

## The Effect of ACE Inhibitor (perindopril) on Peroxidase Activity *in vitro* Conditions

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Hypertension is one of the leading diseases of today. ACE inhibitors are used in the prevention and treatment of hyperthyroidism, as well as other diseases such as pulmonary hypertension, proteinuria, etc. In this study we examined the effect of ACE inhibitors (Perindopril) on peroxidase enzyme activity in *in vitro* conditions. By using electrochemical methods we have determined the type of inhibition. We have proven that perindopril has been noncompetitive bonded to the active site on the enzyme, but also on enzyme/substrate complex. From the Lineweaver-Burk diagram, we obtained the values of the maximum value of the current ( $I_{max}$ ) and the Michaelis-Menten constant ( $K_m$ ) without presence and in the presence of the perindopril inhibitor. Values of  $I_{max}$  vary depending on the concentration of perindopril, while the  $K_m$  values are slightly modified, which is confirmed by the theoretical explanation of the noncompetitive type of inhibition. With this work we came to the conclusion that ACE inhibitors can be linked to different sites on oxidoreductase and thus reduce oxidative stress in the organism.

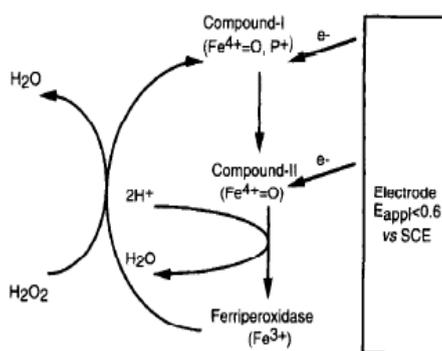
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### 1. INTRODUCTION

Peroxidase-modified electrodes have been developed to detect hydrogen peroxide and other organic and inorganic compounds. These substances have the role of suspending, activating or

inhibiting the reaction of catalysed peroxidase. Immobilization of peroxidase on graphene oxide is a biosensor for the reaction with hydrogen peroxide as an electron acceptor. Also, the immobilization of peroxidase, or its networking in the graphene structure, maintains the catalytic properties, no denaturation of enzymes and no reduction in its activity is observed. For the characterization of HRP, electrochemical methods were used, following the catalytic direct transfer mechanism of the electron described in the work of the author *Ostojic et al.* Direct electron transfer of immobilized HRP is based on the Fe(III)/Fe(II) reduction in the active heme center of the enzyme. The first reaction involves a two-electron oxidation of the ferriheme prosthetic group of the native peroxidase by  $\text{H}_2\text{O}_2$  and the formation of compound I ( $\text{HRP}(\text{Fe}^{+4}=\text{O})^+$ ), which is actually an intermediate. Compound I contains oxyferryl heme ( $\text{Fe}^{+4}=\text{O}$ ) and a porphyrin  $\pi$  cation radical, also is catalytically active and, when receiving one electron from the substrate, converts to Compound II ( $\text{HRP}(\text{Fe}^{+4}=\text{O})$ ). In the third step it is formed HRP (Fe-III) which is the native enzyme, HRP ( $\text{Fe}^{+4}=\text{O}$ )<sup>+</sup> and HRP ( $\text{Fe}^{+4}=\text{O}$ ) are the oxidized forms of the enzyme and  $\text{AH}_2$  and  $\text{AH}\cdot$  are the electron donor substrate and the radical product of its one-electron oxidation [1]. The oxidation products formed during the HRP reaction depend on the nature of the  $\text{H}_2\text{O}_2$  [1,2]. When first reaction mechanism proceeds on an electrode surface compound-I can be reduced into ferriperoxidase by a heterogeneous electron transfer directly from the electrode material as well as by means of redox mediators [3,4]. Other electrodes for the detection of peroxide consists of a layer of peroxidase molecules which is adsorbed onto the electrode surface. Many immobilization studies of HRP were carried out on graphite electrodes, GC electrodes, platinum or gold electrodes. Electrochemical measurements performed on a potential less than 0.6 V vs. SCE shows the proportionality between reduced current and peroxide concentration.



**Figure 1.** Mechanism reduction of hydrogen peroxidase

Hydrogen peroxide detection is very important in many industries. Reactive oxygen compounds can be found in the lungs, during various diseases, and thus the presence of hydrogen peroxide is toxic to the cells [5]. For peroxide detection, three types of peroxidase modifying electrodes are used: electrode surface modified with peroxidase adsorption or covalent bonding; the surface of the electrode modified by the polymer in which the peroxidase is crosslinked; the surface of the electrode is modified where the peroxidase is embedded in conductive and insulating materials [6].

In this paper we used a modified glassy carbon (GC) electrode with a graphene on which the peroxidase was adsorbed. With such a modified electrode, we examined the electrochemical inhibitory

effect of perindopril - ACE inhibitor on peroxidase activity. In pulmonary hypertension the increase in pulmonary artery pressure is a consequence of changes in pulmonary vascular structure and abnormal pulmonary vasoconstriction. Current drug treatment of this disease involves the use of vasodilator drugs that oppose the abnormal vasoconstriction [7, 8]. Perindopril is long-acting ACE inhibitor which belongs to the family of ACE inhibitors that contain a carboxyl group and is a prodrug for perindoprilat [8].

## 2. MATERIALS AND METHODS

**Materials:** Horseradish peroxidase-HRP (250-330 U/mg; Sigma-Aldrich, Buchs, Switzerland);  $\text{KH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4$  (Fisher Chemical, Wien, Austria); Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) p.a. 30% (Sigma-Aldrich, Buchs, Switzerland); Graphene (Sigma-Aldrich, Buchs, Switzerland), Perindopril (Bosnalijek, Bosnia and Herzegovina).

**Methods:** In this paper, all measurements were performed on the potentiostat/galvanostat PAR 263A instrument, and methods of cyclic voltammetry and chronoamperometry were used. The system consisted of three electrodes, a GC working electrode, saturated Ag/AgCl reference electrode and a Pt counter electrode. After preparing the surface of the GC electrode, HRP immobilization was carried out on a graphene carrier, where a biosensor for the detection of hydrogen peroxide was made, as described in the literature [1]. The method of cyclic voltammetry examined the influence of different concentrations of hydrogen peroxide on the activity of the enzyme and the influence of the pH value in the potential range of -1.0 to 0.7 V. Scan rate was of  $50 \text{ mVs}^{-1}$ . The electrochemical cell consisted of 25 mL phosphate buffer (pH 7) in which different concentrations of the substrate (hydrogen peroxide) were added, 0.19 mM, 0.31 mM, 0.49 mM, 0.56 mM. The pH value of the pH (5, 7, 9) was recorded under the same conditions of the potential, but at a substrate concentration of 0.31 mM. Chronoamperometric measurements were performed with constant mixing at 400 rpm and a constant potential of 0.7 V without and in the presence of different concentrations of perindopril 0,16 mg/ml i 0,24 mg/ml.

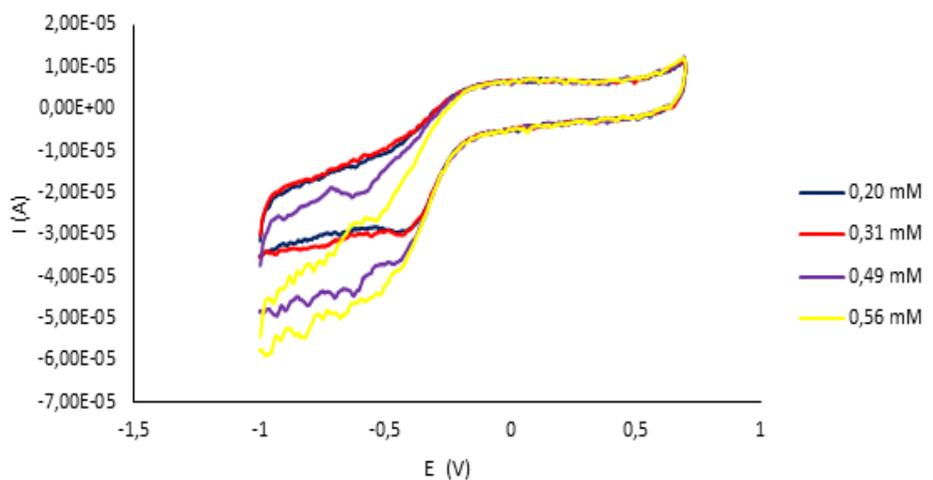
## 3. RESULTS AND DISCUSSION

The purpose of the biosensor kinetic model is to identify factors such as the rate of the enzymatic reaction, the mass transfer rate, which respond to the reaction between the biosensor, the concentration of the electrolyte, and the surface of the electrode. The kinetic processes and reactions involved in the operation of the amperometric biosensor are the reactions between the enzymes and its substrate, between the enzymes and the redox mediators, and between the redox mediator and the electrode. The electrochemical reaction on the contact surface of electrode-electrolyte converts flask substrates or products into electricity. If the reaction of each substrate molecule on the electrode involves  $n$  electrons, the oxidation currents are positive, and the reductions are negative, where the current density is expressed as  $I$  ( $\text{Acm}^2$ ). As the current is directly proportional to the concentration

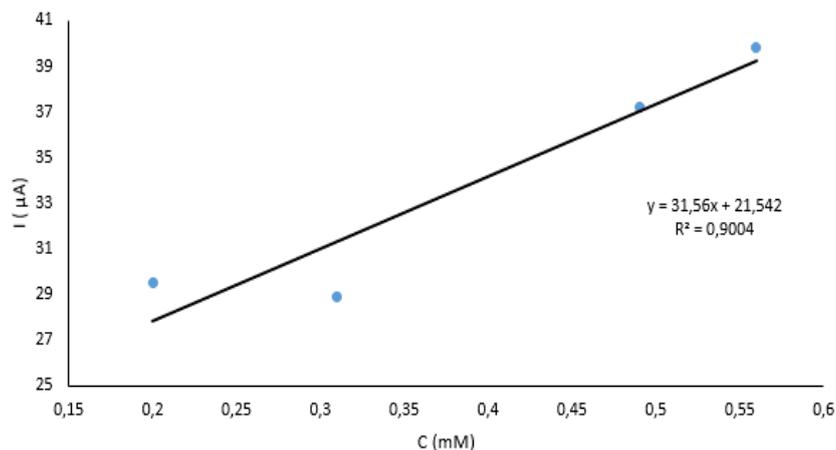
gradient, the current changes follow the changes in the concentration gradient on the surface of the electrode. In many amperometric enzymatic electrodes, a redox mediator is used to link redox reactions to the active site of an enzyme with a redox reaction on the electrode. For peroxidase, the active enzyme is a reduced form and the oxidized form of the mediator is recycled to the cathode. In the general mechanism, the oxidized form of the enzyme reacting with substrate forms the enzyme-substrate complex ES with further decomposition to obtain the reduced form of the enzyme and the product [19].

In Figure 2, shows the cyclic voltammograms the influence of concentrations substrates of 0.20 mM, 0.31 mM, 0.49 mM, 0.56 mM to the enzyme peroxidase. If we compare the cyclic voltammograms of Figure 2 with the cyclic voltammograms (figures 1) in the paper by *Herenda et al.* we see that, for the same potential region, the current values are almost the same [11]. The reason is likely that the enzymes in both papers were from the same oxidoreductase group. However, in this study, we see that oxidation potentials are more pronounced due to the presence of ferric ion in the enzyme and that lower concentrations of substrate are required to produce a reaction between HRP and substrate than is the case with superoxide dismutase and hydrogen peroxide.

We see that at various concentrations of hydrogen peroxide on the surface of the electrode an ES complex is created, as shown in diagram 3. There is a linear dependence of the formation of the ES complex, the proper binding of component I of the enzyme with hydrogen peroxide to its reduced form, which depends on the nature of the substrate. The initial interactions between the enzyme and the substrate molecule are noncovalent.



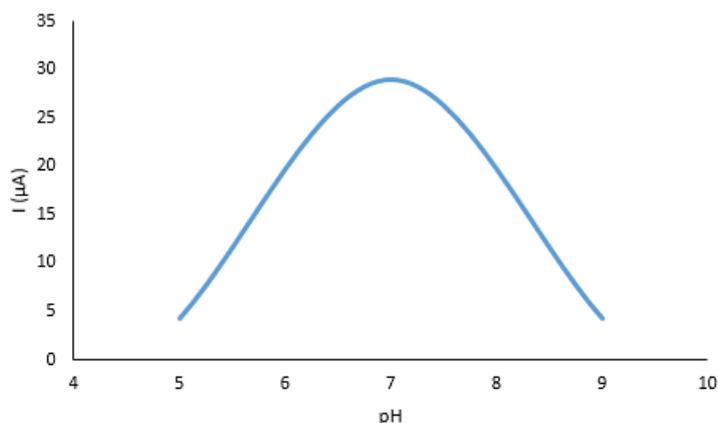
**Figure 2.** Immobilized GC electrode in the presence of different concentrations of substrate ( $H_2O_2$ )



**Figure 3.** Effect of substrate concentration on enzyme activity

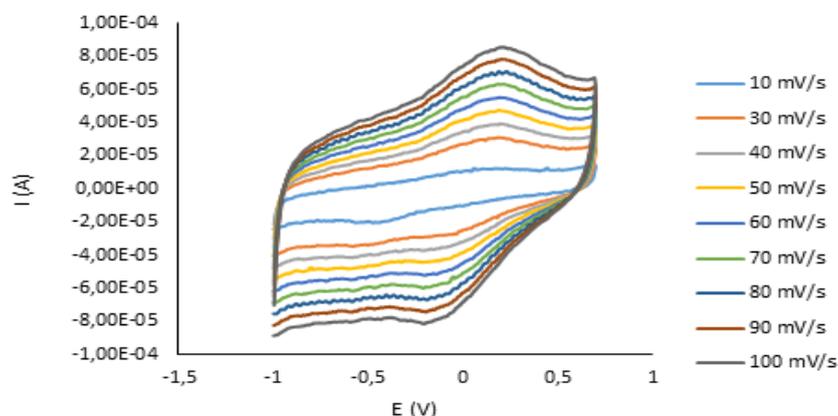
The relationship between pH and enzyme activity depends on the acid-base properties of the enzymes and substrates, especially the type of amino acid residues in the catalytic center. High pH values generally lead to a complete loss of activity for most enzymes. In determining the optimum pH value for an enzyme, the composition of the environment in which the reaction takes place is also very important, and in particular the composition of the buffer mixture used therein.

In this paper, the effect of pH on peroxidase activity was examined by recording a cyclic voltamogram of an immobilized GC electrode immersed in a cell with a phosphate buffer of various pH values of 5, 7 and 9, with the addition of a substrate of 0.31 mM. In Figure 4, we see that for this enzymatic reaction the optimum pH value is 7. For pH 5, the current value was 4.23 μA, for pH 7 28.9 μA, while for pH 9, a current of 4.3 μA was obtained. In research, *Bourdillon et al.* has shown that the pH of the glucose oxidase and the mediator is an optimum pH 8 [10]. In the examination of the influence of dipotassium trioxohydroxytetrafluorotriborate ( $K_2[B_3O_3F_4OH]$ ) on the enzyme superoxidedismutase it was proved that the optimal value of pH 7 [11]. By examining the inhibitory effect of ( $K_2[B_3O_3F_4OH]$ ) on the enzyme from the oxidoreductase group, catalase, it has been concluded that the optimal pH is 6.2, that is ( $K_2[B_3O_3F_4OH]$ ) affects catalase by altering the state of ionization on the amino acids on the active sites of the enzyme. [12].



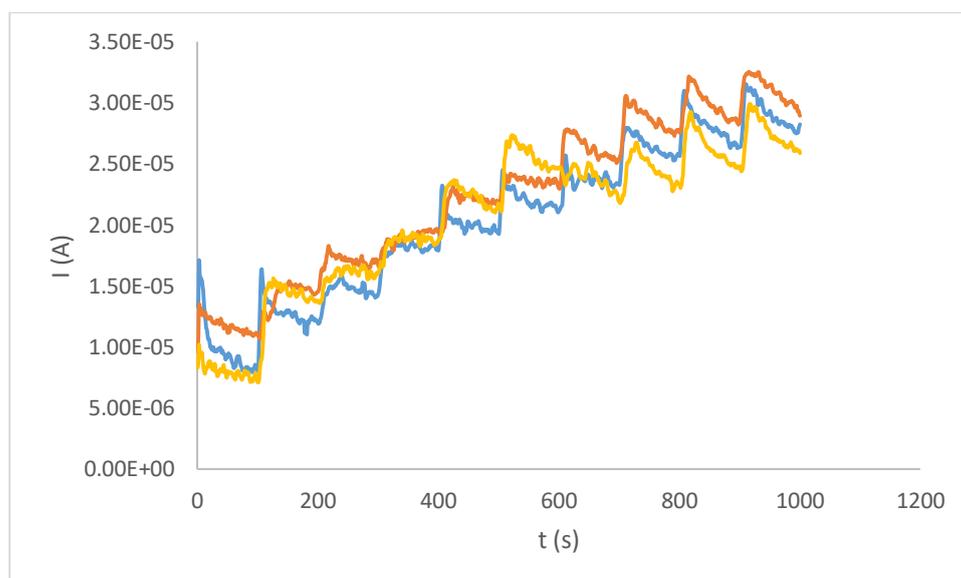
**Figure 4.** The influence of phosphate buffer pH on the enzyme HRP immobilized on GC in the presence of a  $H_2O_2$  concentration of 0.31 mM

An examination of the influence of different scan rates of the GC electrode is shown in Diagram 5. In the diagram we can see that there is a different crude relationship that is regulated by mass transport and the diffusion process from the surface of the electrode and solution. Also, by increasing the speed, there is an increase in cathode and anode currents.



**Figure 5.** Cyclic voltammogram of GC electrodes at different scanning rates

Amperometric measurements were performed at a constant potential of 0.7 V and a scan rate of 50 mV/s for concentrations of perindopril of 0.16 mg/ml and 0.24 mg/ml. Each addition of concentrations of substrate 0.4 mM, 0.8 mM, 2.3 mM, 3.1 mM and 3.8 mM was at intervals of 100 s. Diagram 6.

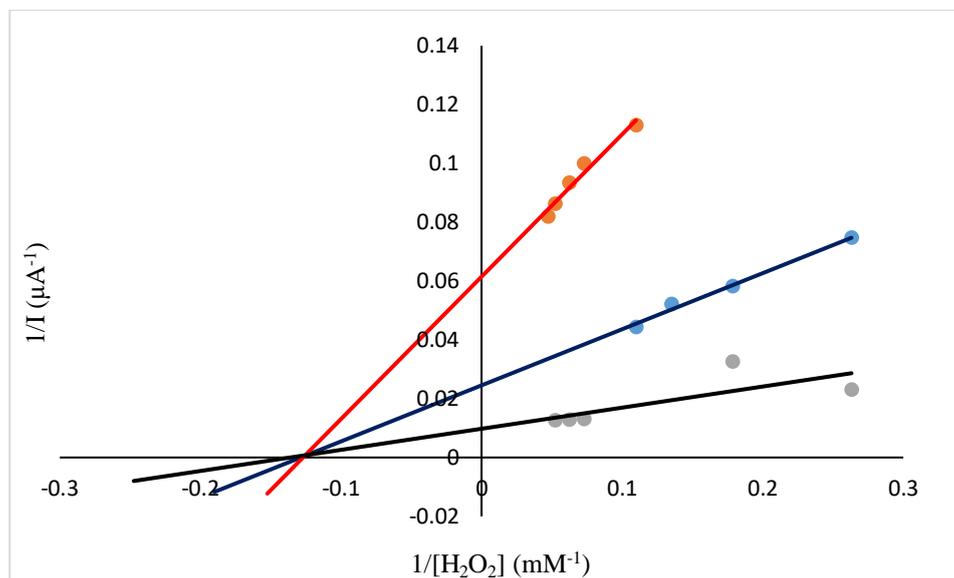


**Figure 6.** Chronoamperogram of an immobilized GC/HRP/Graphene electrode without the presence and with different concentrations of perindopril: (blue line) without inhibitor, (yellow line) 0.16 mg/ml, (orange line) 0.24 mg/ml

From the obtained diagram, it can be seen that with each addition of the substrate an increase in the current occurs, and the reaction can be monitored until the total enrichment of the substrate is saturated. Michaelis-Menten's constant and maximal current are calculated from the Lineweaver diagram and based on the equation [13]:

$$1/I_{ss} = 1/I_{max} + K_m/I_{max} 1/C;$$

$I_{ss}$  is the steady-state current after the addition of substrate,  $C$  is the bulk concentration of substrate and  $I_{max}$  is the maximum current measured under saturated substrate solution.



**Figure 7.** Lineweaver-Burk diagram with different concentrations of perindopril: (black line) without inhibitor, (blue line) 0.16 mg/ml, (red line) 0.24 mg/ml

On Lineweaver-Burke diagram (diagram 7) we see that this is a noncompetitive type of inhibition. This type of inhibition depends on the concentration of the inhibitor relative to the enzyme concentration. According to the obtained results, the  $K_m$  changes slightly, because the saturation of the active center of the enzyme does not interfere with the binding of inhibitors to the second part of the enzyme molecule. The value of the maximum current without inhibitor is  $0.10309 \text{ mol/dm}^3\text{s}$ , while the value is  $K_m 5.32 \text{ mM}$ . From a Lineweaver-Burk plot, the calculated  $K_m$  values were  $7.87$  and  $7.78 \text{ mM}$ , relative to concentration of perindopril, and from the same plot value of  $I_{max}$  were  $0.01623$  and  $0.04081 \text{ mol/dm}^3\text{s}$ . Information on the kinetics of the enzyme immobilized on the electrode or the kinetics of the enzyme-mediator gives us a slower step of the mechanism. The value of the Michaelis-Menten constant depends on the diffusion process on the electrode/enzyme membrane/mediator membrane. Many authors have studied the kinetics of various enzymes and mediators/inhibitors using an electrochemical method. Author *Vilkanauskyte et al.* has described biosensors based on immobilized enzymes and redox mediators on electrophilized films [14]. Monitoring of immobilized enzyme layers on electrode surfaces has been treated by Mell and Malloy [15,16]. In our work we have proven that the value of  $K_m$  without presence and in the presence of perindopril affects many factors

such as mass transport of enzyme substrate and redox cosubstrate in the film and in the adjacent solution, local pH in the enzyme film, partition of substrate and redox mediator between the enzyme film and the solution etc. The same factors were also proved by the authors of *Gough at all* and *Bourdillon at all* [17,18]. The kinetics of the enzyme/ substrate complex or the enzyme/substrate/complex medium mediator depend on whether the mediator is trapped in the film with an enzyme or is in solution [19]. A complete theoretical treatment of the steady-state diffusion and kinetics in amperometric immobilised enzyme electrodes where the mediator is also entrapped within the film is described in the work of the author Bartlett and Pratt [20]. Spectrophotometric determination of the type of inhibition in the reaction between dipotassium trioxohydroxytetrafluorotriborate and peroxidase enzymes in the presence of two substrates of peroxide and guaiacol, alternately showed that it was a competitive type of inhibition, which was confirmed by the electrochemical method [21]. ACE inhibitors do not fully block angiotensin II production, but in this paper we have proven to be associated with an active site on HRP as well as on the enzyme-substrate complex, and therefore can reduce proteinuria, pulmonary hypertension and many other diseases related to cardiac diseases.

#### 4. CONCLUSION

The ability of organisms to spontaneously develop collateral vessels represents an important response to vascular occlusions and operates to improve perfusion of ischemic tissues. The reaction between the reduced form of the enzyme (HRP) and the oxidized mediator (Perindopril) has been considered as irreversible. The reaction was achieved by diffusion processes and the transfer of electrons from the surface of the electrode and solution. Perindopril has proven to be a noncompetitive inhibitor of peroxidase, which means that *in vivo* studies can reduce heart and pulmonary hypertension. The values of Michelis-menten's constants speak of the method of binding perindopril to the active site of the enzyme, but also to the enzyme-substrate complex. Electrochemical studies of the effect of ACE inhibitors (Perindopril) on the activity of immobilized HRP *in vitro* conditions can be the basis for further *in vivo* research.

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