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Short Communication

Electrochemical Investigation of the Influence of $K_2[B_3O_3F_4OH]$ on the Activity of Immobilized Superoxide Dismutase

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It is known that oxidoreductase is responsible for the regulation of oxidative stress in organisms, and pathological changes occur within the cell in the form of accumulation or lack of superoxide and peroxide radicals if the oxidoreductase activity is disturbed. Currently, the development of drugs that target the affected cells while leaving healthy cells unaffected is of great interest. The action of potential drugs is based on the inhibition / activation of oxidoreductase. In this work, we studied the electrochemical parameters of superoxide dismutase as well as the action of the potential drug of boroxine - dipotassium trioxohydroxytetrafluorotriborate ($K_2[B_3O_3F_4OH]$) as a target therapeutic for enzyme activity. Electrochemical tests were carried out in a classical three-electrode system using cyclic voltammetry and chronoamperometry techniques, and the obtained results were presented in the form of the kinetic parameters with the maximum value of the current obtained when the solution was saturated with the substrate (I_{max}) and the Michaelis-Menten constant (K_m). $K_2[B_3O_3F_4OH]$ was proven to be a reversible inhibitor, and the obtained I_{max} without inhibitor value of 0.014 mM and K_m = 12.09 mM. The results from the Lineweaver - Burk diagram show that the inhibition is a partial non-competitive inhibition type.

Keywords: oxidoreductase, enzyme kinetics, boroxine derivative, cyclic voltammetry, chronoamperometry

1. INTRODUCTION

Enzymes that function inside cells may be the best indicators of the health of an organism. A particular focus for elucidating enzyme mechanisms is the class of enzymes that regulate the level of oxidative stress in an organism, which are known as oxidoreductases. Superoxide dismutase (SOD) catalyses the degradation of the O^{2-}free radical:

• Cu^{2+}-SOD + O^{2-} → Cu^{+}-SOD + O_2 (copper reduction, superoxide oxidation)

• Cu^{+}-SOD + O^{2-} + $2H^{+}$ → Cu^{2+}-SOD + H_2O_2 (oxidation of copper, reduction of superoxide)

This enzyme protects the cells that metabolize oxygen from the harmful effects of superoxide free radicals [1]. SOD protects the hyaluronate from depolymerisation caused by free radicals. Exogenous SOD may have an anti-inflammatory effect. The O^{2-} ion, which plays a very important role in the ageing of organisms, peroxidation of the lipids and peroxidation of the erythrocyte membrane, is generated by the univalent reduction of O_2 during various enzymatic reactions or ionising radiation. Activated phagocytes (neutrophils, monocytes, macrophages) in larger amounts release the superoxide anion, and the lack of SOD in an organism can cause haemolytic anaemia. The effects of SOD disturbances significantly accelerate the progression of various types of cancer, which doctors, biologists, chemists and pharmaceutical companies consider to be one of the biggest contemporary challenges. The development of drugs that will have the ability to "recognize" diseased cells and completely inactivate them without an effect on healthy cells is currently of great interest. High drug concentrations do not affect the target enzyme activity, but they reduce the catalytic effect of the therapeutic effect [2].

Halogenated boric acid derivatives - boroxines are currently being investigated as potential enzyme modifiers and therapeutics. Dipotassium trioxohydroxytetrafluorotriborate, $K_2[B_3O_3F_4OH]$ or IUPAC name boroxin(2-hydroxy-4,6-tetrafluoro-1,3,5,2,4,6-trioxatriborinane), is particularly important as an enzyme inhibitor that can be used for therapy in cancer patients [3,4]. It has recently been discovered that $K_2[B_3O_3F_4OH]$ can be used for the prevention and treatment of benign and malignant skin changes, manifesting in the form of neuroma or tumours [5-7]. The effect of boroxine on enzyme peroxidase was studied, and the inhibitory action of boroxine was observed [8-11]. The aim of this study was to investigate the influence of dipotassium trioxohydroxytetrafluorotriborate, $K_2[B_3O_3F_4OH]$, on the activity of immobilized superoxide dismutase using the electrochemical methods of cyclic voltammetry and chronoamperometry and to determine the kinetic parameters and the type of inhibition of $K_2[B_3O_3F_4OH]$.

2. MATERIALS AND METHODS

Materials: Superoxide dismutase (EC 1.15.1.1; Sigma-Aldrich, Buchs, Switzerland); KH_2PO_4 and Na_2HPO_4 (Fisher Chemical, Wien, Austria); hydrogen peroxide (H_2O_2) p.a. 30% (Sigma-Aldrich, Buchs, Switzerland); Nafion 5% (Sigma-Aldrich, Buchs, Switzerland).

Dipotassium trioxohydroxytetrafluorotriborate, $K_2[B_3O_3F_4OH]$, is a boron inorganic derivative synthesised by the reaction of potassium hydrofluoride (KHF_2) with boric acid in a molar ratio of 2:3 at room temperature as described in the literature [12].

Methods: The instrument used for the measurements is a potentiostat/galvanostat PAR 263A with a conventional three-electrode system in which a saturated Ag/AgCl electrode is used as the reference electrode, a Pt electrode is used as the counter electrode, and a GC electrode is used as the working electrode. An amperometric biosensor for the determination of H_2O_2 was formed by the immobilized enzyme (SOD) captured in a Nafion layer, as described in the literature [13], on the surface of the GC electrode. Electrochemical methods of cyclic voltammetry and chronoamperometry were used for the measurements. Cyclic voltammetry was used to examine the effect of different substrate concentrations on the enzyme activity as well as to examine the thickness of the enzyme film on the surface of the electrode. All cyclic voltammetry tests were carried out in a phosphate buffer (pH 7) in the potential range of -1.2 to 0.7 V and at a scan rate of 50 mVs^{-1}. In the electrochemical cell containing 25 ml of phosphate buffer, different concentrations of hydrogen peroxide of 1.96 mM, 3.84 mM, 5.66 mM, 7.4 mM were added. A chronoamperometric technique was used to determine the kinetic parameters, i.e., the Michaelis-Menten constant (K_m) and the maximum current value at which the substrate is saturated with the substrate (I_{max}), which is equivalent to the maximum reaction rate (V_{max}) [14]. Chronoamperometric measurements were carried out in the electrochemical cell containing 25 mL of the phosphate buffer solution with a constant potential of 0.9 V applied to the working electrode as well as constant mixing at 400 rpm. The reaction was monitored with and without the presence of different concentrations of $K_2[B_3O_3F_4OH]$, i.e., 0.3 mM, 0.5 mM, 0.6 mM and 0.8 mM.

3. RESULTS AND DISCUSSION

In many cases, the approach of immobilization allows the direct study of the electrochemical properties of redox enzymes and enzyme catalysis by direct voltammetry without mediators. Polymers or other materials are often used to help immobilize enzymes on the electrodes. The efficiency of redox conversion in the resulting films then depends on the thermodynamics of the potential-driven redox reaction, the kinetics of electron transfer at the film-electrode interface, the rate of charge transport within the film, which may depend on the counterion entry and exit rates, the electron self-exchange between the redox sites and the structural transformations coupled to the electron transfer, such as conformational changes. One particularly useful approach involves plots of the reduction and oxidation peak potentials against the log of the scan rate over a wide scan rate range, which can be used for mechanistic studies in both the absence and presence of the substrate. Such a plot for an enzyme with reversible thin-film voltammetry at low scan rates with electron-transfer kinetic limitations at high scan rates shows nearly equal and constant oxidation-reduction peak potentials at the low scan rate that change symmetrically in the opposite directions at higher scan rates [15-18].

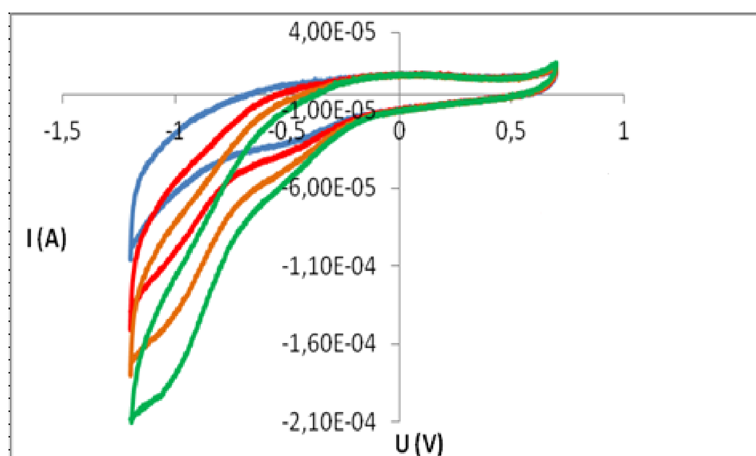

Figure 1. Cyclic voltammograms of the immobilized GC electrode in the presence of different concentrations of hydrogen peroxide: (blue line) 1.96 mM, (red line) 3.84 mM, (orange line) 5.66 mM, and (green line) 7.4 mM

The oxidation potential depended on the scan rate [19]. The increase of the anodic peak current depends on the H_2O_2 concentration. Moreover, the oxidation potential was almost the same at scan rates below 50 mV s^{-1}, which suggested facile charge transfer kinetics over this range of sweep rates. Kamyabi and Hajari examined the chemical reaction between H_2O_2 and CuO. Nano-copper oxide particles were found to be suitable as mediators for shuttling electrons between H_2O_2 and the working electrode. Depending on the immobilization of the working electrode, the oxidation potential is 0.65 V [20]. Additionally, the linear dependence of the current on the substrate concentration and the different scan rates was obtained by Giovanni et al. when testing the immobilization of the catalase enzymes on the GC electrode modified with different nanomaterials. Depending on the type of modification, different values of the oxidation potential of the peroxide have been obtained [21].

In these electrochemical measurements, the electron transfer was observed in the reaction between the hydrogen peroxide substrates and the superoxide dismutase immobilized on the GC electrodes. Figure 1 shows the influence of different concentrations of $[H_2O_2]$ = 1.96, 3.84, 5.66, and 7.4 mM on the immobilized GC electrode with Nafion and superoxide dismutase (SOD) (GC/Nafion/SOD). Cyclic voltammograms were recorded in a 100 mM phosphate buffer solution (pH 7.0) at a scan rate of 50 mVs^{-1}. In the same figure, an increase in the current peak in the area of the oxidized intermediates caused by the hydrogen peroxide decomposition at a potential of 0.533 V can be seen with the increasing substrate concentration in the solution. Electron transfer involving peroxide-activated horseradish peroxidase and other peroxidase enzymes adsorbed onto carbon electrodes has been studied [22] because of their importance in the detection of hydrogen peroxide and as labels in electrochemical DNA and immunosensor assays. Figure 2 shows that there is a proper sequence of the increase in the current with the increasing concentration of hydrogen peroxide in the solution. The increase in the current is observed with the increase in the hydrogen peroxide concentration in the range between 1.96 mM and 7.4 mM.

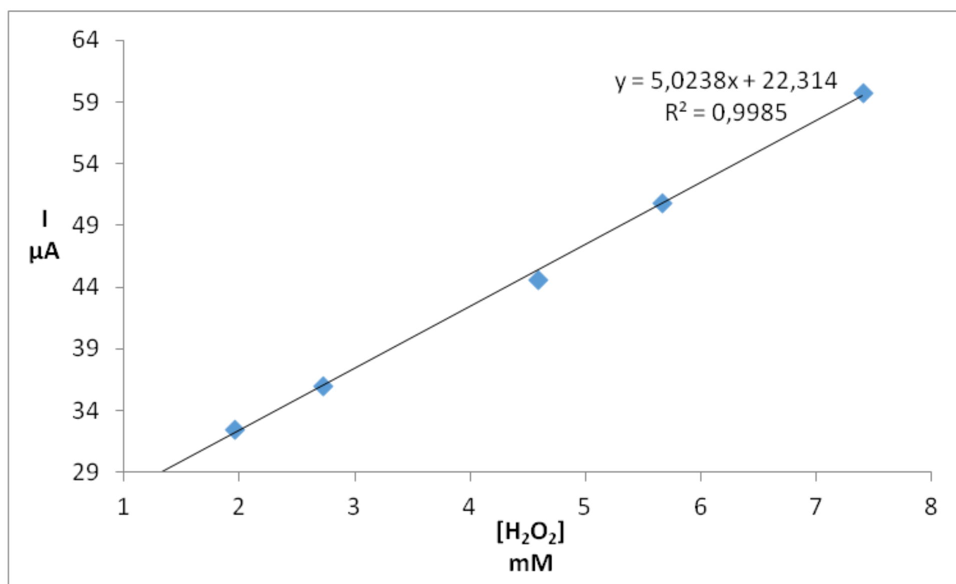

Figure 2. Linear dependence of the current on the concentration of the hydrogen peroxide substrates

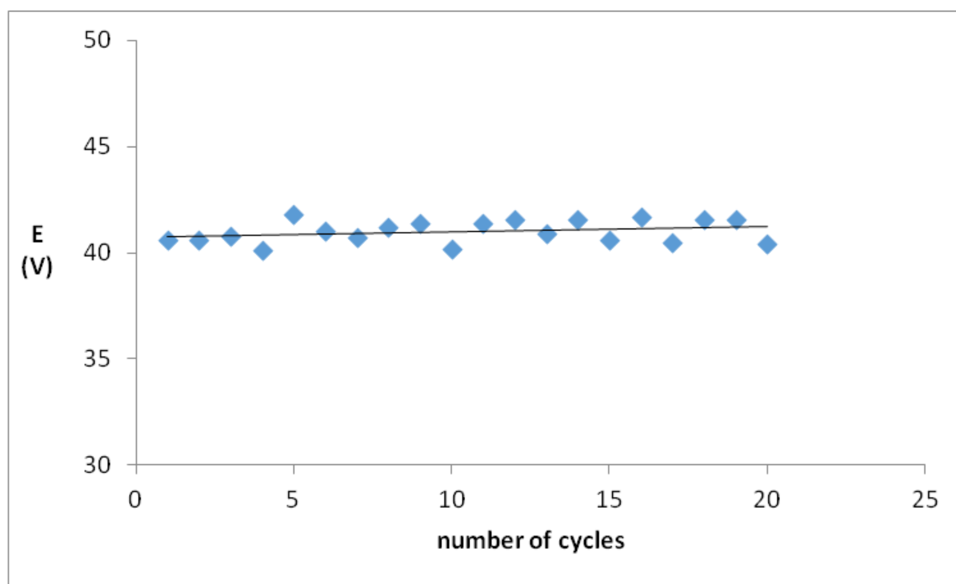

Figure 3. Dependence of the current peak of the electrode (GC/Nafion/SOD) in the phosphate buffer on the number of cycles

To test whether the enzyme layer on the surface of the GC electrode was correctly arranged, 20 cycles with the GC/Nafion/SOD electrodes in a phosphate buffer were recorded at a scan rate of 50 mV s^{-1}. The results showed that there are no deviations in the appearance of the curve for each of the cycles, so we present this phenomenon in Figure 3. The values of the currents in the anode oxidation field are presented at the potential of 0.534 V. Since the current is directly proportional to the thickness of the enzyme film, the deviation of the curve towards larger or smaller values with the increase in the number of cycles indicates the uneven distribution of the enzyme film on the surface of the electrode

[23]. Ideal, reversible voltammograms form a monolayer of electroactive enzyme on an electrode and for a simple electron transfer reaction are similar to those of any ultrathin electroactive film. Films thicker than a monolayer of electroactive enzyme can often provide a larger enzyme loading per unit electrode area resulting in larger peak currents. For the complete electrolytic conversion throughout these films achieved on the CV timescale, the thin-layer electrochemistry model can describe the CVs approximately. Charge transport through these films may involve physical diffusion of the enzymes and/or electron self-exchange reactions between the enzyme redox centres [24]. It can be clearly seen in Figure 3 that the enzyme layer is aligned equally throughout the surface of the GC electrode.

Amperometric measurements were performed at a constant potential of 0.9 V and with the successive addition of hydrogen peroxide in 100 mM phosphate buffer (pH 7.0) every 7 seconds. The values of the Mihaelis-Menten constants (K_m) and the maximum current (I_{max}) were calculated from the famous Lineweaver-Burk equation [25]. As shown in Figure 4, with the increasing concentration of $K_2[B_3O_3F_4OH]$, there is an increase in the current response for each hydrogen peroxide addition. The concentration range of 0.1 mM boroxine was not randomly selected. Namely, in the range of given concentrations of $K_2[B_3O_3F_4OH]$, the targeted action of the potential drug on tumour cells is obtained without any effect on the healthy cells. Higher concentrations of $K_2[B_3O_3F_4OH]$ can cause damage and death of healthy cells, and smaller concentrations are negligible in the treatment process.

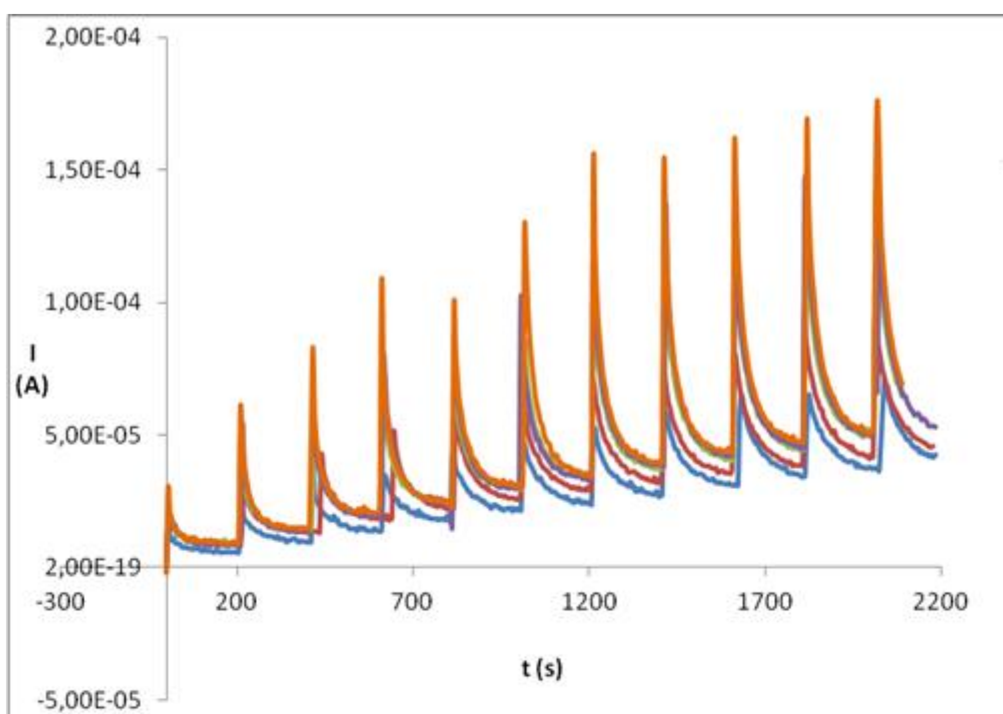

Figure 4. Amperometric response of an immobilized GC/SOD/ Nafion electrode in the presence of successive additions of the substrate without the presence and with different concentrations of $K_2[B_3O_3F_4OH]$: (blue line) without inhibitor, (red line) 0.3 mM, (green line) 0.5 mM, (violet line) 0.6 mM, and (orange line) 0.8 mM

The Mihaelis-Menten constant (K_m) is calculated from the slope obtained from a reciprocal flow diagram depending on the reciprocal values of the various hydrogen peroxide concentrations [25]. Mihaelis-Menten's constant (K_m) was calculated with and without the presence of boroxine $K_2[B_3O_3F_4OH]$. The value of the constant (K_m) of the superoxide dismutase can depend on the isolation, the metal ion in the enzyme structure, and the manner of binding of boroxine to the active site of the enzyme. Additionally, molecular diffusion has an effect on the values of the kinetic constants of the immobilized enzyme because they are different from the free enzyme constants. The inhibitory effect of boroxine $K_2[B_3O_3F_4OH]$ is shown in diagram 5, where the fixed concentrations of $K_2[B_3O_3F_4OH]$ as a function of different concentrations of H_2O_2 are presented. From the Lineweaver-Burk diagram, the values of I_{max} = 0.014 mA and K_m=12.09 mM were calculated without the presence of boroxine.

Calculated values were also obtained in the presence of different concentrations of boroxine that indicate that in this case, $K_2[B_3O_3F_4OH]$ behaves as a partial non-competitive inhibitor, which implied that the enzyme is still active in the presence of inhibitors [26]. The values of the maximum currents (I_{max}) with the inhibitor are 0.017, 0.023, 0.025, and 0.028 mA, while the calculated values are K_m 11.70, 12.43, 12.15, and 11.45 mM, respectively, which provided additional confirmation that boroxine is a partial non-competitive inhibitor because I_{max} (without inhibitor) < I_{max} (with inhibitor) as the concentration of boroxine increases.

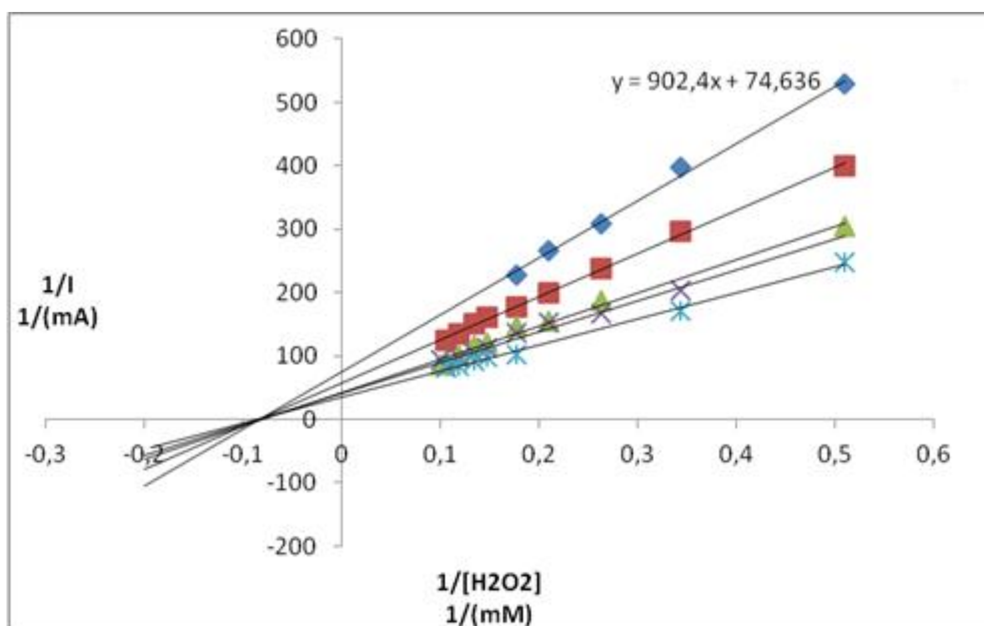

Figure 5. Lineweaver-Burk diagram for the determination of kinetic constants (I_{max} and K_m) without and in the presence of different concentrations of boroxine $K_2[B_3O_3F_4OH]$: (◊) without inhibitor, (□) 0.3 mM, (Δ) 0.5 mM, (x) 0.6 mM, and (Ж) 0.8 mM

4. CONCLUSION

We examined the kinetic action of boroxine on SOD by electron transfer by using electrochemical methods under *in vitro* conditions. The electrochemical method and biosensors are

more suitable for this research than the spectrophotometric method because of their simplicity, high selectivity, sensitivity, accuracy, speed, and low cost. This study shows that halogenated boroxine on the enzyme superoxide dismutase (SOD) acts as a reversible inhibitor because I_{max} with the inhibitor is greater than the I_{max}-free inhibitor. In the case of reducible inhibitors in the enzyme reaction, the substrate was not completely reacted to produce the product, and binding of the inhibitors for the enzyme was observed as well as the formation of an enzyme/substrate/inhibitor of the complex. Since in our reaction the SOD is still active in the presence of boroxine, we concluded that this effect is a partial non-competitive inhibition, which is very important in the regulation of cell metabolism. By examining boroxine as a potential drug, it was concluded that it has inhibitory properties on SOD activity, and therefore, it can be used to regulate and prevent oxidative stress and to stop the further development of many types of diseases.

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