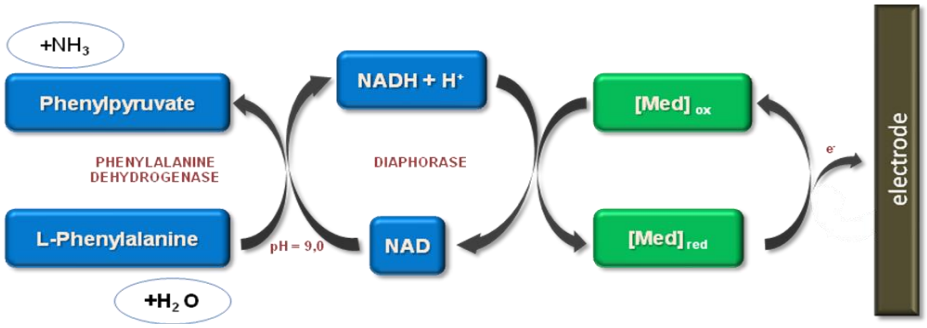

Fig. 1. L-phenylalanine bioelectrode reaction scheme

Finally, ferrocyanide is re-oxidized to ferricyanide on the electrode surface and the resulting current proportional to the analyte concentration is measured.

2.1. Optimization of working conditions. The amperometric response of biosensor is commonly a function of some working conditions, such as the enzyme loadings, working potential, temperature and pH. The temperature parameter was not optimized in this work because we considered the use of this biosensor for a home monitoring with expected ambient room temperature.

The quantities of PDH and DP on the electrode surface were optimized from 0.1 to 1 U and from 0.5 to 4 U, respectively. Although high enzyme loadings led to the current sensitivity increase, the biosensor responses became very sluggish. This effect could be probably attributed to the blocking of the electrode surface by the increased amount of immobilized protein. On the other hand, the low enzyme quantities resulted in the decrease of biosensor sensitivities and narrow linear ranges. The optimum activities of 1.1 U of DP and 0.25 U of PDH were found for the immobilization on the working electrode, taking into account the acceptable response time less than 1 min. These loadings were consequently used for the biosensors preparation.

The optimum concentration of the coenzyme NAD^+ in the working media was found to be 2 mM for studies in the range from 0.02 to 5 mM. Similarly, the best responses of biosensors were obtained using 2.5 mM ferricyanide for the determinations in the range from 0.5 to 10 mM.

The working potential was optimized within the range of +100 to +400 mV against the Ag/AgCl reference electrode. Higher values of the working potential were not further investigated because of the risk of possible interferences during analyses of real samples. Biological fluids often contain electroactive compounds which can be oxidized on the electrode giving a false current response. With increasing potential the amperometric response logically grew and the highest one was achieved at an applied potential of +400 mV. However, we have selected the value of +250 mV for further experiments taking into account the possible interferences. This potential makes it possible to measure with satisfactory sensitivity and simultaneously prevents from unwanted interferences.

Only phosphate buffer solution (PBS) containing 2 mM NAD^+ and 2.5 mM ferricyanide was used as a measuring medium for the optimization of the pH value considering our previous experiences and also recommendations of the enzyme suppliers. The study was performed by successive addition of 10 μL of 50 mM phenylalanine standard solution into 1 mL of measuring solution to see the pH effect at a high substrate concentration level. As expected the biosensor response was pH dependent. From Fig. 2 it can be seen that the maximum current response of the biosensor was obtained at pH 9.0. However, the responses at pH 9.5 and 10.0 were almost identical.

It is necessary to note that the use of ferricyanide in this electrochemical process might also affect the optimal pH value as well as the polymer matrix used for immobilization. Since the pH values higher than 9.0 can lead to the reduced enzymes stabilities according to the suppliers, this pH could be considered as optimal. Omidinia et al. [18] applied pH 10.4 at the biosensor based on PDH without DP, which was the pH value recommended by the supplier for those enzymes. Similarly, the pH value of 11.0 was found to be optimal when applying only PDH in a packet-bed bioreactor [22]. In our case, when using also DP the optimal pH is lower.

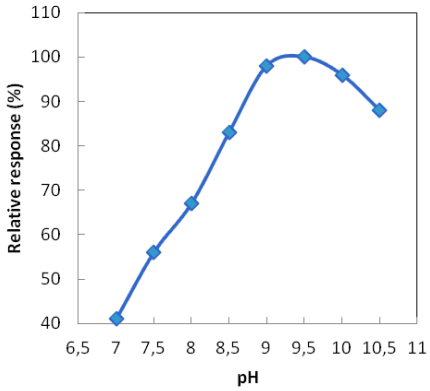

Fig. 2. Effect of pH on the response of L-phenylalanine biosensor (the response at pH 9.5 = 100%). Experimental conditions: 2 mM NAD^+, 2.5 mM ferricyanide, 100 μM L-phenylalanine in 0.4 M phosphate buffer, applied potential +250 mV vs. Ag/AgCl

2.2. Analytical characteristics. The amperometric response to increasing concentration of phenylalanine is plotted in Fig. 3. The biosensor showed a good linearity in the range of 12–1250 μM with a correlation coefficient of 0.992 (n = 21). The corresponding detection limit was 6.4 μM (based on signal/noise = 5) and the sensitivity of 22.3 nA μM^{-1} cm^{-2}. The current of the sensor started to increase approximately 6 s after the injection of substrate standard solution reaching a 90% steady-state current within 45 s. Final response times were found to be about 60 s.

These results characterizing then biosensor performance were in most cases better than those found in literature. Weiss et al. [17] described reagentless biosensor having the detection limit of 0.5 mM and the linear range of 10 to 80 mM. The same useful range was described by Ominidia et al. [18], without a presentation of the detection limit and sensitivity. Only Khadilkar et al. [19] reached lower detection using the optical biosensor. On the other hand, the optical biosensor showed very long response time of 30 min. In comparison to other biosensors described previously, we obtained similar analytical characteristics [20, 21]. Generally used spectrophotometric tests have detection limits from 2 to 30 μM [19, 23, 24], but they are more time consuming.

The measurement reproducibility with the same biosensor was tested by a current response to 100 μM of phenylalanine. The average response of 54.29 ± 2.63 nA (n = 8) represents RSD = 4.8% which is an acceptable value.

The presented biosensor was subsequently tested for the operation and storage stabilities. No loss of activity after at least 60 successive addition of 100 μM phenylalanine with the biosensor was observed. When the biosensor was stored in the 0.4 M PBS of pH 9.0 at 5 °C after use, it conserved its response ability above 90% after 5 days. Long-term storage stability of biosensors plays also one of the key roles in case of their potential commercial and home-monitoring uses. Humidity and high temperature are considered as the most negative natural factors which can affect the storage stability of enzymatic biosensors. When the biosensors were held in a desiccator without any previous use for 12 months, their sensitivity remained about 90% of the initial value. This excellent long-term storage stability was observed in our laboratory also for other biosensors based on the chitosan sandwich. The positive effect of the chitosan biopolymer matrix on the biosensor stability was discussed in our previous

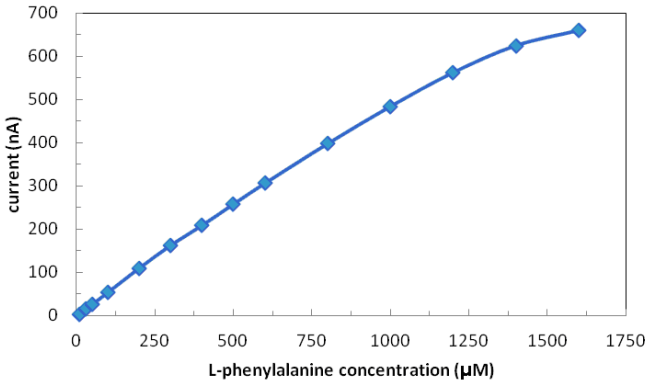

Fig. 3. Calibration curve obtained for the L-phenylalanine biosensor. Experimental conditions: 2 mM NAD^+, 2.5 mM ferricyanide, 0.4 M phosphate buffer of pH 9.0, applied potential +250 mV vs. Ag/AgCl

works [20, 21]. These findings are very positive for the planned expected use in home monitoring, because the stability contributes to a reliability of biosensors and a potential use of one biosensor for many measurements during several days could significantly reduce the operational costs of potential users. No data are known for the storage stability of other phenylalanine biosensors discussed above. Their operational stabilities were from 3 to 14 days.

2.3. Real samples analysis. The presented biosensor was preliminary tested for its potential use in phenylalanine determination in urine. The results are shown in Table 1. The content of phenylalanine in the urine of 3 healthy persons was measured before and after spiking with phenylalanine. The measured concentrations of phenylalanine in the urine of healthy persons were very low. Practically no significant signal was monitored by the biosensor. It means also that no interferences coming from the urine could be registered. Recovery experiments demonstrated a good reliability of the biosensor giving the values of about 100%.

Tab. 1

L-phenylalanine determination in urine of healthy donors

No	Phenylalanine (mM)	Phenylalanine spike (mM)	Phenylalanine found (mM)	Recovery (%)
1	< 0.1	20	19.42	97.1
2	< 0.1	30	31.20	104.7
3	0.12	40	38.66	96.7

The spiking concentrations from 20 to 40 mM phenylalanine in urine reflect the real range of phenylketonurics being between 20 and 60 mM. Langenbeck et al. [14] and Boulos et al. [25] recently demonstrated a feasibility of noninvasive prediction of phenylalanine blood concentrations from its analysis in urine. This opens the possibilities for patients to perform home self-monitoring directly by phenylketonurics which could result in improving their quality of life. The results presented here show that the developed biosensor is suitable for application on real samples.

Conclusions

In this work, we present a novel amperometric enzymatic biosensor based on nanocomposite electrode consisting of multi-walled carbon nanotubes for the detection of phenylalanine. Bi-enzymatic composition of L-phenylalanine dehydrogenase and diaphorase was immobilized between the chitosan layers. This simple and effective layer by layer immobilization technique provided long-term storage stability, low fabrication costs, and good analytical performance. The biosensor performed wide linear range, low detection limit, high sensitivity, short measuring time and interference-free measurements.

The results obtained from the tests of the real urine samples indicate that the biosensor presented here have a potential for self-monitoring directly by phenylketonurics at home. Our future work will be addressed to more complex tests with a number of patients. Moreover, subsequent biosensor improving, simplifying measurement procedure and development of easy-to-use and cheap analyzer similar to those for glucose monitoring by diabetics are necessary and remain our next challenge.

This work was supported by the Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic (Project 1/0361/14) and the Competence Center for SMART Technologies for Electronics and Informatics Systems and Services (Project ITMS 26240220072) funded by the Research & Development Operational Programme from the ERDF.

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Received
June 30, 2014

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БИОСЕНСОР НА ОСНОВЕ L-ФЕНИЛАЛАНИН ДЕГИДРОГЕНАЗЫ И ДИАФОРАЗЫ, ИММОБИЛИЗОВАННЫХ НА НАНОКОМПОЗИТНОМ ЭЛЕКТРОДЕ, ДЛЯ ОПРЕДЕЛЕНИЯ L-ФЕНИЛАЛАНИНА В МОЧЕ

М. Стредьянски, Я. Лабуда

Аннотация

Разработан биферментный амперометрический сенсор на основе L-фенилаланин дегидрогеназы и диафоразы для определения L-фенилаланина. Иммобилизацию ферментов проводили между слоями хитозана на поверхности планарных нанокомпозитных электродов, включающих многослойные углеродные нанотрубки. Диапазон линейной зависимости составляет 12–1250 мкМ L-фенилаланина на фоне фосфатного буферного раствора pH 9.0 в присутствии 2 мМ $НАД^+$ и 2.5 мМ феррицианид-ионов в качестве медиатора. Предел обнаружения составил 6,4 мкМ, чувствительность – 22.3 нА/мкМ·$см^2$ и время отклика – около 60 с. Биосенсор показал хорошую стабильность при хранении (90 % от исходной чувствительности после 12 месяцев) и эксплуатации (более 90% после 5 дней работы). Сенсор может применяться пациентами с фенилкетонурией для самостоятельного мониторинга фенилаланина в моче. Для образцов мочи здоровых людей получен низкий сигнал биосенсора, что свидетельствует о низкой чувствительности по отношению к потенциальным мешающим компонентам. Для образцов с внесенным фенилаланином величины степени открытия близки к 100%.

Ключевые слова: биосенсор, L-фенилаланин дегидрогеназа, диафораза, определение L-фенилаланина, нанокомпозит, фенилкетонурия, многослойные углеродные нанотрубки, моча.

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Поступила в редакцию
30.06.14

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