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Determination of total creatine kinase activity in blood serum using an amperometric biosensor based on glucose oxidase and hexokinase

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ABSTRACT

Creatine kinase (CK: adenosine-5-triphosphate–creatine phosphotransferase) is an important enzyme of muscle cells; the presence of a large amount of the enzyme in blood serum is a biomarker of muscular injuries, such as acute myocardial infarction. This work describes a bi-enzyme (glucose oxidase and hexokinase based) biosensor for rapid and convenient determination of CK activity by measuring the rate of ATP production by this enzyme. Simultaneously the biosensor determines glucose concentration in the sample. Platinum disk electrodes were used as amperometric transducers. Glucose oxidase and hexokinase were co-immobilized via cross-linking with BSA by glutaraldehyde and served as a bioreognition element of the biosensor. The biosensor work at different concentrations of CK substrates (ADP and creatine phosphate) was investigated; optimal concentration of ADP was 1 mM, and creatine phosphate – 10 mM. The reproducibility of the biosensor responses to glucose, ATP and CK during a day was tested (relative standard deviation of 15 responses to glucose was 2%, to ATP – 6%, to CK – 7–18% depending on concentration of the CK). Total time of CK analysis was 10 min. The measurements of creatine kinase in blood serum samples were carried out (at 20-fold sample dilution). Twentyfold dilution of serum samples was chosen as optimal for CK determination. The biosensor could distinguish healthy and ill people and evaluate the level of CK increase. Thus, the biosensor can be used as a test-system for CK analysis in blood serum or serve as a component of multibiosensors for determination of important blood substances. Determination of activity of other kinases by the developed biosensor is also possible for research purposes.

1. Introduction

Creatine kinase (CK) is an important intracellular enzyme that is located in the muscle cells of the humans (first of all in the skeletal muscles and myocardium). During active muscular work, reserves of ATP in the muscle cells become depleted, and then CK synthesizes new ATP molecules by transferring phosphate group from creatine phosphate to ADP. Thus, CK supplies additional energy to muscles that is stored in the form of creatine phosphate. During a rest period, CK catalyzes a reverse reaction and renews stockpile of creatine phosphate.

CK level in the blood serum of healthy people is very low: 0.038–0.174 U/ml in the male serum and 0.026–0.14 U/ml in the female serum. CK concentration in child serum is several times bigger than in the case of adults. After intensive physical work and muscular injuries (because of different inflammation processes, myocardial infarction and muscular dystrophy) CK enters blood and its activity increases up to 2.0 U/ml or 39–185 ng/ml [1–3]. Furthermore, CK activity in blood serum increases in the case of hypothyroidism. Usually CK serves as a biomarker of acute myocardial infarction (AMI) and in the modern clinical diagnostics CK activity (or concentration) is determined for confirmation of other symptoms of this disease. CK level in blood starts to increase immediately after AMI and becomes abnormal after 4–6 h; maximal CK concentration is observed after 18–24 h after AMI [3]. Level of CK increase is used for assessment of AMI severeness in order to choose an appropriate treatment [4]. In addition, CK

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determination in blood serum is useful for assessment of physical qualities of athletes [1].

The most precise and selective methods of biomarker determination (i.e., CK) are immunological methods [3,5]. They are based on binding of CK with a specific antibody and further detection of this interaction by using chemoluminescense or enzyme immunoassay; also electrochemical detection of CK–antibody binding was described [6]. These methods determine the weight concentration of CK (in nanograms); thus, activity of CK is not important and CK determination can be carried out in samples that were stored inappropriately or during long periods. Possibility of separate determination of different isoforms of CK is another advantage of immunological methods; this is useful for finding of the source tissue from which CK was released into blood. Disadvantages of these methods are high price, requirement of animals for antibody production, impossibility of multiple usages of main reaction components.

Medical laboratories usually use much simpler and cheaper spectrophotometric methods of CK determination. These methods assess CK activity by measuring the rate of formation of the products of CK-catalyzed reaction [7,8]. Often an enzymatic system based on hexokinase and glucose-6-phosphate dehydrogenase is used. CK synthesizes ATP that is used by hexokinase for synthesis of glucose-6-phosphate. The latter is oxidized by glucose-6-phosphate dehydrogenase and NADH is formed. NADH is registered spectrophotometrically at 340 nm; the amount of NADH is proportional to CK activity in the sample. Such methods cannot distinguish between different isoforms of CK and total activity of all isoforms is registered. However, in most cases it is enough for diagnostic purposes.

Electrochemical biosensors are a perspective alternative to immunological and spectrophotometric methods of CK determination. The biosensors are potentially cheap, simple in production and application, and need no sophisticated equipment or large space to operate [9]. Single biosensor can be used for multiple measurements of different samples. Furthermore, the biosensors can be combined into multibiosensor systems for simultaneous determination of different components of a sample. However, few biosensors for CK determination were described up to date. The first biosensor was proposed in 1986; it was amperometric and was based on graphite disk electrodes, and glucose oxidase–hexokinase enzyme system was used to determine the rate of ATP production by the CK [2]. This work for the first time demonstrated determination of CK by using an ATP-sensitive biosensor. However, the authors did not propose a procedure for CK determination in real samples, and hexokinase was added to working cell before each measurement (only glucose oxidase was immobilized). Proposed in the article principle of CK determination was taken as a basis for our work. Later disposable amperometric ATP-sensitive biosensors were applied for CK determination. The biosensors were based on glycerol kinase and glycerol phosphate oxidase [1,10].

Completely different principle of CK determination was proposed in work [3]. It describes an electrochemical biosensor in which creatine phosphate was immobilized onto the surface of amperometric transducer. After sample addition, CK bonded to the creatine phosphate and dephosphorylated it. This resulted in changes of electrochemical characteristics of the electrode surface what were registered by using electrochemical impedance spectroscopy and square wave voltammetry. The biosensor had good sensitivity to CK and selectivity; it was successfully applied for measurements of synthetic blood. On the other hand, the biosensor was disposable and was difficult to prepare; the procedure of measurements was also complicated.

Thus, most of enzyme biosensors for creatine kinase detection are completely disposable, so they could be used to measure only one sample. Other biosensors require addition of enzymes to the working cell before measurement. This results in larger amounts of enzymes needed for one measurement. Aim of our work was to develop a reusable amperometric biosensor for rapid and simple determination of CK activity in blood serum. In our case enzymes were immobilized onto the surface of electrode and are used for multiple measurements. For this purpose, we used previously developed biosensor based on glucose oxidase–hexokinase; its characteristics can be found in the work [11].

2. Materials and methods

2.1. Materials

Creatine kinase (CK, EC 2.7.3.2) from rabbit muscle with activity of 32 U/mg was obtained from Serva Feinbiochemica (Heidelberg/New York). Enzymes hexokinase (HEX, EC 2.7.1.1) from *Saccharomyces cerevisiae* with activity 30.6 U/mg (Sigma-Aldrich, Germany) and glucose oxidase (GOD, EC 1.1.3.4) from *Aspergillus niger* with activity 272 U/mg (Genzyme, UK) were used in biorecognition elements of the biosensor. Bovine serum albumin (BSA, fraction V), glucose, ATP (adenosine 5′-triphosphate disodium salt), ADP (adenosine 5′-diphosphate sodium salt), creatine phosphate, glycerol, HEPES, $MgCl_2$, and 50% aqueous solution of glutaraldehyde (GA) were purchased from Sigma-Aldrich Chemie (Germany, France). All other chemicals were of p.a. grade.

2.2. Design of amperometric transducers

In this work platinum disk electrodes of own production served as amperometric transducers. Platinum wire 0.4 mm in diameter and 3 mm long was sealed in the end of a glass capillary with an outer diameter of 3.5 mm. An open end of the wire served as the transducer working surface. An inner end of the platinum wire was connected to a silver wire, placed inside the capillary, using fusible Wood's alloy. A contact pad for connecting to the potentiostat was placed at the other end of the silver wire. The working surface of platinum electrodes was obtained by grinding using alumina powder (particle size 0.1 and 0.05 μm). This surface was treated with ethanol prior to immobilization of the biorecognition element.

2.3. Immobilization procedure

Biorecognition elements of the biosensors were obtained by covalent immobilization of enzymes and auxiliary substances on the surface of amperometric transducer. The initial solution for preparing the bioselective membrane of the biosensors contained 5% GOD (hereafter – w/w), 5% HEX, 3% BSA in 20 mM phosphate buffer, pH 6.5, with 10% glycerol. This solution was mixed with 0.6% aqueous solution of glutaraldehyde (cross-linking agent) in a ratio of 1:1 and immediately deposited onto the transducer working surface. Afterwards, the created biosensors were dried for 40 min in air at room temperature, and then immersed in the working buffer for 10 min for washing unbound components of biorecognition elements and GA excess.

2.4. Method of measurement

The biosensor was placed in an electrochemical cell with an auxiliary electrode (platinum wire) and a reference electrode (Ag/AgCl in saturated KCl) that were connected to the PalmSens potentiostat (Palm Instruments BV, Netherlands). Usage of the 8-channel multiplexer (from the same producer), connected to the potentiostat, allowed simultaneous monitoring of signals from