Available online at www.sciencedirect.com
ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 49 (2007) 55–60
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

A novel biosensor based on L-homocysteine desulfhydrase enzyme immobilized in eggshell membrane

Sencer Alacam, Suna Timur*, Azmi Telefoncu
Ege University, Faculty of Science, Biochemistry Department, 35100 Bornova, Izmir, Turkiye

Received 7 April 2007; received in revised form 15 August 2007; accepted 20 August 2007
Available online 24 August 2007

Abstract

A novel biosensor for homocysteine determination has been developed. The biosensor was fabricated with L-homocysteine desulfhydrase immobilized on the ammonium selective electrode by means of eggshell membrane. The measurement principle is based on determination of ammonia due to the enzymatic reaction in the medium by ammonium selective electrode. The effects of enzyme loading, glutaraldehyde concentration, pH, buffer concentration, temperature, dithiotreitol (DTT) concentration and ionic strength adjustment buffer (ISA) on the biosensor response were investigated in detail. The linear detection range and limit of detection (LOD) for homocysteine were found to be 0.15–1.8 mM and 55 μM, respectively. Finally, the homocysteine biosensor has been applied to plasma samples for determination of total homocysteine contents.
© 2007 Elsevier B.V. All rights reserved.

Keywords: Homocysteine biosensor; L-Homocysteine desulfhydrase; Eggshell membrane; Ammonium selective electrode

1. Introduction

Homocysteine is a non-protein forming sulfur amino acid and formed during methionine metabolism. It has a key role in the sulfur amino acid metabolism and in one-carbon transfer reactions, therefore, information about the efficiency of these reactions as well as about the vitamin cofactors involved (folic acid, B_{12}, B_6, B_2) can be obtained from homocysteine plasma levels [1]. In biological systems, homocysteine is present as the disulfide homocystine (RSSR), as a mixed disulfide with other low-molecular weight thiols (homocysteine/cysteine- and homocysteine-reduced glutathione mixed disulfides) and as homocysteine disulfide cross-linked to proteins. The term total homocysteine, as applied to biological samples such as plasma, refers to the sum of concentrations of all the homocysteine species present in the plasma [2]. For a healthy person plasma total homocysteine concentration is in the range of 5–16 μM [3]. Elevated levels of homocysteine are strongly correlated with an increased risk of coronary artery disease, cerebrovascular disease and peripheral vascular disease. Recent clinical studies have demonstrated that plasma homocysteine levels correlate better than cholesterol levels with an increased risk of arteriosclerosis [4]. Because of the relationship between elevated homocysteine and several diseases, determination of homocysteine has gained high interest within biomedical community [5]. For this purpose a lot of methods are now available for homocysteine determination such as high performance liquid chromatography [6–9], immunoassay [10,11], enzymatic [12–15], capillary electrophoresis [16,17], molecular imprinting [18] and electrochemical [19].

Biosensors are analytical devices incorporating a biological material in an intimate contact with a suitable transducer device that converts the biochemical signal into quantifiable electric signals [20]. Enzyme-based biosensors are currently a major area for research and development since essentially all chemical reactions in living systems are catalysed by enzymes. For a reusable enzyme-based biosensor, most commonly, an enzyme required to be immobilized. It has been reported that some biomaterials consisting of silk, collagen and eggshell membrane were employed as platforms for the immobilization of enzymes. An eggshell membrane, having excellent gas and water permeability, can be an ideal biomaterial for enzyme immobilization [3,21–24].

In this study, we explore the possibility of fabricating a homocysteine biosensor based on a L-homocysteine desulfhydrase enzyme-immobilized eggshell membrane. To our knowledge,

* Corresponding author.
E-mail address: sunatimur@yahoo.com (S. Timur).

1381-1177/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2007.08.005

this study describes for the first time preparation and evaluation of a biosensor based on L-homocysteine desulfhydrase for homocysteine determination.

2. Experimental

2.1. Chemicals and reagents

L-Homocysteine desulfhydrase (E.C. 4.4.1.2 from *Trichomonas vaginalis*) with a specific activity of 2 units/mg powder was obtained from BioCatalytics Inc. (Pasadena, CA, USA). DL-Homocysteine, 1,4-dithio-DL-threitol (DTT) and potassium phosphate dibasic (anhydrous) were obtained from Fluka Chemicals (Buchs, Switzerland). L-Cysteine (hydrochloride, anhydrous) was purchased from Sigma (St. Louis, MO, USA). 50% (w/w) glutaraldehyde solution in water was purchased from Aldrich (Milwaukee, WI, USA). L-Methionine was purchased from Ajinomoto Co. Inc. (Tokyo, Japan). One hundred percent of acetic acid and potassium phosphate monobasic (anhydrous) was from Riedel-de Haen (Germany). Bovine serum albumin (factor V) was obtained from Merck (Damstadt, Germany). All chemicals were used as received and aqueous solutions were prepared with distilled water. Chicken eggs were provided from local markets.

Plasma samples were obtained from a local hospital and incubated with 3 mM DTT for 30 min before determination.

2.2. Instrumentation

Model 720A + pH/ionmeter, ammonium selective electrode (model 9318) and Ag/AgCl reference electrode (model 90-02) were purchased from Thermo-Orion (Chicago, IL, USA).

2.3. Preparation of eggshell membrane

The chemical compositon (by weight) of eggshell has been reported as follows: calcium carbonate 94%, magnesium carbonate 1%, calcium phosphate 1% and organic matter 4% [25]. Calcium carbonate (main component) is soluble in acetic acid. Therefore, eggs were incubated in 100% acetic acid at 4 °C for 18 h to obtain whole eggshell membranes. After incubation, weakened eggshell was easily peeled off. Obtained eggshell membranes were cut into two equal parts and cleaned with copious amount of distilled water after albumen and yolk had been removed. The clean eggshell membranes were finally stored at 4 °C in a pH 7.0 potassium phosphate buffer (5 mM) until further use.

2.4. Immobilization of L-homocysteine desulfhydrase on eggshell membrane

For enzyme immobilization, circular (radius nearly 2 cm) eggshell membrane was removed from the phosphate buffer. Twenty microlitres of (100 mg/mL) L-homocysteine desulfhydrase solution in a phosphate buffer (pH 7.0, 5 mM) was added and membrane was kept for 90 min at 4 °C for adsorbsion. After adsorbsion, 10 μL of a 1% (w/w) glutaraldehyde solution as the crosslinking agent was dropped onto the surface of the membrane and stood for 5 min. A glass rod was gently used to spread the glutaraldehyde solution thoroughly on the membrane surface. After 5 min, the membrane was immersed and washed with phosphate buffer solution (pH 7.0).

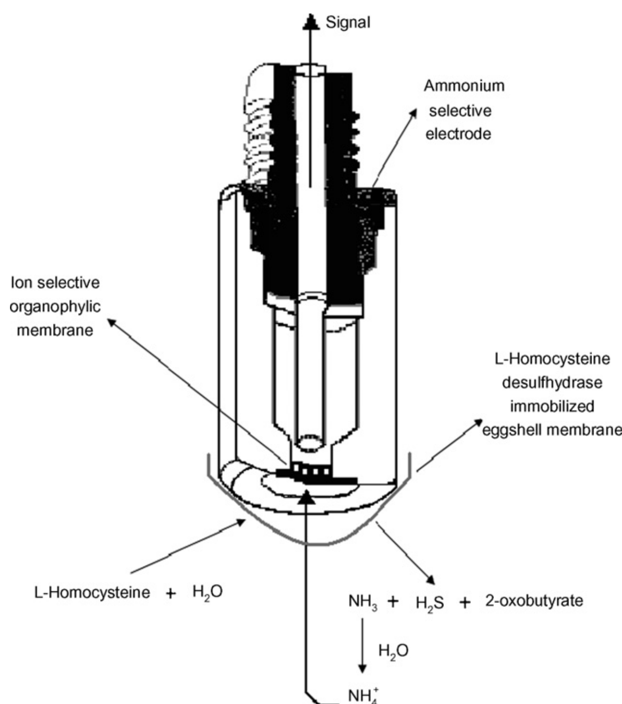


Fig. 1. Principle of operation.

2.5. Assembly of homocysteine biosensor and determination

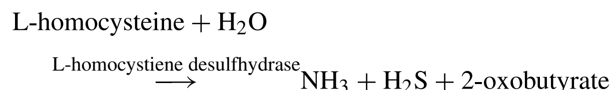
L-Homocysteine desulfhydrase immobilized eggshell membrane was positioned on the surface of a Thermo-Orion ammonium selective electrode and kept in a steady position by an O-ring. Then, ammonium selective electrode and reference electrode (Orion, model 90-02) were immersed together into a stirred reaction media containing phosphate buffer solution (10 mL). Standard DL-homocysteine or sample solution was injected into the reaction media and ammonium concentration signal was measured and processed by a Thermo-Orion 750A + pH/ionmeter (Fig. 1).

3. Results and discussion

3.1. Measuring procedure

The ammonium selective electrode acting as an ammonium transducer was employed to measure the rate of ammonium formation in the enzymatic consumption of homocysteine. The analytical signal of the homocysteine biosensor is the increase in the millivolts (mV) upon exposure to a homocysteine solution. The enzymatic oxidation of homocysteine by L-homocysteine

desulfhydrase is shown in the following equation [26]:



$$\text{L-homocysteine} + H_2O \xrightarrow{\text{L-homocystiene desulfhydrase}} NH_3 + H_2S + \text{2-oxobutyrate}$$

The increase in the signal (mV) was found to be proportional to the homocysteine concentration. Hence, the biosensor responses were defined as ΔmV and the increase in the signal (mV) was plotted against the homocysteine concentration. Moreover, relative (%) biosensor response was estimated by assuming current value 100% at the optimal working conditions.

3.2. Optimization of biosensor response

3.2.1. Effect of enzyme loading and glutaraldehyde amount

Since the response of the homocysteine biosensor strongly depends on the enzymatic activity of L-homocysteine desulfhydrase, any change in enzyme and cross-linker concentration on immobilization would affect the sensitivity of the biosensor. Enzyme-immobilized eggshell membranes were prepared by adding various amounts of L-homocysteine desulfhydrase enzyme on the eggshell membranes. Higher biosensor response was obtained with an increase in enzyme loading (1 and 2 mg which were equal to 2 and 4 units, respectively). Hovewer, when 3 mg of the enzyme (6 U) was applied, a sharp decrease was observed in the response (Fig. 2). This is due to the diffusion problem, which is caused by higher protein molecules. As a consequence, 2 mg of the enzyme, which was corresponding to 4 U was chosen as the optimal enzyme amount and used in further studies.

The effect of glutaraldehyde concentration on the response of the biosensor was investigated (Fig. 3). Because of porous structure of the membrane and long absorbsion time enzyme membrane responds well without glutaraldehyde but enzyme leaching occurs during operation. It is a fact that, when glutaraldehyde concentrations lower than 1% (w/w) was applied, cross-linker amount could be insufficient to bind all the enzyme

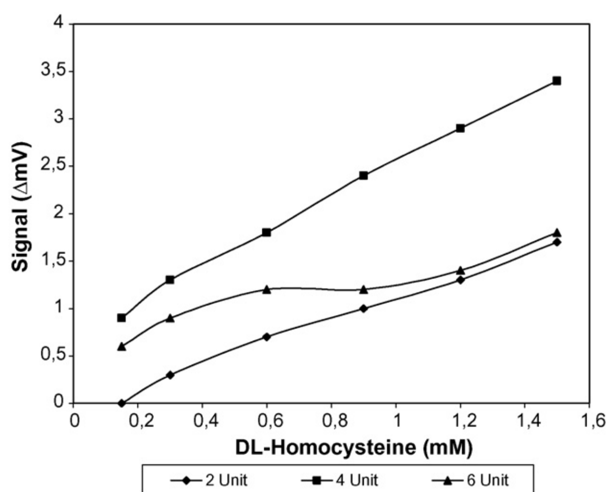


Fig. 2. Effect of enzyme loading on response of the biosensor (pH 7.0; 5 mM phosphate buffer, 35 °C, 3 mM DTT into 30 mM DL-homocysteine).

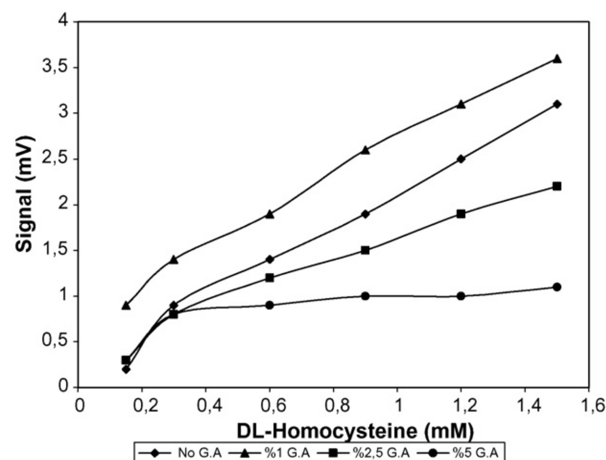


Fig. 3. Effect of glutaraldehyde on response of the biosensor (pH 7.0; 5 mM phosphate buffer, 35 °C, 3 mM DTT into 30 mM DL-homocysteine).

molecules on the membrane. On the other hand when glutaraldehyde was larger than 1% (w/w), the excess glutaraldehyde may cause denaturation of the enzyme and the response time may also be prolonged. As a result, the optimum concentration for 10 μL glutaraldehyde was determined to be 1% (w/w).

3.2.2. Effect of pH and buffer concentration

The pH range of ammonium selective electrode is specified as 2.0–7.0 by manufacturer that is why the effect of pH on the biosensor response was studied in the range 5.5–7.0 as shown in Fig. 4. The biosensor was subjected to 900 μM DL-homocysteine in phosphate buffer solutions in various pHs. The biosensor response is pH-dependent since ionization of amino acids at enzyme's active site and homocysteine in aqueous solutions varies with pH [27]. On the basis of Fig. 4, the optimal pH of the biosensor was chosen to be pH 7.0.

The effect of buffer concentration on the response of the biosensor was investigated by subjecting the biosensor to 900 μM DL-homocysteine concentration in pH 7.0 phosphate buffer solutions in various concentrations, shown in Fig. 5. Ammonium selective electrode must be used with ionic strength adjustment buffer (ISA), consisting of 0.25 M magnessium acetate and 0.5 M acetic acid. But ISA could not be used because

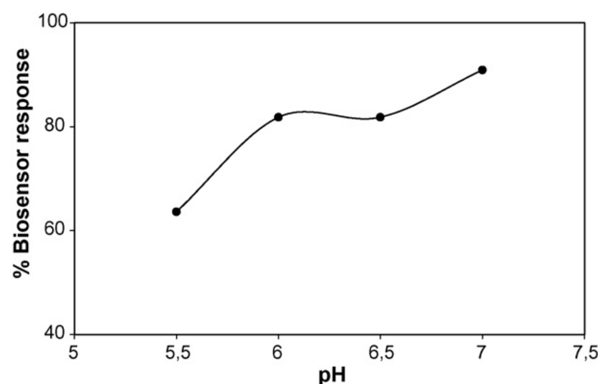


Fig. 4. Effect of pH on the biosensor response (pH 5.5–7.0; 5 mM phosphate buffer, 35 °C, 6 mM DTT into 30 mM DL-homocysteine).

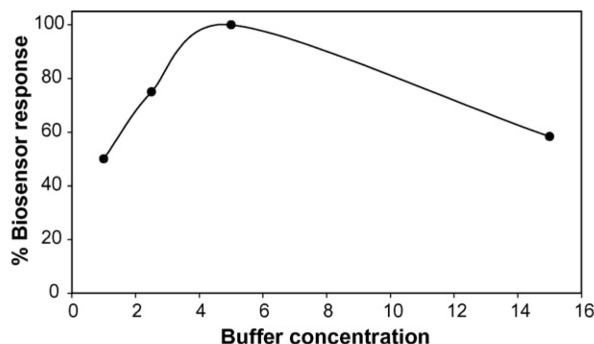


Fig. 5. Effect of buffer concentration on the response of the biosensor (pH 7.0; 1, 2.5, 5, 15 mM phosphate buffers, 35 °C, 6 mM DTT into 30 mM DL-homocysteine).

it affects pH of reaction media. Therefore the investigated buffer concentrations are kept low. Eventually optimum buffer concentration was chosen as 5 mM.

3.2.3. Effect of temperature

Temperature range of ammonium selective electrode is 0–40 °C so the effect of temperature on the homocysteine biosensor was studied in the range 20–40 °C as shown in Fig. 6. It is well known that the analytical performance of an enzyme-immobilized membrane is highly sensitive to variation of temperatures. The response rate of the biosensor increased sharply at higher working temperatures. This implies that the activity of an immobilized enzyme is governed by the kinetics of the enzymatic reaction. Although the analytical sensitivity was highest at 40 °C, for practical purpose, the working temperature was chosen to be 35 °C to increase the lifetime of the biosensor.

3.2.4. Effect of DTT concentration

The only substrate of L-homocysteine desulfhydrase is L-homocysteine so DTT is necessary for reduction of oxidized homocysteine. For this purpose the effect of DTT concentrations (0.3, 1.5, 3 and 7.5 mM) that were added to 30 mM of DL-homocysteine stock solution were investigated (Fig. 7). The results showed that response of homocysteine biosensor did not show significant change with DTT concentrations in the range of 0.3–7.5 mM. Therefore to avoid enzyme structure and also keeping homocysteine reduced, the optimum DTT concentration was selected as 3 mM.

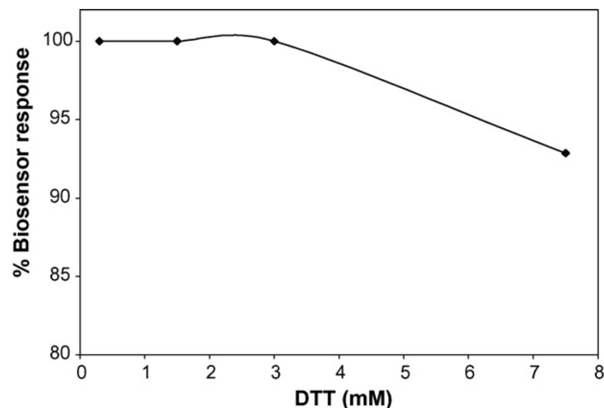


Fig. 7. Effect of DTT concentration added into 30 mM substrate on the biosensor response (pH 7.0; 5 mM phosphate buffer, 35 °C).

3.3. Analytical characteristics of the biosensor

3.3.1. Linear detection range

Linear detection range of the biosensor has been investigated after optimum conditions found. Linearity was obtained in a concentration range of 0.15–1.8 mM for DL-homocysteine with the equation $y = 1.793x + 0.719$ and correlation coefficient, R^2; 0.998. At higher concentrations calibration curve showed a deviation from linearity (Fig. 8).

3.3.2. Accuracy

Repeatability is a crucial factor for any bioanalytical technique, especially for a biosensor. The signal changes of the biosensor were investigated when it was alternately exposed to a phosphate solution and a 0.6 mM homocysteine solution for

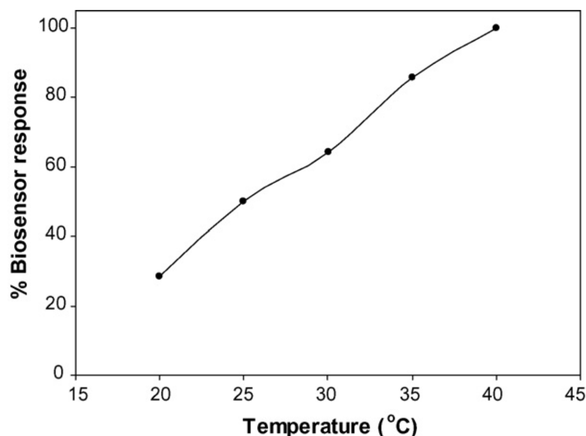


Fig. 6. Effect of temperature on the biosensor response (pH 7.0; 5 mM phosphate buffer, 20–40 °C, 3 mM DTT into 30 mM DL-homocysteine).

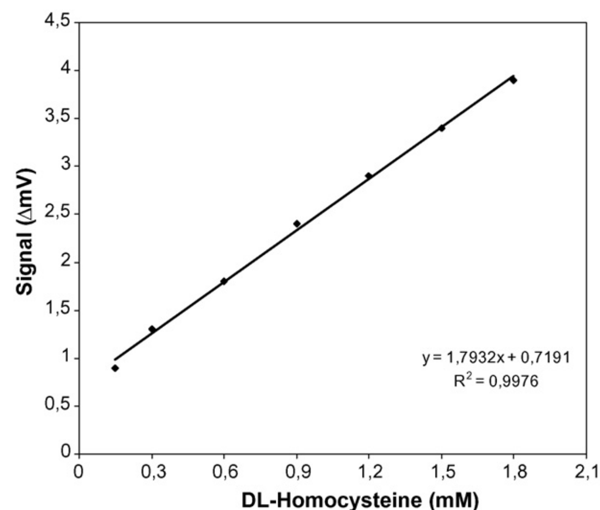


Fig. 8. Calibration curve for DL-homocysteine (pH 7.0; 5 mM phosphate buffer, 35 °C, 3 mM DTT into 30 mM DL-homocysteine).

Table 1
Repeatability of the proposed biosensing system (pH 7.0; 5 mM phosphate buffer, 35 °C, 0.6 mM DL-homocysteine added to reaction media)

	Number of measurements (*n*)						
	1	2	3	4	5	6	7
Obtained signal (ΔmV)	1.7	1.8	1.7	1.8	1.7	1.7	1.7
Concentration (μM)[a]	547	602	547	602	547	547	547

[a] Concentrations were calculated from calibration graph (Fig. 8).

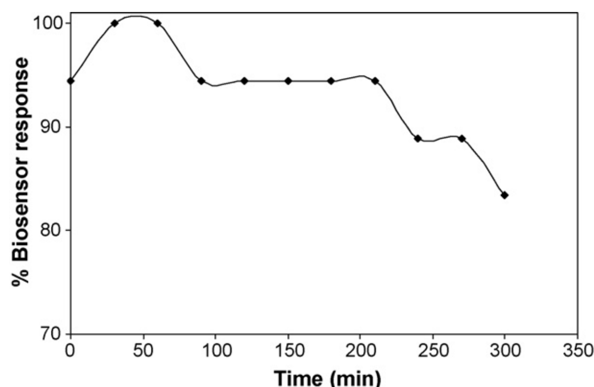


Fig. 9. Operational stability of the biosensor (pH 7.0; 5 mM phosphate buffer, 35 °C, 3 mM DTT into 30 mM DL-homocysteine).

seven times (Table 1). The biosensor exhibited a fairly desirable analytical feature of repeatability (*n*, 7; R.S.D., 4.76%) and coefficient of variation ($\pm 26.83 \times 10^{-3}$ mM).

3.3.3. Operational stability

The operational stability of the biosensor was tested in every 30 min during 6 h. The biosensor was stored in reaction media (phosphate buffer) at 35 °C when it was not being used. The response of the biosensor started decreasing after 210 min and decreased to 83.3% of its initial value at the end of the period (Fig. 9).

3.3.4. Interference study

The interference study was evaluated by exposing the biosensor to L-cysteine and L-methionine in a 5 mM phosphate buffer at pH 7.0 (Table 2). The response rate obtained was calculated as the homocysteine concentration equivalence (i.e., the concentration of homocysteine that can produce the same response as the interferant). The homocysteine biosensor was not affected by several concentrations of L-cysteine (0.3, 0.9 and 1.5 mM) and L-methionine (0.3, 0.9 and 1.5 mM). L-Cysteine could form disulfide bonds so 3 mM DTT was added to its 30 mM stock solution. This conclusion could be supported by the data that the only known substrate of the enzyme is L-homocysteine.

Table 2
Interference study results (pH 7.0; 5 mM phosphate buffer, 35 °C, 3 mM DTT into 30 mM stock solutions of DL-homocysteine and L-cysteine)

Added amount (mM)	DL-Homocysteine signal (mV)[a]	L-Cysteine signal (mV)[a]	L-Methionine signal (mV)[a]
0.3	1.3	0	0
0.9	2.4	0.1	0.1
1.5	3.5	0.2	0.1

[a]All measurements were performed two to three times and data were given as the average values.

Table 3
Recovery values for total homocysteine determination in plasma by standard addition method (pH 7.0; 5 mM phosphate buffer, 35 °C, 3 mM DTT into 30 mM DL-homocysteine)

Added DL-homocysteine (μM)	Obtained signals (ΔmV)[a]	Expected signals (ΔmV)[b]	Recovery (%)
300	1.2	1.3	92.3
600	1.6	1.8	88.9
900	2.1	2.4	87.5

[a] All measurements were performed three times and data were calculated by using average values.
[b] Expected signals were calculated from the standard calibration graph.

3.3.5. Sample application and recovery

The homocysteine biosensor was applied to the determination of homocysteine in human plasma. As the level of plasma homocysteine in healthy human is below the detection limit of the biosensor, the recovery test for homocysteine was investigated by adding known amounts of homocysteine to the plasma and by diluting them 10-fold with phosphate buffers in reaction media. The amounts of added homocysteine were evaluated by using the proposed homocysteine biosensor.

In these experiments, the signals obtained from homocysteine added plasma samples were compared with the calibration curve that was given as Fig. 8. Moreover, the own effect of plasma on the ammonium electrode was searched by using an eggshell membrane prepared with 2 mg bovine serum albumin (BSA) instead of enzyme and it was observed that plasma creates 1.5 mV signals by itself. This value includes all interferants in plasma and was also taken into the consideration while recoveries were calculated. Table 3 summarises the results of the recoveries. As is shown in Table 3, sample matrix effects were observed while the homocysteine concentration was increasing. But in lower standard concentrations, higher recoveries that were closed to 100% were obtained.

4. Conclusion

The developed homocysteine biosensor has a very simple principle and it is cost-effective. The biosensor's linear detection range was found for DL-homocysteine but our main target is L-homocysteine that is practically half of the DL-homocysteine

fraction. This means that linearity was obtained in a concentration range of 75–900 μM for L-homocysteine.

During the development of biosensor the main problem was selection of suitable conditions for both ammonium selective electrode and enzyme. For example the optimum pH of the enzyme was given as 7.5 by the manufacturer but in this study pH 7.0 was selected as optimum pH because ammonium selective electrode's pH working range is 2.0–7.0.

The improved biosensor could be good alternative for determination of the moderate and severe hyperhomocysteinemia levels. It is possible to do a cheap scanning of severe hyperhomocysteinemia over a chosen population. Moreover the obtained recovery values were not acceptable but further experiments, such as finding better immobilization platform or electrode, are in progress to improve the recoveries and stability of the biosensor.

Acknowledgements

The work described in this paper was supported by research fund of Ege University (2005 FEN 003). Dr. I. Alacam was also acknowledged for his supports.

References

[1] S.H. Mudd, L.H. Levy, F. Skobvy, Disorders on transsulfuration, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic Basis of Inherited Disease, 6th ed., McGraw Hill, New York, 1989, pp. 693–734.
[2] G. Zhang, D. Liu, S. Shuang, M.M.F. Choi, Sens. Actuator B: Chem. 114 (2006) 936–942.
[3] O. Nekrassova, N.S. Lawrence, R.G. Compton, Talanta 60 (2003) 1085–1095.
[4] D.A. Cramer, Lab. Med. 29 (1998) 410–417.
[5] N.S. Lawrence, R.P. Deo, J. Wang, Talanta 63 (2004) 443–449.
[6] S.T. Chou, L.E. Ko, C.S. Yang, Anal. Chim. Acta 429 (2001) 331–336.
[7] A.R. Ivanov, I.V. Nazimov, L. Baratova, J. Chromatogr. A 895 (2000) 157–166.
[8] O. Chailapakul, W. Siangproh, B.V. Sarada, C. Terashima, T.N. Rao, D.A. Tryk, A. Fujishima, Analyst 127 (2002) 1164–1168.
[9] A. Pastore, R. Massoud, C. Motti, A.L. Russo, G. Fucci, C. Cortese, G. Federici, Clin. Chem. 44 (1998) 825–832.
[10] M.T. Shipchandler, E.G. Moore, Clin. Chem. 41 (1995) 991–994.
[11] K. Demuth, V. Ducros, S. Michelsohn, J.-L. Paul, Clin. Chim. Acta 349 (2004) 113–120.
[12] T.-F. Fu, M. di Salvo, V. Schirch, Anal. Biochem. 290 (2001) 359–365.
[13] B. Zappacosta, S. Persichilli, A. Minucci, D. Scribano, S. Baroni, S. Fasanella, P. Neri, P.D. Daloiso, B. Giardina, P. De Sole, Clin. Biochem. 39 (2006) 62–66.
[14] N. Matsuyama, M. Yamaguchi, M. Toyosato, M. Takayama, K. Mizuno, Clin. Chem. 47 (2001) 2155–2157.
[15] Y. Tan, L. Tang, X. Sun, N. Zhang, Q. Han, M. Xu, E. Baranov, X. Tan, X. Tan, B. Rashidi, Z. An, A.W. Perry, R.M. Hoffman, Clin. Chem. 46 (2000) 1686–1688.
[16] C. Chassaing, J. Gonin, C.S. Wilcox, L.W. Wainer, J. Chromatogr. B 735 (1999) 219–227.
[17] S.H. Kang, W. Wei, E.S. Yeung, J. Chromatogr. B 744 (2000) 149–156.
[18] C.F. Chow, M.H.W. Lam, H.K.P. Leung, Anal. Chim. Acta 466 (2002) 17–30.
[19] K. Gong, Y. Dong, S. Xiong, Y. Chen, L. Mao, Biosens. Bioelectron. 20 (2004) 253–259.
[20] S.K. Sharma, N. Sehgal, A. Kumar, Curr. Appl. Phys. 3 (2003) 307–316.
[21] D. Xiao, M.M.F. Choi, Anal. Chem. 74 (2002) 863–870.
[22] M.M.F. Choi, W.S.H. Pang, D. Xiao, X.J. Wu, Analyst 126 (2001) 1558–1563.
[23] B. Wu, G. Zhang, S. Shuang, M.M.F. Choi, Talanta 64 (2004) 546–553.
[24] B. Wu, G. Zhang, S. Shuang, C. Dong, M.M.F. Choi, A.W.M. Lee, Sens. Actuator B: Chem. 106 (2005) 700–707.
[25] W.T. Tsai, J.M. Yang, C.W. Lai, Y.H. Cheng, C.C. Lin, C.W. Yeh, Bioresour. Technol. 97 (2006) 488–493.
[26] K.W. Thong, G.H. Coombs, Exp. Parasitol. 63 (2) (1987) 143–151.
[27] H.D. Yakobke, H. Jeschkert, Amino Acids, Peptides and Proteins: An Introduction, Akademie Verlag, Berlin, 1977, p. 25.