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Process Biochemistry 41 (2006) 1371–1377

Process Biochemistry

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Potentiometric bienzymatic biosensor based on PVC membrane containing palmitic acid for determination of creatine

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Received 13 July 2005; received in revised form 28 January 2006; accepted 31 January 2006

Abstract

Creatine bienzymatic potentiometric biosensors were prepared by immobilizing urease and creatinase with four different procedures on poly(vinylchloride) (PVC) ammonium membrane electrode containing palmitic acid prepared by using nonactine as an ammonium-ionophore. The analytical characteristics were investigated and were compared with those of the biosensor prepared by using carboxylated PVC. The effects of pH, buffer concentration, temperature, enzyme concentration and stirring rate, and enzyme immobilization procedures on the response to creatine of the biosensor were investigated. The linear working range and sensitivity of the biosensor were also determined. The creatine biosensor prepared by using the PVC membranes containing palmitic acid showed more effective performance than those of the carboxylated PVC based biosensors. Additionally, creatine assay in serum was successfully carried out by using the standard addition method without any pretreatment. The results were good agreement with those obtained from Jaffe methods at 95% confidence level.

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Keywords: Creatine biosensor; Potentiometric; Creatine determination; Palmitic acid

1. Introduction

The determination of creatine in biological fluids, which is important factor in the evaluation of muscular function, is of great interest in clinical diagnosis. The creatine level in blood serum and urine is clinically used as a parameter of muscle damage. The physiological concentration of creatine is between 40 and 150 μmol/L in serum, but pathological values due to muscle disorder may rise to above 1000 μmol/L. Creatine is commonly determined enzymatically or by means of the Jaffe reaction both employing colorimetric indication. Other various instrumental methods such as HPLC [1,2], capillary electrophoresis [3], HPLC–mass spectrophotometry [4,5], IR [6] were also proposed for the determination of creatine. There has been much recent progress in the study of enzyme electrodes [7–12]. Motonaka et al. had prepared an enzyme electrode for creatine by immobilization of creatine kinase on the surface of a glass electrode but the use of this biosensor required the presence of ATP and Mg^{2+} in the analysed solution and generally the use of pH-based enzyme electrode has a disadvantage in respect to pH and buffer capacity of the analysed solutions [13]. A bienzymatic enzyme electrode containing urease and creatinase enzymes for creatine has been developed by Koncki et al. by using only carboxylated poly(vinylchloride) based on an ammonium membrane electrode [14]. However, ammonium electrodes prepared by using PVC have more advantages than those of prepared by carboxylated PVC: high stability, short response time, good reproducibility, high lifetime, easy for biosensor construction [15]. But, these type electrodes are not suitable for biosensor construction [16–18] since the enzymes cannot chemically immobilize on the hydrophobic surface of PVC membrane. The more effective enzyme immobilization can be made on PVC membrane with containing functional groups such as –COOH, $–NH_2$ [14,19]. In the literature, the biosensors prepared by immobilizing enzymes on ammonium electrode containing nonactin as ammonium ionophore and carboxylated PVC as a polymer membrane matrix material were seen to be low sensitivities against ammonium ions [14]. The electrode slopes for creatine and arginine obtained from these type electrodes (so electrode sensitivity) were 29.6 mV/p[creatine] and 26.3 mV/p[arginine], respectively. It was shown that these slopes were lower than Nernstian value (theoretical slope is nearly 59 mV).

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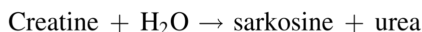
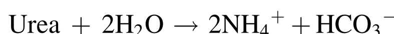
doi:10.1016/j.procbio.2006.01.017

The aim of this work was to develop new bienzymatic creatine biosensors by using PVC membrane ammonium-selective electrode containing nonactine as an ammonium ionophore and palmitic acid (a long-chain fatty acid). Its sensitizing membrane contains creatinase and urease enzymes. First enzyme creatinase catalyses the creatine hydrolysis:



$$\text{Creatine} + H_2O \rightarrow \text{sarkosine} + \text{urea}$$

A nonelectroactive intermediate product, urea, is decomposed by urease to electroactive species (ammonium ion) according to the reaction:



$$\text{Urea} + 2H_2O \rightarrow 2NH_4^{+} + HCO_3^{-}$$

We also determined lifetime, response time, optimum working range, and other response characteristics of the prepared creatine biosensors. Furthermore, we investigated whether these biosensors can be used to determine creatine level in human serum.

2. Materials and method

2.1. Reagents and apparatus

Creatinase (E.C.3.5.3.3), nonactin, palmitic acid, bis-(2-ethyl)hexylsebacate (DOS), poly(vinylchloride) (PVC), carboxylated poly(vinylchloride) (PVC–COOH) were obtained from Fluka. Urease (E.C.3.5.1.5) was purchased from Merck (Darmstadt, Germany). 1-Ethyl-3-(3-dimetylaminopropyl)carbodiimide (EDC) was obtained from Sigma Chem. Co. (St. Louis, USA). All other chemicals used were of analytical reagent grade. Standard solutions and buffer solutions were prepared with deionized water.

The blood serum samples were provided by Biochemistry Laboratory at İbn-i Sina Hospital, Ankara University.

Potential and pH measurements were carried out with an ORION 720A model pH-ionmeter.

The potential values were given against the saturated Ag/AgCl double junction reference electrode (ORION 90-02).

2.2. Preparation of ammonium-selective membrane electrodes

The membrane compositions of ammonium-ion selective electrodes prepared by using PVC containing palmitic acid and carboxylated PVC are shown in Table 1.

Each membrane according to the compositions in Table 1 was prepared by dissolving about 400 mg membrane components in 5 mL of tetrahydrofurane (THF). The solutions were poured on a glass plate inside a glass ring (42 mm diameter). After 24 h drying at room temperature, the membrane was formed (approximately 0.5 mm thickness). Disks of 5 mm diameter were cut out and attached to the glass electrode body. The inner electrode solution was 1.0×10^{-2} M ammonium chloride (pH 7.0).

2.3. Preparation of creatine biosensors

Enzymes were chemically immobilized on all membrane electrode surfaces by four different procedures according to the similar procedures given by Koncki et al. [20] and Karakuş et al. [21].

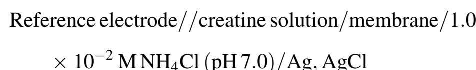
(A) Twenty milligram creatinase, 30 mg urease, and 5 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide were dissolved in 1 mL deionized water. Fifty microliter of this solution was dropped on the membranes of ammonium-selective electrodes and left overnight (biosensor A).
(B) Fifty microliter of 2.5% glutaraldehyde solution was deposited on the surface of biosensor A and the biosensor was left for 0.5 h. Then the surface was washed with deionized water to remove the excess of glutaraldehyde (biosensor B).
(C) Thirty milligram urease and 20 mg creatinase were dissolved in 1 mL deionized water. Fifty microliter of this solution was deposited on the biosensor B surface. The biosensor was left overnight (biosensor C).
(D) Biosensor C was placed in phosphate buffer at pH 7.0 (10 mM) and 20 mg sodium borohydride was added by stirring vigorously. Biosensors were left for 1 h (biosensor D).

To remove the excess of unbounded enzymes all types of biosensors were left for 1 h vigorously stirred phosphate buffer (pH 7.0, 10 mM). When not in use, biosensors were stored in a refrigerator at 4 °C.

Moreover, the corresponding urea biosensors were prepared with only urease immobilization on the ammonium-selective electrode by using the procedures above mentioned for creatine biosensors [21].

2.4. Measurement of the biosensor response to creatine

Potential measurements for creatine biosensors were carried out by varying creatine concentration in steady-state condition. Five millimolar of TRIS buffer was used as a working buffer solution (pH 7.0). The following electrochemical cell was formed with the proposed creatine biosensors by using a reference electrode:



$$\text{Reference electrode}//\text{creatine solution}/\text{membrane}/1.0 \times 10^{-2}\,\text{M}\,NH_4Cl\,(\text{pH}\,7.0)/\text{Ag, AgCl}$$

Measurements were made with the proposed creatine biosensor and reference electrode. Biosensor was immersed to a depth of 1.5 cm in creatine solution that stirred by a magnetic stirrer. The pH values were determined using an Orion combinated glass-pH electrode. All the experimental works were carried out at 20 ± 1 °C.

The calibration curves were obtained by plotting the potential values of a series of standard creatine solution against the logarithm of creatine concentration.

2.5. Procedure for determination of creatine in serum

Human serum samples were collected from hemodialysis patients. The creatine levels in these serum samples in a concentration range of 0.3–3.2 mg/dL were determined by using the standard addition method. The creatine biosensor was immersed in the 5 mM TRIS buffer containing certain amount

Table 1
The membrane compositions of ammonium-selective electrodes prepared by using PVC containing palmitic acid and carboxylated PVC

Membrane components	Membrane composition (%)			
	IC and ID	IIC and IID	IIIC and IIID	IVC and IVD
PVC	30.0	30.0	–	–
Palmitic acid	3.0	2.0	–	–
Carboxylated PVC	–	–	32.5	31.5
Nonactin	3.0	4.0	3.0	4.0
Bis-(2-ethyl)hexyl sebacate (DOS)	64.0	64.0	64.5	64.5

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