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Enzyme Immobilization Onto Carbon Fiber Electrodes by Electrochemical Polymerization

Elektrokimyasal Polimerizasyonla Karbon Fiber Elektrotlara Enzim Tutuklaması

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Abstract

New enzyme electrodes were fabricated by using carbon fiber as electrode substrate. Electrodes were obtained by immobilization of invertase in the conductive polypyrrole matrix during electrochemical polymerization onto laboratory–made carbon fiber electrodes. Maximum reaction rate, (V_{max}) and substrate affinity, (K_m), of immobilized enzyme were determined as 56.7 μmol min^{-1} $electrode^{-1}$ and 1.57 mM respectively. The effect of conditions on enzyme activity was investigated. It was found that the optimum temperature, optimum pH value, linear working range are 30 ºC, pH 5.0 and 0.0025–0.0200 M respectively. The electrodes were examined for operational stability. The results were compared with the previous studies in which invertase was immobilized onto platinum substrate.

Keywords: Carbon fiber electrode, Conducting polymer, Enzyme immobilization, Invertase

Öz

Elektrot malzemesi olarak karbon fiber kullanılarak yeni enzim elektrotları hazırlandı. Elektrotlar, laboratuvarda hazırlanan karbon fiber elektrotlar üzerine elektrokimyasal polimerizasyon esnasında invertazın iletken polipirol matrisinde tutuklanmasıyla elde edildi. Tutuklanan enzimin kinetik parametreleri, V_{max} (maksimum reaksiyon hızı) ve K_m (substratın enzim ilgisi) 56,7 μmol dak^{-1} $elektrot^{-1}$ ve 1,57 mM olarak elde edildi. Reaksiyon koşullarının enzim aktivitesine etkisi incelendi. Tutuklanmış enzimin optimum sıcaklığı, optimum pH'ı ve doğrusal çalışma aralığı 30 ºC, pH 5, ve 0,0025–0,0200 M olarak bulundu. Elektrotların ardışık stabilitesi saptandı. Sonuçlar invertazın polipirol kaplı platin elektrotlara tutuklandığı daha önceki çalışmalarla karşılaştırıldı.

Anahtar Kelimeler: Karbon fiber elektrot, İletken polimer, Enzim tutuklaması, İnvertaz

1.Introduction

Carbon fiber, as an electrode substrate, is used in biosensors for *in vivo* and *in vitro* detection of species owing to its unique advantages such as chemical stability, biocompatibility and possibility for miniaturization. Carbon fiber electrodes (CF) attract great interest since they have larger surface area than other type of electrodes and are very convenient for surface modifications (Fei et al. 2005). Here, it has been used first time for invertase immobilization through the electrochemical polymerization.

Physical entrapment of enzyme in conducting polymer matrices by electrochemical polymerization is a considerable way of immobilization since it is a rapid, reliable, simple and an economical way of enzyme deposition. Through the conducting polymers, polypyrrole has a special place with its superior properties (Deepa and Ahmad 2008, Tokonami et al. 2012) such as easy synthesis, longterm stability, resistance to air and water (Minkstimiene et al. 2011), stability to heating (Mehdinia et al. 2012) and being biocompetitive (Ferraz et al. 2012). Owing to its solubility in water, the enzyme deposition becomes possible in aqueous media.

Invertase or β-D-fructofuranosidase (E.C.3.2.1.26) catalyzes the hydrolysis of sucrose to glucose and fructose which is known as invert sugar used widely in the production of noncrystallizing sugary foods. Although invertase has a lower possibility of finding a commercial use in its immobilized form since the soluble enzyme is available at little cost, it is one of the most studied enzymes because of its utilization as a model enzyme to give idea about the immobilization of high cost enzymes (Kiralp et al. 2003, Waifalkar et al. 2016, Cabrera et al. 2017).

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The present work describes new enzyme electrodes based on carbon fiber electrode material. In order to investigate the performance of this substrate, a common polymer matrice (PPy) and a model enzyme (invertase) were used. Optimization and characterization of enzyme electrodes are given in following sections.

2. Materials and Methods

High modulus Yutai T300 6K carbon fiber was used as electrode material. Invertase (E.C.3.2.1.26) Type V, pyrrole and sodium dodecyl sulphate (SDS) were supplied from Sigma. Constituents of Nelson reagent were of analytical grade. Wenking POS-88 potentiostat, GAMRY Instruments Interface 1000 Potentiostat/Galvanostat/ZRA, Shimadzu UV-1201-V spectrophotometer and JEOL Scanning Microscope Model JSM-6400 scanning electron microscope (SEM) were used for electrode preparation and characterizations.

2.1. Synthesis of Polymer and Immobilization

Carbon fiber with diameter of 30 um and steel wire were connected to each other with teflon tape. Carbon fiber was inserted into the conical micropipette tip, secured by a cured epoxy resin. A glass tube was used to cover the wire. Typical three–electrode cell was used for electropolymerization, consisting of carbon fiber as working electrode, Pt foil as counter electrode and a Ag/Ag^{+} as reference electrode. Immobilization of enzyme was achieved via electropolymerization of Py on carbon fiber substrate in 10 mL buffer solution. Acetate buffer (pH 5.0) including 0.6 mg/mL SDS (supporting electrolyte), 5.0 μL/mL pyrrole, 0.6 mg/mL invertase was used for immobilization. Codeposition of enzyme and PPy was carried out by constant potential at 1.0 V for 30 min at room temperature.

2.2. Determination of Invertase Activity

Somogyi-Nelson method was used for determination of immobilized invertase activities (Hatanaka and Kobara 1980), in which different concentrations of sucrose were prepared (1.0 mL), enzyme electrode was immersed for 2, 4, 6 min, 1.0 mL of Nelson's reagent was added and the tubes were incubated into boiling water for 20 min. 1.0 mL of arsenomolibdate reagent and 7 mL water were added for total volume of 10.0 mL. Activities were calculated by reaction rates obtained from absorbances measured at 540 nm.

2.3. Kinetic Studies

Kinetic parameters of enzyme electrodes; maximum enzyme activity, V_{max} and Michaelis-Menten constant, K_m were obtained at optimum pH and 25 °C by using Michaelis-Menten method and Lineweaver-Burk graph (Lineweaver and Burk 1934).

2.4. pH and Temperature Examination and Daily Stability

Immobilized invertase activities were measured by scanning pH between 2 and 12 and incubation temperature between 10 °C and 80 °C. Consecutive 40 activity measurements were performed in order to investigate stability of enzyme electrodes. 5 K_m substrate concentration was used throughout the optimization and stability studies.

3. Results

3.1. Morphology of Carbon Fiber Electrodes

Morphological study of carbon fiber electrodes clearly proves the polymerization of pyrrole. SEM micrographs were used to examine the surface of electrodes. As seen on Figure 1, CF coated with polymer is almost two times thicker than bare CF. Electrode surface exhibits standard cauliflower structure of polypyrrole and average diameter of globules is 1 μm.

3.2. Polymerization

Electropolymerization on CF substrates was performed by cyclic voltammetry between +0.7 and -1.7 V for 14 cycles. Resulting voltammogram shows an incline in peak currents as the cycle number increases (Figure 2). Polymer thickness on the surface of CF increases as the polymerization proceeds, which resulted in an increase in current.

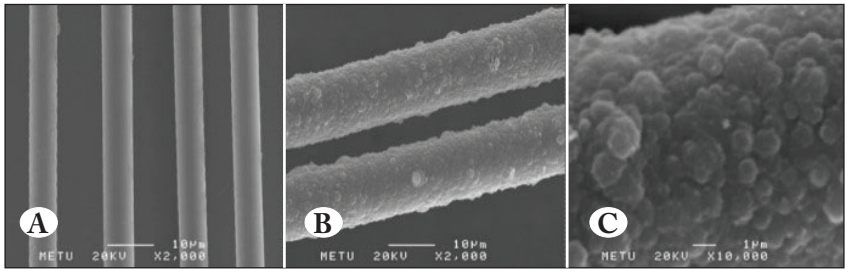

Figure 1. SEM micrographs of **(A)** bare CF magnified by 2,000 times, **(B)** polymer coated CF magnified by 2,000 times, **(C)** polymer coated CF magnified by 10,000 times.

3.3. Kinetic Parameters of Immobilized Invertase

Determination of kinetic parameters of immobilized invertase was performed by measuring the enzyme activities according to the procedure described previously.

Substrate concentration was raised until a constant rate of product formation was reached as shown on Figure 3A. It is considered as maximum speed of enzyme reaction, V_{max}. The substrate concentration corresponding half of the maximum speed is referred as K_m and this parameter represents the affinity between enzyme and its substrate. Lower K_m value indicates higher affinity. From this curve, working range is obtained between 0.0025 M and 0.0200 M. K_m and V_{max} are determined from Lineweaver-Burk plot (Figure 3B) as 56.7 mM and 1.57 μmol min^{-1} $electrode^{-1}$ respectively.

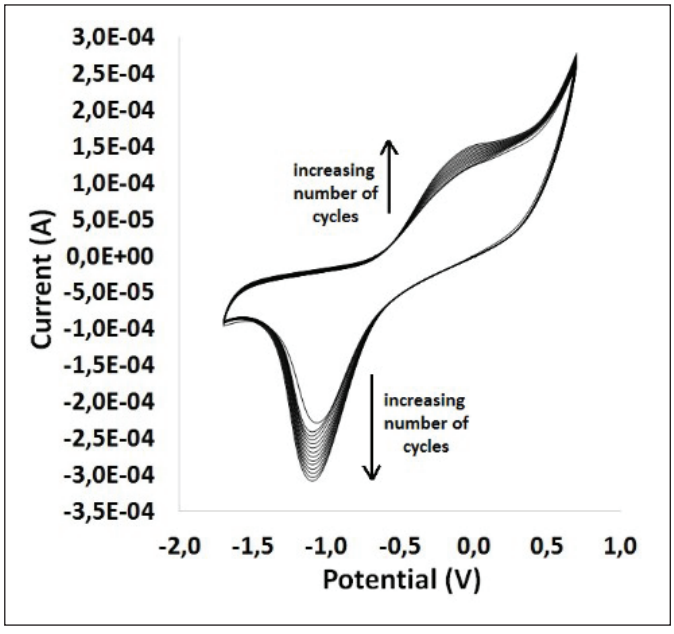

Figure 2. Polypyrrole synthesis on CF electrodes by cyclic voltammetry.

3.4. pH and Temperature Stability

The activity of the enzyme was followed between pH 2 and pH 12 (Figure 4A). At pH 3, the electrode exhibits a maximum activity. After pH 3, the activity declined and was lost at pH 9. In this study, pH 5 acetate buffer was used for practical reasons.

Changes in temperature has an influence on enzymes since they are heat sensitive protein molecules. This effect was demonstrated in Figure 4B. The maximum enzyme activity was revealed at temperature of 30 °C. Enzyme activity showed a decline after 30 °C because of denaturation, however, the electrodes can be used for a wider temperature ranges between 20 °C and 50 °C with a 75% activity.

3.5. Daily Stability

The stability study followed on Figure 5 shows a decline in activity during first 5 measurements. This situation is generally observed because of unentrapped enzyme molecules which exist on the surface of electrode only by adsorbtion. These nearly free enzymes pour out to the solution during the first measurements. After electrode loses these unbounded enzyme molecules, it shows a steady activity. After those initial steps, the retained enzyme activity of 70% is kept until 40th measurement.

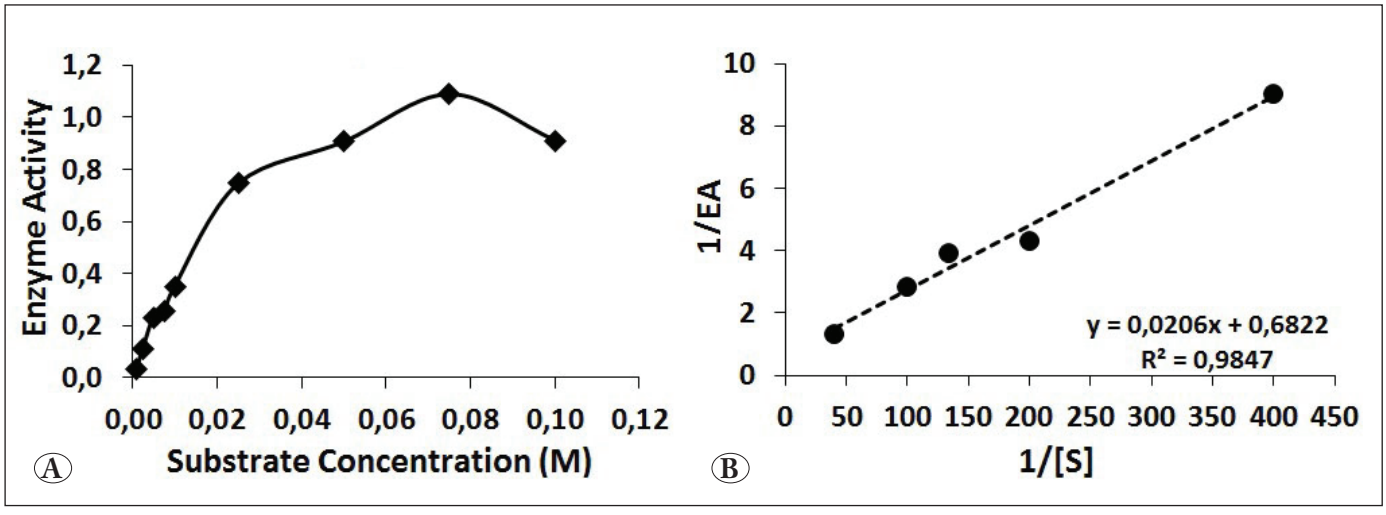

Figure 3. (A) Michaelis-Menten graphic, **(B)** Lineweaver-Burk plot of invertase.

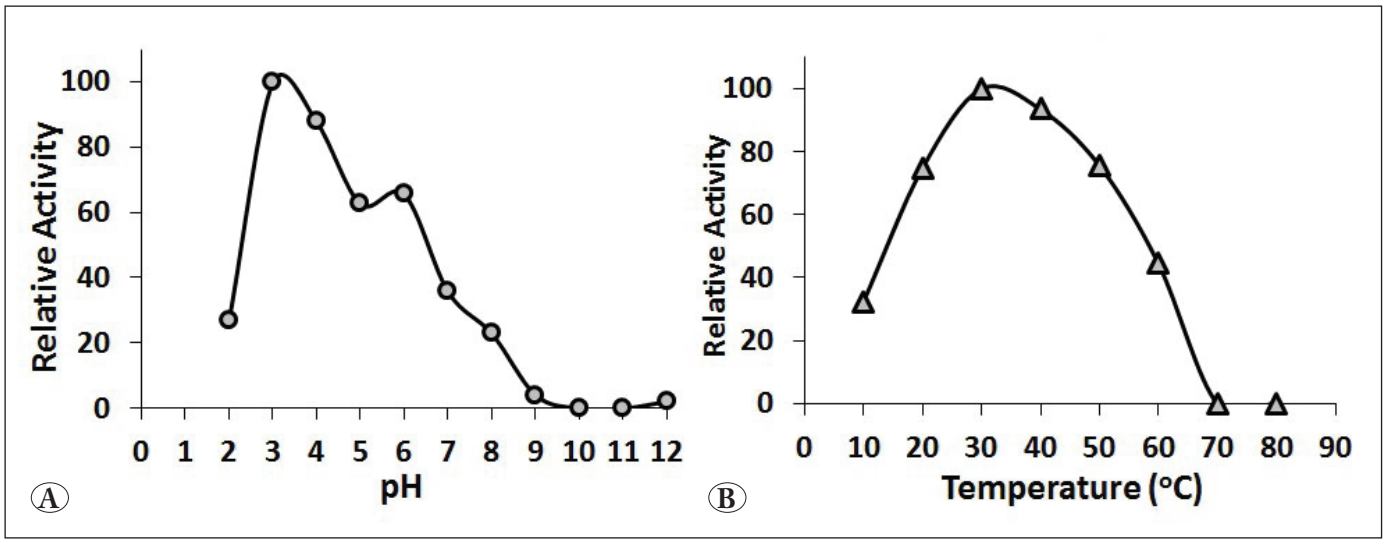

Figure 4. Effect of **(A)** pH and **(B)** incubation temperature on invertase activity.

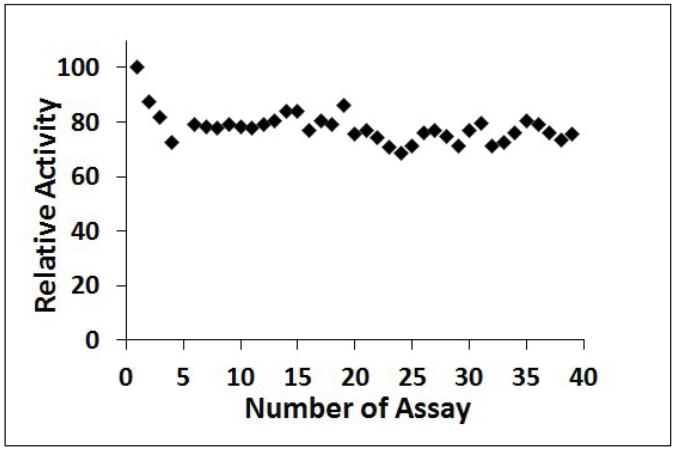

Figure 5. Stability of enzyme electrode upon consecutive measurements.

4. Discussion

Among the immobilization matrices, conducting polymers, particularly polypyrrole attract attention since they can be employed by entrapment method of immobilization during electrochemical polymerization. Entrapment, as being just confinement, is a method providing stability to immobilized enzyme. Several conducting polymers have been synthesized and used for invertase immobilization employing platinum plates as electrode material. Ak et al. performed a comparative study and found V_{max} and K_m of free enzyme as 82.3 μmol min^{-1} mL^{-1} and 26 mM, quite different than immobilized invertase into polypyrrole matrice that has V_{max} and K_m as 3.0 μmol min^{-1} $electrode^{-1}$ and 58.0 mM (Ak et al. 2014). A drastic decrease in reaction rate upon immobilization is a common result due to the presence of much lower amount of enzyme in immobilized form relative to free enzyme in solution. Besides, conformational changes of enzyme molecules formed through the immobilization eventuate in inactivation of some active sites of enzyme that results in a decline in V_{max}. On the other hand, higher K_m observed for immobilized enzyme indicates a restricted diffusion of substrate towards enzyme located in a matrice which creates a diffusion barrier.

Sahmetlioğlu et al. entrapped invertase into copolymer of thiophene functionalized vinyl alcohol and polypyrrole on a platinum plate with dimension of 1cm x 1cm (Sahmetlioğlu et al. 2006). Recorded V_{max} and K_m are 0.40 μmol min^{-1} $electrode^{-1}$ and 60 mM and V_{max} is quite lower than the one obtained by Ak et al. for PPy matrice. Thiophene bonded side groups of vinyl alcohol provides more space between copolymer chains which causes invertase molecules entrapped loosely. Therefore, enzyme in this matrice may be lost back easily. The situation resulted in a lower amount of enzyme immobilized and consequently a lower V_{max}. Invertase, in another study, was immobilized into Poly(SNS(NO_2)-*co*-Py) on the same dimension platinum electrode, in which the polymer chains are composed of alternating thiophene and pyrrole (SNS) with nitrobenzene side groups (Tuncagil et al. 2008). They obtained V_{max} and K_m as 1.6 μmol min^{1-} $electrode^{1-}$ and 50 mM. V_{max} here, is higher than Sahmetlioglu's copolymer since the side group nitrobenzene is a shorter group relative to thiophene bonded vinyl alcohol, a situation resulted in a more closely packed polymer chains that keeps more enzyme molecules.

Another SNS type polymer with alternating thiophene and pyrrole units in the main chain and a benzene in the side group position was used and coated on the 1cm x 1cm platinum electrode (Celebi et al. 2009). V_{max} and K_m represented in this study are 2.6 μmol min^{-1} $electrode^{-1}$ and 40 mM. Side group benzene is a smaller substituent than nitrobenzene, which gives a much more closely packed structure and a higher V_{max} with respect to Tuncagil's polymer. Invertase molecules are entrapped more and more as the polymer chains are packed more closely. Aydar et al. used Poly(EDOT-benzothiadiazole-EDOT) on the same type of platinum electrode. It is a copolymer with large main units in the skeleton, large benzothiadiazole units and large EDOT units which prevent formation of packed structure in a considerable degree (Aydar et al. 2011). Therefore, 0.96 μmol min^{-1} $electrode^{-1}$ and 31.8 mM were obtained as V_{max} and K_m. Through these examples, polypyrrole gives the most closely packed structure due to its lineer chains without side group. This provides to the polypyrrole the highest quantity of V_{max} as 3.0 μmol min^{-1} $electrode^{-1}$. If it comes to comparison of K_m values, a remarkable difference is not seen between the first three polymers discussed above and polypyrrole which means that these matrices have almost similar diffusion barrier for substrate. Celebi and Aydar groups observed lower K_m values thus higher affinity between enzyme and substrate, which indicates an easier diffusion and a lower barrier in these polymer matrices.

V_{max} and K_m obtained in the present study are 1.57 μmol min^{-1} $electrode^{-1}$ 56.7 mM. V_{max} is almost half of the value obtained by Ak et al. which is 3.0 μmol min^{-1} $electrode^{-1}$. Among the studies in this area, present study is unique owing to using CF as microelectrode material with a diameter of 50 μm. Although dimension of CF electrode is considerably smaller than its platinum counter, the kinetic parameter V_{max} of CF is just half of the one obtained by platinum. This means that the quantity of entrapped enzyme with CF is also half of the platinum electrode which indicates that surface area of the CF is half of its counter. It may be possible only if carbon fiber has a surface structure remarkably porous that results in a high surface area relative to platinum. When comparing different types of polymer, it should be considered with two criteria, polymer packing degree and electrode surface area, which makes the comparison difficult. Our V_{max} is higher than the ones obtained by Sahmetlioglu et al. and Aydar et al., which implies a higher amount of enzyme immobilized to PPy coated CF electrode, which may be a result of higher surface area of CF or more closely packed structure of polymer. For other studies which has higher V_{max} than our value, there must be higher amount of immobilized enzyme again, because of the reasons above. V_{max} presented by Tuncagil et al. is almost same with the one we obtained, indicating a balance between surface areas and polymer packing, which gives comparable V_{max}'s. On the other hand, K_m, the affinity between enzyme and substrate is almost same with two types of electrode, polypyrrole coated platinum electrode and carbon fiber electrode, prepared in this study, coated with the same material. Here, electrode material does not affect the affinity because the microenvironment where enzyme and substrate meet each other is the polymer matrice. Since the polymer is same for both electrodes, affinity is almost same for two electrode types. When the stabilities of the electrodes above are considered, it is found that repeatibility of the enzyme electrodes constructed by platinum is better than their CF counter.

By these results, it can be concluded that CF electrodes are superior to platinum substrate by being less expensive, ability to be used as microelectrode and entrapping relatively higher amount of enzyme. On the other hand, its repeatibility is the parameter that needs to be improved.

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