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# Amperometric bienzyme screen-printed biosensor for the determination of leucine

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**Abstract** Leucine plays an important role in protein synthesis, brain functions, building muscle mass, and helping the body when it undergoes stress. Here, we report a new amperometric bienzyme screen-printed biosensor for the determination of leucine, by coimmobilizing *p*-hydroxybenzoate hydroxylase (HBH) and leucine dehydrogenase (LDH) on a screen-printed electrode with $NADP^+$ and *p*-hydroxybenzoate as the cofactors. The detection principle of the sensor is that LDH catalyzes the specific dehydrogenation of leucine by using $NADP^+$ as a cofactor. The product, NADPH, triggers the hydroxylation of *p*-hydroxybenzoate by HBH in the presence of oxygen to produce 3,4-dihydroxybenzoate, which results in a change in electron concentration at the working carbon electrode, which is detected by the potentiostat. The sensor shows a linear detection range between 10 and 600 μM with a detection limit of 2 μM. The response is reproducible and has a fast measuring time of 5–10 s after the addition of a given concentration of leucine.

**Keywords** Biosensor · Amperometric · Leucine · *p*-Hydroxybenzoate hydroxylase · Leucine dehydrogenase · Screen-printed electrode

## Introduction

Leucine is one of the three essential amino acids [1] that cannot be synthesized by the human body in the required quantities and is obtained from diet or supplements [2, 3]. Leucine is the only dietary amino acid that has the capacity to stimulate muscle protein synthesis, and it plays an important function in increasing muscle mass and helping in muscle recovery after exercise or any strenuous activity [4]. It supports blood sugar regulation and supplies energy to the body. These functions make leucine essential, and leucine is invaluable when the body is stressed or in shock from events such as surgery, trauma, infection, fever, malnutrition, etc. [4]. In addition, leucine also plays an important role in brain functions [5]. Thus, developing a miniaturized and economical device for the sensitive, specific, and rapid determination of leucine is highly desired.

Various methods have been used for its determination, including high-performance liquid chromatography [6–8], liquid chromatography–mass spectroscopy [1], gas chromatography–mass spectroscopy [9–11], spectrophotometry [12], chemiluminescence [13], ELISA [1], etc. However, these methods are expensive, are time-consuming, or require skilled personnel and a complex protocol. Recently, biosensor methods have been developed for the detection of leucine, including fluorescent biosensors with UV-LED excitation [14], cell-based biosensors [15, 16], and biosensors based on transcriptional regulators [16]. However, they require complex optical instrumentation or result in nonspecific detection.

The importance of amperometric 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 measurements of target analyte in complex matrices such as blood, food products, and environmental samples. However, to date, there are only leucine oxidase-based biosensors [17, 18] being reported for the determination of leucine. Recently, we have shown the coimmobilizations of a $NAD(P)^+$-dependent dehydrogenase with *p*-hydroxybenzoate hydroxylase (HBH) on electrodes for developing a general type of dehydrogenase-

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based biosensors [19, 20], which show high-performance characteristics.

In this work, we report a new amperometric determination of leucine with a bienzyme screen-printed biosensor based on coimmobilization of leucine dehydrogenase (LDH) and HBH. As shown in Fig. 1, the principle is as follows: LDH catalyzes the specific dehydrogenation of leucine by consuming $NADP^+$. The product, NADPH, initiates the irreversible decarboxylation and 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.

## Experimental

### Apparatus

A potentiostat, Autolab PGSTAT101 (Metrohm USA, Riverview, FL), and a computer installed with Autolab NOVA software were used. The screen-printed electrode purchased from Bio Sensor Technologie GmbH, Berlin, Germany with a two-electrode configuration was composed of a thick-film carbon working electrode (diameter 1 mm) which was electroactive for sensing and an Ag/AgCl reference/counter electrode. The screen-printed electrode was fitted in a stirred beaker (volume 10 ml), and the terminals were connected to the potentiostat and insulated with epoxy to avoid disturbance during electrical measurement. A Synergy 2 multimode microplate reader (BioTek Instruments, Inc., Winooski, VT) was used for the spectrophotometric measurement.

### Chemicals

HBH with an activity of 31.2 U $mg^{-1}$ (1 U is defined as the amount of the enzyme that catalyzes the conversion of 1 μmol of substrate per minute; the value is provided by the supplier) and LDH with an activity of 38.6 U $mg^{-1}$ were purchased from Toyobo (Osaka, Japan). Sodium *p*-hydroxybenzoate, different L-amino acids, and tris(hydroxymethyl) aminomethane were purchased from Sigma-Aldrich (St. Louis, MO). Nicotinamide adenine dinucleotide phosphate sodium salt ($NADP^+$) was purchased from Applichem (St. Louis, MO), and glutaraldehyde solution was purchased from Fisher Scientific (Pittsburgh, PA). Human serum was purchased from Zen-Bio, Inc. (Research Triangle Park, NC). All the solutions were prepared in ultrapure water obtained from Barnstead NANOpure® DI*amond*™ Water Systems (Thermo Scientific, Asheville, NC).

### Biosensor preparation

Enzyme solutions of HBH and LDH were mixed with diluted glutaraldehyde (3 %) with a ratio of 1:1:1, and 1 μl of this mixture was spread over the working carbon electrode of the screen-printed electrode and then stored at 4 °C overnight. The enzyme solution is dissolvable in glutaraldehyde solution, which is efficient for immobilizing the enzyme on the electrode. Six kinds of enzyme electrodes, listed in Table 1, were prepared from different mixtures of LDH and HBH for the optimization of enzyme loadings. The sensor was then fitted into a beaker filled with Tris–HCl solution (100 mM, pH 8.0) and rehydrated for around 30 min at room temperature (21 °C). The leucine solutions were prepared by dissolving L-leucine in ultrapure water to obtain different concentrations of leucine solutions.

### Electrical measurement

Experiments were carried out at room temperature by applying +0.4 V to the screen-printed electrode and magnetically stirring the solution at 160 rpm to obtain a uniform distribution of leucine after its addition. After a steady background current was achieved, measurements were started by adding various volumes (0.01–1 ml) of leucine solutions (20–200 mM) into the buffer solution, and steady-state current difference was recorded for plotting the calibration curve. Sensing experiments were also performed using human serum (1:5 dilution in PBS) spiked with different concentrations of leucine.

### Spectrophotometric measurement

Spectrophotometric measurement for the determination of leucine in human serum was performed to compare to that using the biosensor method; 200 μl of human serum without or with different concentrations of spiked leucine, 500 μM of NADP, and 1 U of LDH were mixed and the enzymatic reaction generates NADPH, which resulted in an absorbance at 340 nm. A calibration curve was plotted for leucine in

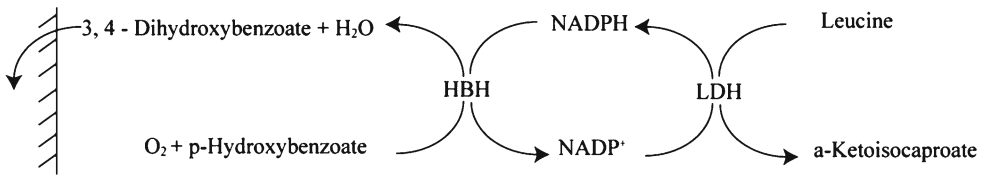


**Fig. 1** Schematic illustration of bienzyme leucine screen-printed sensor (*HBH p*-hydroxybenzoate hydroxylase, *LDH* leucine dehydrogenase)

**Table 1** Sensitivities of the bienzyme leucine sensors with different loadings of HBH and LDH

| HBH (U) | LDH (U) | Sensitivity (nA $\mu M^{-1}$) |
|---|---|---|
| 0.5 | 1.0 | 1.24 |
| 1.0 | 2.0 | 1.34 |
| 2.0 | 4.0 | 1.72 |
| 4.0 | 6.0 | 1.81 |
| 6.0 | 6.0 | 1.81 |
| 6.0 | 8.0 | 1.81 |

ultrapure water with the spectrophotometry, which was further used to determine the concentration of leucine in the serum.

## Results and discussion

A two-step optimization of the working conditions was performed before the sensor characterization in order to improve the sensor performance for the determination of L-leucine.

The first step was the optimization of the enzyme loadings, as shown in Table 1. The loadings of HBH were varied from 0.5 to 6 U, and the loadings of LDH were varied from 1 to 8 U. With a varying loading of HBH from 0.5 to 4 U and an increasing loading of LDH from 1 to 6 U, the sensitivity increased (~38 %). While changing HBH loading from 4 to 6 U, the sensitivity changed very slightly with a LDH loading of 6 U. Similarly, while keeping LDH loading constant at 6 U, the sensitivity changed very slightly (~0.08 %) with a HBH loading varying from 4 to 6 U. The maximum signal was found with the loading combination of 4 U HBH and 6 U LDH, which was used as the standard enzyme loading for further experiments.

After the enzyme optimization, the optimal working buffer condition was investigated, including the buffer pH value and cofactor concentrations. Various loadings of cofactors $NADP^+$ and *p*-hydroxybenzoate (*p*-HB) and working buffer pH were tested to improve the biosensor performance as shown in Fig. 2. The amounts of cofactors $NADP^+$ and *p*-HB must be sufficient during the enzymatic reaction to obtain a good linear range. The linear range of the biosensor ranging from 10 to 600 μM was determined by using excess amounts of $NADP^+$

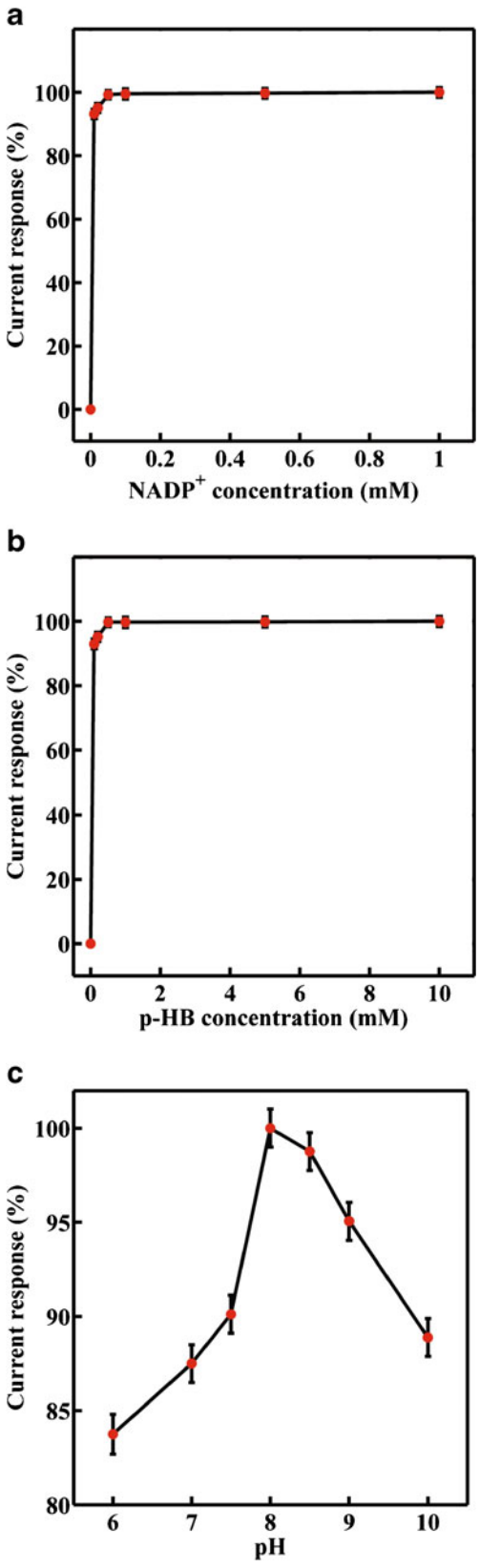


**Fig. 2** Determination of working condition ($n=3$). **a** Optimum loading of $NADP^+$ (buffer: 100 mM Tris–HCl buffer at pH 8.0, 500 μM *p*-HB; substrate: 600 μM leucine). **b** Optimum loading of *p*-hydroxybenzoate (buffer: 100 mM Tris–HCl buffer at pH 8.0, 50 μM $NADP^+$; substrate: 600 μM leucine). **c** Optimum pH of working buffer (buffer: 100 mM Tris–HCl buffer at pH 6.0–10.0, 500 μM *p*-HB, 50 μM $NADP^+$; substrate: 40 μM leucine). Sensors: 4 U HBH and 6 U LDH in the enzyme matrices. The relative response (percent) was calculated by normalizing the signal to the maximum signal

(500 μM) and *p*-HB (5 mM) in the measurement of leucine. For the optimization of $NADP^+$, the biosensor response to

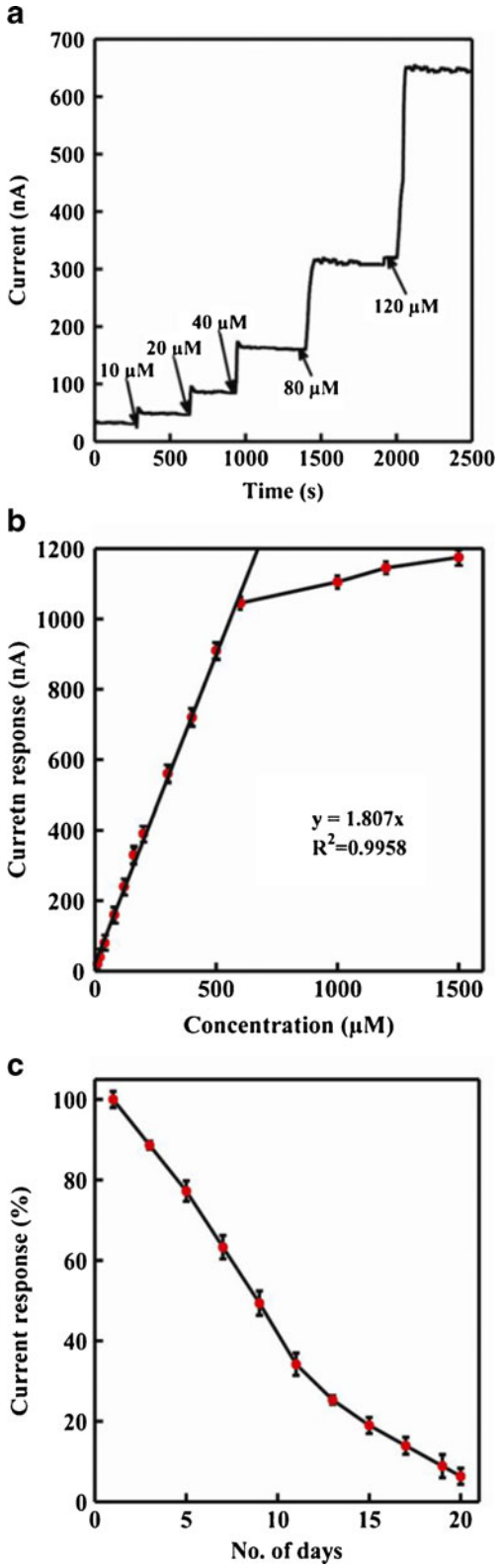


**Fig. 3** Characterization of the sensor performance for leucine. **a** Current response to 10, 20, 40, 80, and 80 μM of leucine. **b** Calibration curve for the determination of leucine ($n$ =3). **c** Storage stability of the sensor (the relative response (percent) was calculated by normalizing the signal to the initial signal obtained on the first day of measurements). Sensor: 6 U HBH and 8 U LDH in the enzyme matrix. Buffer: 100 mM Tris–HCl buffer at pH 8.0, 500 μM $p$-HB, 50 μM $NADP^+$

600 μM of leucine was investigated with various $NADP^+$ loadings. The response increased with increasing $NADP^+$ loading and started saturating at a loading of 50 μM. Similarly, the response to 600 μM of leucine with various loadings of $p$-HB was tested. The response increased with increasing loading of $p$-HB and became saturated at a loading of 500 μM. For the investigation of the working buffer pH, the sensor was tested in Tris–HCl buffer with pH values ranging from 6.0 to 10.0. The signal was obtained by measuring the response to 20 μM of leucine. The maximum response was obtained between pH 8.0 and 8.5. Therefore, the standard working buffer was determined to be Tris–HCl buffer (100 mM, pH 8.0) containing 500 μM $p$-HB and 50 μM $NADP^+$.

The sensor performance for the determination of leucine was characterized. Figure 3a shows the current–time response of the bienzyme sensor, obtained by adding various amounts of leucine. The measurements of leucine concentrations were performed by the biosensor with the optimal enzyme matrix and working condition. After the addition of leucine, the increase in the current was proportional to the concentration of leucine. The sensor had a response time of a few seconds after the addition of a given concentration of leucine and achieved a new steady state in 5–10 s. The signal response is also reproducible and reliable.

Figure 3b shows the calibration curve for the detection of leucine with the amperometric bienzyme biosensor. A linear relationship was observed between the current response and the concentration of leucine from 10 to 600 μM with a detection limit of 2 μM (slope 1.81 nA $\mu M^{-1}$, $R^2$=0.9958, $n$ =3). The limit of detection (LOD) was calculated by using a signal-to-noise ratio of 1.0. The response was saturated for a leucine concentration larger than 600 μM.

Further experiments were performed to test the selectivity towards other amino acids. The sensor shows no response to different concentrations (50 μM, 1 mM) of other L-amino acids (L-alanine, L-arginine hydrochloride, L-asparagine, L-aspartic acid, L-cysteine hydrochloride, L-cystine, L-glutamic acid, L-glutamine, glycine, L-histidine hydrochloride, *trans*-4-hydroxy-

**Table 2** Comparison of sensor and spectrophotometric measurement of human serum spiked with different concentrations of leucine

| Spiked amount (μM) | Sensor detection (μM) | Spectrophotometric detection (μM) |
|---|---|---|
| 0 | 159.9 | 161.7 |
| 10 | 170.1 | 171.0 |
| 100 | 261.0 | 260.2 |
| 200 | 358.8 | 360.8 |
| 300 | 460.4 | 462.0 |
| 400 | 561.0 | 560.5 |
| 500 | 660.7 | 660.3 |

L-proline, L-isoleucine, L-lysine hydrochloride, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine). Therefore, this L-leucine sensor has a high specificity for the determination of L-leucine.

Sensing performance was also performed using human serum spiked with different concentrations of leucine. Leucine concentrations in human serum could range from several micromolars to several hundred micromolars under different physiological conditions or diseases, such as healthy people sera (123±25 μM) [21] and patient sera samples which might have the maple syrup urine disease (MSUD) (643–667 μM) [22], epilepsy (97.8–108.0 μM) [23], and heart failure (273± 10 μM) [24]. Table 2 summarizes the result of the determinations of leucine in seven different serum samples, which were spiked with 0, 10, 100, 200, 300, 400, and 500 μM of leucine, respectively. The results were compared with those obtained through spectrophotometric measurement. The regression equation between the results obtained by the spectrophotometric measurement ($x$-axis) and those by the leucine sensor ($y$-axis) was $y=0.9958x$, and $R^2=0.9999$. The agreement was excellent for these serum sample measurements, and the good agreement shows that the sensor is accurate for measuring leucine in serum.

The long-term storage stability of bienzyme electrode was evaluated by measuring its performance every alternate day for 20 days with storage at 4 °C in Tris–HCl buffer (100 mM, pH 8.0). The stability was studied by monitoring its current response to 40 μM of leucine (a concentration within the linear detection range) with intermittent usage (every alternate day). It can be seen from the Fig. 3c that the biosensor retains around 49 % of its initial activity after 10 days. After 20 days, the biosensor samples were not very active and retained around 6 % of the initial response for leucine. The reduction of sensor performance was probably mainly due to the reduced lifetimes of enzymes during storage, the electrode surface fouling during amperometric measurements, and the easier leakage of enzymes from the enzyme matrix.

In addition, the sensing performance was evaluated in the same day for testing the storage stability of the sensors in and outside the working buffer. The performance of the sensor was investigated by testing the response to 200 μM of leucine (a concentration within the linear detection range). The sensor shows excellent performances. For storage in the buffer, the sensor was able to show around 95 % of the initial response after usage of 12-h usage. For storage outside the buffer, the sensor's response reduced to around 86 % of the initial response after 12-h usage.

## Conclusions

In this work, we demonstrated the development of an amperometric screen-printed bienzyme sensor for the detection of leucine. The sensor showed a high level of performance for the detection of leucine, with high sensitivity and a rapid response. The approach described here may open up new avenues for a variety of applications in medical science, sports, microbiology, and nutrition studies.

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