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A new methodology for the determination of enzyme activity based on carbon nanotubes and glucose oxidase

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ABSTRACT

In this research, a novel enzyme activity analysis methodology is introduced as a new perspective for this area. The activity of elastase enzyme, which is a digestive enzyme mostly of found in the digestive system of vertebrates, was determined by an electrochemical device composed of carbon nanotubes and a second enzyme, glucose oxidase, which was used as a signal generator enzyme. In this novel methodology, a complex bioactive layer was constructed by using carbon nanotubes, glucose oxidase and a supporting protein, gelatin on a solid, conductive substrate. The activity of elastase was determined by monitoring the hydrolysis rate of elastase enzyme in the bioactive layer. As a result of this hydrolysis of elastase, glucose oxidase was dissociated from the bioactive layer, and following this the electrochemical signal due to glucose oxidase was decreased. The progressive elastase-catalyzed digestion of the bioactive layer containing glucose oxidase decreased the layer's enzymatic efficiency, resulting in a decrease of the glucose oxidation current as a function of the enzyme activity. The ratio of the decrease was correlated to elastase activity level. In this study, optimization experiments of bioactive components and characterization of the resulting new electrochemical device were carried out. A linear calibration range from 0.0303 U/mL to 0.0729 U/mL of elastase was reported. Real sample analyses were also carried out by the new electrochemical device.

1. Introduction

Elastase enzyme is one of the hydrolases and especially catalyzes hydrolysis of an important protein, elastin which plays a crucial role in ensuring mechanical stability of connective tissues in vertebrates [1]. Determination of elastase activity in many medical circumstances is vitally important because elastase activity can be a biomarker for certain health threats. For example, many efforts have been made to identify the role of elastase in progressive lung disorders [2] and, cystic fibrosis [3]. A novel model study demonstrated that externally applied forces can affect the structure and function of the lung matrix. This model was developed on the basis of lung elastase activity [4]. In another study it was shown that the neutrophilic release of elastase has been implicated in host defense [5]. Most importantly, over the last decade, a large number of studies explaining the relationship between elastase levels and diabetes have been reported [6–8]. Moreover, the fecal elastase test is an important clinical parameter used for identifying pancreatic functions. The studies found that determination of elastase levels in fecal matter is highly useful in the diagnosis of pancreas-related diseases [9].

Because of the medical importance of elastase as a biomarker for many health threats, its activity determination in physiological fluids is vitally significant. Because of this, there are a few developed methods for activity determination of elastase enzyme reported in medical literature. One of the most used methods is based on the well-known ELISA method [10–12]. The other most used methods in elastase activity determination are based on enzymatic activity assays. A fluorogenic substrate is commonly used in these enzyme assays as a colored indicator which is hydrolyzed by elastase enzyme [13–16]. Both ELISA and enzyme assay methods involve really time-consuming steps and are also expensive.

In this new study, to achieve the label-free detection of elastase, glucose oxidase and carbon nanotubes were combined to produce an amplified-electrochemical signal which was negatively modulated by elastase activity. In this measurement system, glucose oxidase (GOD) enzyme and carbon nanotubes (CNT) were immobilized with the help of gelatin as a carrier protein on a solid, conductive substrate. Glucose oxidase was used to generate an electrochemical signal which was related to the elastase activity in a sample. CNTs were used to enhance and amplify the electrochemi-

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cal signal generated by GOD. Significant optimization experiments such as gelatin, GOD, and CNT amounts were carried out. The effects of working potential, pH, and temperature on the biosensing system were also investigated. Statistical analyses were performed on the experimental data as well. Finally, the biosensing system was used to determine the elastase activity of real samples.

2. Experimental

All reagents and carbon nanotubes (multi-walled carbon nanotubes), glucose oxidase (from *Aspergiullus niger*), elastase (from human leukocytes), and gelatin (type B, from bovine skin) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Real serum samples were collected from healthy people from a local hospital, in Tekirdağ. Artificial serum solutions (4.5 mM KCl, 5 mM $CaCl_2$, 1.6 mM $MgCl_2$, 4.7 mM (D+)-glucose, 2.5 mM urea, 0.1% human serum albumin, and 145 mM NaCl) spiked with elastase were also analyzed. In a biosensing system, a glassy carbon based solid substrate was used as a working electrode material on which GOD, CNTs, and gelatin were immobilized. The conductive surface area of this was 2.01 mm^2. An Ag/Cl (saturated with KCl) and a Pt electrode were used as a reference and counter electrodes, respectively. For all electrochemical measurements, a potentiostat/galvanostat system (Gamry, Reference 600, Warminster, USA) was utilized. A solution containing 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) and 0.1 M KCl was used as a redox probe in all electrochemical experiments.

The first stage of preparation of the biosensing system was the cleaning of the glassy carbon electrode. The glassy carbon-based working electrode was first polished with alumina (0.05 μm) on a microcloth pad and then rinsed with pure ethanol in an ultrasonic bath to remove physically adsorbed alumina particles. After that, the electrodes were dried under an Argon stream. In the next step, 7.5 mg gelatin was dissolved in 50 μL phosphate buffer (50 mM, pH 7) in a water bath at 38 °C. Following that, 0.5 mg CNT and 10 μL GOD solution (containing 18 units of GOD) were subsequently added to 5 μL of this gelatin solution. 10 μL of the resulting mixed solution was gently applied onto the surface of the cleaned working electrode and left for 30 min to dry at 4 °C. Finally, the bioactive layer on the surface was cross-linked by glutaraldehyde (1%, v/v) to prevent dissociation of GOD from the electrode surface to the working solution.

The working principle of the new biosensing system was based on a decrease in the electrochemical signal caused by glucose oxidase. When a sample was applied to the biosensing system, the elastase enzyme in the sample started to degrade the bioactive layer of the working electrode. Consequently, part of the GOD in the bioactive layer was dissociated to the working solution. This dissociation of the GOD resulted in a decrease in the electrochemical signal. Finally, the ratio of decrease in the signal was attributed to the amount of elastase activity in the sample. In a measurement setup, first of all, the biosensing system was operated in a glucose solution (10 mM glucose in citrate buffer (50 mM, pH 5.5) containing 25 mM $K_3[Fe(CN)_6]$) without elastase enzyme, and the electrochemical signal caused by the glucose oxidation by the GOD was recorded under a working potential of 0.4 V. In the next step, the same working electrode was immersed in the elastase sample solution prepared in an $NaHCO_3$ buffer (pH 8.5, 50 mM) and was left in this solution for an hour. Following this step, the working electrode was again applied to the same glucose solution and the electrochemical signal was recorded under the same conditions mentioned above. Finally the variation in the electrochemical signal between the first and the second measurements was derived from elastase activity in the sample solution. Elastase activity calibration graphs were prepared with the help of these electrochemical signal differences. Preparation of the new system and the measurement setup are summarized in Fig. 1.

Table 1
Optimization studies: part I.

The effect of gelatin amount on the biosensing system		
Gelatin amounts (mg)	Linear equation	R^2
2.5	$y = -0.693x + 15.541$	0.3961
5	$y = -1.9383x + 16.732$	0.8747
7.5	$y = -3.0074x + 25.641$	0.9923
10	$y = -1.5719x + 12.171$	0.9601
Optimization of glucose oxidase activity amount		
GOD activities (U)	Linear equation	R^2
9	$y = -0.6583x + 4.975$	0.9885
18	$y = -0.7795x + 7.706$	0.9848
36	$y = -0.4355x + 4.9648$	0.7715
72	$y = -0.7114x + 7.3926$	0.9169
108	$y = 0.053x + 2.5289$	0.1513
The effect of CNT amount on the system		
CNT amounts (mg)	Linear equation	R^2
0.2	$y = -1.2207x + 10.952$	0.898
0.5	$y = -1.1151x + 11.228$	0.9918
1	$y = -0.7795x + 7.706$	0.9848
2	$y = -0.7694x + 7.6983$	0.9801
Optimization of elastase incubation period		
Incubation periods (min)	Linear equation	R^2
10	$y = -0.3885x + 5.9589$	0.9324
20	$y = -1.1073x + 10.725$	0.9342
30	$y = -0.3159x + 7.6913$	0.9859
40	$y = -0.6118x + 8.4997$	0.9416
60	$y = -1.1532x + 8.5318$	0.9939

3. Results and discussion

Optimization parameters such as gelatin, GOD activity, CNT amounts, elastase incubation period, working potential, the effect of the pH value of the $NaHCO_3$ buffer, and working temperature were investigated in detail. After the optimization experiments, in the characterization studies of the new biosensing system, the linear calibration range of the system, repeatability of the signals, electrochemical impedance spectroscopy studies, and the performance of the system in real sample analyses were evaluated.

3.1. Optimization parameters of the biosensing system

To investigate the effect of the amount of gelatin on the measurement system, 2.5 mg, 5 mg, 7.5 mg, and 10 mg gelatin amounts were tested during the preparation of the biosensing system. The linear equations related to elastase calibration graphs are given in Table 1. From the results, it was concluded that 2.5 mg gelatin was not sufficient to make a physically strong bioactive layer. Moreover, an increase in the gelatin amount resulted in favorable electrochemical signals. A gelatin amount more than 7.5 mg caused a considerable decrease in the signals because higher gelatin amounts formed a denser bioactive layer which was prevent to diffuse substrate into the bioactive layer. Consequently, 7.5 mg gelatin was used as an optimum amount for the construction of the biosensing system.

Another important parameter was the amount of GOD activity, which was directly related to the electrochemical signal obtained. For this reason, a detailed optimization experiment was carried out. In these experiments, five different GOD portions containing 9, 18, 36, 72, and 108 U were separately tested. The linear equations of