IMMOBILIZATION OF GLUCOSE OXIDASE AND POLYPHENOL OXIDASE IN CONDUCTING COPOLYMER OF PYRROLE FUNCTIONALIZED POLYSTYRENE WITH PYRROLE

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

OLÇUN EKİNCİ

IN PARTIAL FULFILLMENT OF THE REQUIREMENT
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
CHEMISTRY

JULY 2006

Approval of the Graduate School of Natural and Applied Sciences.

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This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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ABSTRACT

IMMOBILIZATION OF GLUCOSE OXIDASE AND POLYPHENOL OXIDASE IN CONDUCTING COPOLYMER OF PYRROLE FUNCTIONALIZED POLYSTYRENE WITH PYRROLE

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July 2006, 76 pages

Electrochemical polymerization of pyrrole functionalized polystyrene (PStPy) with pyrrole was carried out in water-sodium dodecyl sulfate solvent-electrolyte couple. Characterization of the resulting copolymer was performed via Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and four probe conductivity measurements. Glucose oxidase and polyphenol oxidase enzymes were immobilized in polypyrrole (PPy) and conducting copolymer of pyrrole functionalized polystyrene with pyrrole (P(PStPy-co-Py). Resulting enzyme electrodes were characterized by kinetic parameters; V_{max} and K_m. Behavior of enzyme electrodes upon temperature and pH changes were investigated. Glucose oxidase electrode was used for the determination of glucose in orange juice and polyphenol oxidase electrode was used for the determination of polyphenolic compounds in red wine.

Keywords: Electrochemical polymerization, conducting copolymer, immobilization of enzymes, polyphenol oxidase, glucose oxidase, glucose determination, wine analysis.

ÖZ

GLUKOZ OKSİDAZ VE POLİFENOL OKSİDAZ ENZİMLERİNİN PİROL UÇLU POLİSTİRENİN PİROLLE OLUŞTURULAN İLETKEN KOPOLİMERİNDE TUTUKLANMASI

Ekinci, Olçun
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Temmuz 2006, 76 sayfa

Pirol uçlu polistirenin pirolle kopolimeri, su-sodyom dodesil sülfat çözücü-elektrolit çiftinin içinde oluşturuldu. Elde edilen kopolimerin karakterizasyonu Fourier transform infrared spectroskopisi (FTIR), taramalı electron mikroskobu (SEM) ve dört milli iletkenlik ölçümü yöntemiyle yapıldı. Glukoz oksidaz ve polifenol oksidaz enzimleri polipirol (PPy) ve pirol uçlu polistirenin pirol ile oluşturulan kopolimerinde (P(PStPy-co-Py) tutuklandı. Oluşan enzim elektrotları V_{max} ve K_m kinetik parametreleri ile karakterize edildi. Enzim elektrotlarının sıcaklık ve pH değişimine göre davranışları araştırıldı. Glukoz oksidaz elektrodu portakal suyunda glukoz tayini ve polifenol oksidaz enzimi şarapta polifenolik bileşiklerin tayini için kullanıldı.

Anahtar Kelimeler: Elektrokimyasal polimerleşme, iletken kopolimer, enzim tutuklaması, polifenol oksidaz, glukoz oksidaz, glukoz tayini, şarap analizi.

To My Family

ACKNOWLEDGMENTS

I would like to express my gratitude to my supervisor Prof. Dr. Levent Toppare for his continuous support and guidance throughout this study. With his encouragements, I gain enthusiasm and motivation in persuing this research.

I would like to send my special thanks to Dr. Senem Kıralp for her help and support.

I would like to thank Dr. A. Elif Böyükbayram for her comments on enzymes.

I would like to thank my family, my brother Orkunay for their everytime support and moral aid.

I would like to send my special thanks to my friends Mahmut Çil, Oğuzhan Yıldız, Simge Tarkuç, Vedat Şimşek and Ayça Özoğul.

I would also like to thank group members.

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ABREVIATIONS

BSA	Bovine serum albumin
FTIR	Fourier transform infrared spectrometry
GOD	Glucose oxidase
HOMO	Highest occupied molecular orbital
LUMO	Lowest unoccupied molecular orbital
MBTH	3-methyl-2-benzothiozolinone
Pan	Polyaniline
POD	Peroxidase
PPO	Polyphenol oxidase
PPy	Polypyrrole
P(PStPy-co-Py)	Conducting copolymer of pyrrole functionalized polystyrene with pyrrole
Py	Pyrrole
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy

CHAPTER I

INTRODUCTION

1.1 Historical Background of Conducting Polymers

Polymers have good mechanical and attractive processing properties due to the long chain molecules that they are composed of. They are used as insulators in electrical and electronic applications. By combining the properties such as mechanical strength, stability, flexibility and ease of processing with electrical conductivity, a new research area was opened in polymer science called as conducting polymers. This new area gained the interests of both scientists and industry in the last twenty years. There are some methods in order to obtain conducting polymers such as incorporation of a transition metal atom into polymer backbone, incorporation of conductive fillers and electrochemical oxidation of resonance stabilized aromatic molecules. The latter method is the one that has been used widely. For a polymer to be conductive upon oxidation and reduction it must be conjugated. Conjugated organic polymers can be classified in two groups, polyenes and polyaromatics. In 1916 polypyrrole was first synthesized chemically [1]. In 1968 Dall'Ollio [2] described electrochemical synthesis of polypyrrole. By oxidation of pyrrole at a platinum electrode in the

presence of supporting electrolyte produces a free standing electronically conductive film [3,4]. After electropolymerization of pyrrole, many other aromatic monomers were polymerized electrochemically. These include aniline [5], indole [6], thiophene [6,7], furan[6], azulene [6,8], phenol [9,10], and carbazole [11,12]. Moreover, substituted derivatives of these monomers were also used.

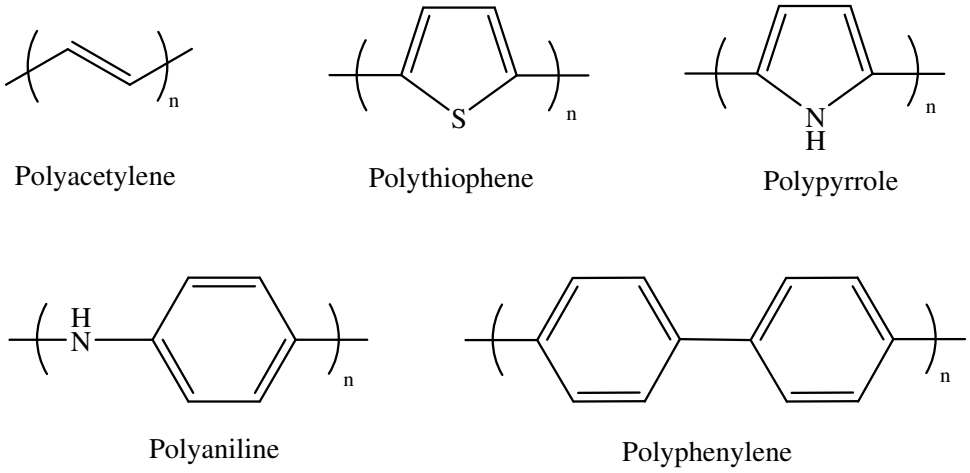


Figure1.1 Some conducting polymers

Mechanical properties of conducting polymers are poor. As compared with plastics, conducting polymers have no melt processability, they are brittle and they have low elongation at breaking point. Synthesizing an organic polymer that possesses the electrical, electronic, magnetic, and optical properties of a metal while retaining the mechanical properties, processibility [13] became the target of scientists. Thus, blends, composites and copolymers were made. Lately soluble conducting polymers were

synthesized via the modification of structure of heteroaromatic compounds [14-16].

1.2 Conductivity

Materials are classified as being insulator, semiconductor and conductor with respect to their electrical conductivity at room temperature. Metals such as copper and aluminum are the most conductive materials.

The conduction mechanism can be explained by using the band gap model as shown in figure 1.2. E_g, energy gap, denotes the energy spacing between the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO). The value of this parameter gives information whether the corresponding material is an insulator, semiconductor or a conductor.

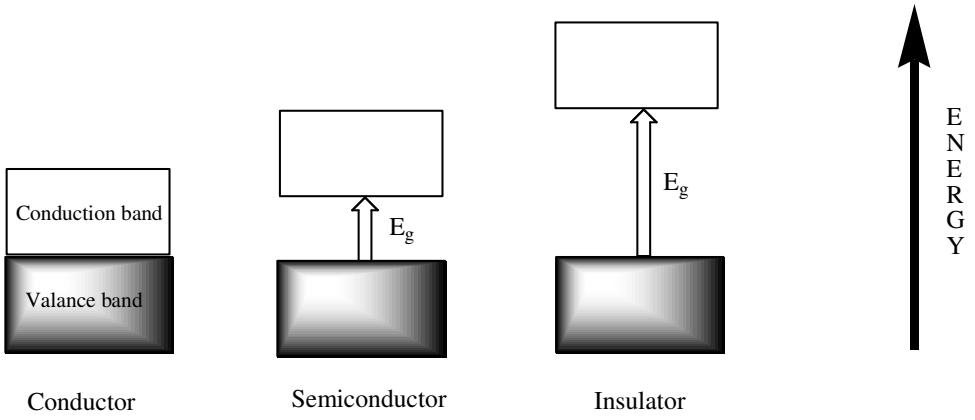


Figure 1.2 Band gaps in conductors, semiconductors and insulators

If an electron promotes from the valence band to the conduction band, conduction occurs. In conductors (metals), the spacing between valence and conduction bands are zero since the highest energy level of the valence electron and the lowest energy level of the conduction band are of similar energy, hence the motion of electrons between these bands becomes very easy. In semiconductors, E_g is narrow whereas in insulators, it is too wide. Thus, thermal excitation of electrons from valence band to conduction band does not occur. Increasing the temperature of semiconductors promotes excitation of electrons and enhances the conductivity. When an electron is excited from the valence band to the conduction band, it leaves vacancy (hole) in valence band. Electrons carrying negative charge named as n-type carriers and holes carrying positive charge named as p-type carriers.

1.2.1 Conduction Mechanism in Conducting Polymers

Conduction occurs by the motion of charge carriers through a medium with the influence of electric field. Electrons, holes and polarons are the charge carriers whose motions produce conductivity.

There are various theories [17] suggested for the conduction mechanism in conducting polymers. All carbon atoms in a conjugated polymer possess four electrons in their outer shell. One of them makes a bond with hydrogen, two of them participate in σ bond formation and the last electron occupies a p_z orbital which forms the π double bond. Delocalization of these π bonds along the chain of the polymer creates a one-dimensional band which is half-filled since each orbital has one electron. Thus, this makes the material to have metallic character along the chain.

Conjugation or bond alternation among the conducting polymers are responsible for the conductivity according to another theory [17,18]. For

instance, in polyacetylene the lengths of the bonds are slightly different from each other (called as Peierls distortion) (Fig. 1.3). This alternating difference creates a band gap between the HOMO (fully occupied π-band) and LUMO (empty π^*-band) (Fig. 1.4). The resulting material would be a semiconductor as in the case of polyacetylene.

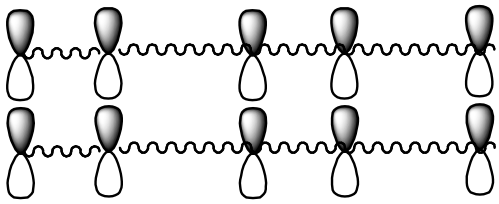


Figure 1.3 Peierls distortion

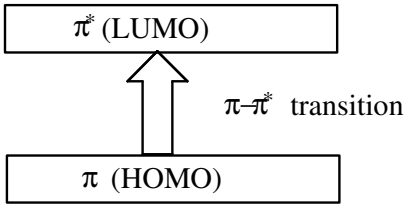


Figure 1.4 HOMO-LUMO transitions

Conduction occurs via the motion of the charge carriers in organic polymeric materials. The conductivity is expressed as,

$$\sigma = nq\mu$$

where μ is the carrier mobility, q is the charge of carrier and n is the number of carriers or the concentration of them.

In conducting polymers, conduction mechanism occurs by the conformational defects such as solitons, polarons and bipolarons which are produced during the doping process.

1.2.2 Doping Process

Having conjugated structure is not adequate for a polymer to conduct electricity. There must be a defect in the chain of the polymer also. In conjugated polymers the concentration of charge carriers are too low.

Doping is the process which increases the concentrations of charge carriers by oxidation (p-type doping) or reduction (n-type doping) of the conjugated polymers. Electron acceptors are used in the oxidation of conjugated polymers. By oxidation, an electron is removed from the conjugated polymer creating a hole or a cation on the chains of the polymer. In the reduction of conjugated polymer, an electron is added to the system which creates an anion. When the coulomb binding energy between the hole and the counterion (anion) is overcomed by the hole, it will travel through the chain and conduction occurs.

A conducting polymer can be doped either electrochemically or by exposure of polymer to the solutions or vapors of the dopant.

The concept of doping is a unique, central, underlying, and unifying theme which distinguishes conducting polymers from all other types of polymers [19,20]. The organic polymer whose conductivity is too low (between 10^{-10}

– 10^{-5} S/cm) before the doping process may reach the conducting regime (1 – 10^{4} S/cm). Figure 1.5 shows the conductivities of some materials;

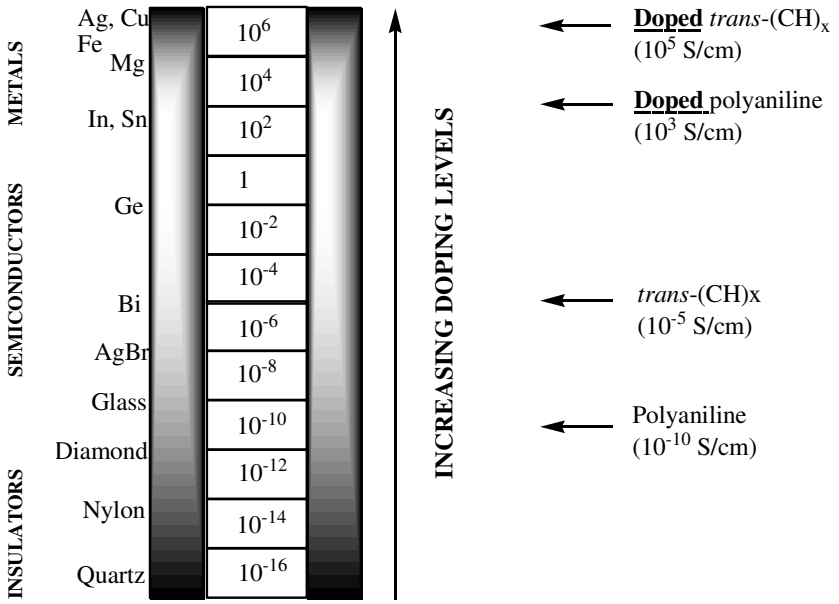


Figure 1.5 Conductivities of some materials

1.3 Applications of Conducting Polymers

Doped conjugated polymers are used in a variety of applications not only due to their conduction properties but also their excellent mechanical properties that they possess. They are used in the preparation of light emitting diodes [21], rechargeable batteries, electromagnetic shielding (EMI) applications, sensors and membranes.

In rechargeable batteries conducting polymers can be used as electrodes as a result of the redox reactions that occur in these polymers [17,22].

Electromagnetic shielding (EMI) is another application of conducting polymers. Polythiophene blends [23,24], doped polyaniline (Pan) and blends [25,26] are used in EMI applications.

Sensor technology is another area where conducting polymers are utilized. Methanol [27,28], benzene [29], and water [30] sensors using polypyrrole (PPy) and its derivatives were already mentioned. Electrically conducting polymers have also been used as matrices in the immobilization of enzymes [31,32].

Electrochromic displays [33] and Schottky barrier diodes [34] are yet other application areas of conducting polymers.

1.4 Electrochemical Polymerization

Via electrochemical oxidation, corresponding polymer is obtained as a free standing film on the electrode surface. Upon applying an appropriate potential, oxidation of monomer is achieved forming a radical cation. Two radical cations will couple and by elimination of two protons, rearomatized dimer is formed in the propagation step. Since the oxidation potential of the dimer is lower than that of the corresponding monomer, the polymerization continues by oxidative coupling of the monomer units to the growing chain. Polymer is formed on the surface of the electrode as the molecular weight increases.

The morphology of the resulting polymer depends on the conditions of the electrochemical polymerization. Type of the solvent, electrode material and the counter ion affect the morphology.

Solvents with high dielectric constants must be used in order to allow ionic conductivity. Nucleophilic solvents must not be used, since they inhibit the film formation by giving side reactions with radical cations. Lastly, solvent must be stable over a broad potential range.

Inert electrode materials such as gold or platinum must be used in order to prevent oxidation of the electrode.

Supporting electrolyte must be chosen considering its solubility, dissociation and nucleophilic characters.

Polypyrrole is the widely used conducting polymer. The main reasons behind are its excellent thermal stability in air, environmental stability and good electrical conductivity [35].

The electropolymerization of polypyrrole is as follows:

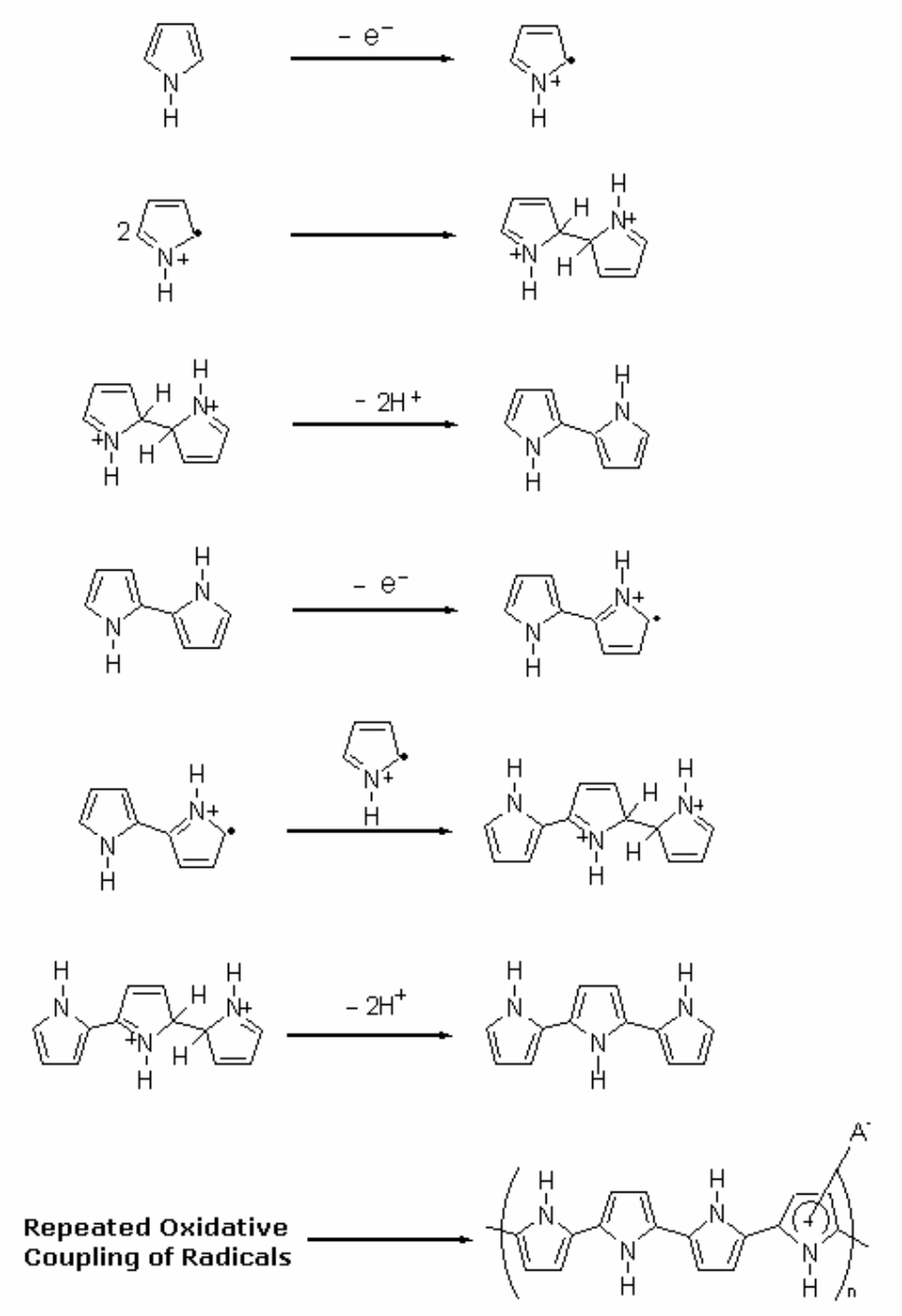


Figure 1.6 Electropolymerization of pyrrole

1.4.1 Advantages of Electrochemical Polymerization

By the electrochemical polymerization technique, film thickness can easily be controlled by changing potential or current with time. The method is simple and reproductive. Molecular weight control is possible. Polymerization occurs at room temperature and it is also possible to obtain copolymers**.**

1.5 Enzymes

Catalysts are the substances that increase the reaction rate by decreasing the activation energy of reactions. Enzymes are the biological catalysts that speed up the reactions in living organisms. Operating at very mild conditions and the specifity they show against substrates (compounds which are converted to products by the action of enzymes) are the major differences compare to inorganic catalysts.

The catalytic actions of enzymes were first recognized by Jon Jakob Berzelius, Swedish chemist. Later, in 1926, urease enzyme is obtained from jack bean by James B. Sumner who was awarded with Nobel prize in 1947 as a result of his work.

All enzymes are proteins. They are composed of chains of aminoacids that are linked to each other by peptide bonds (figure 1.7). Hence, they are high molecular weight substances with molecular weights varying between 10,000 and 2,000,000.

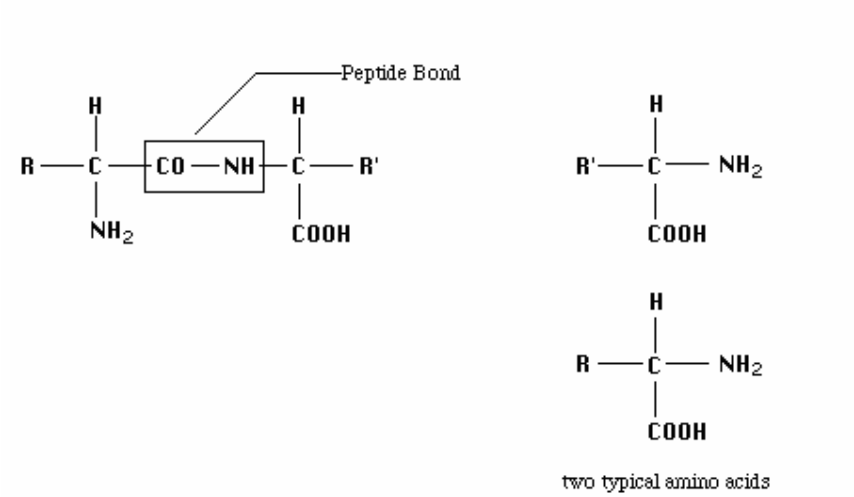


Figure 1.7 Structure of a peptide bond

Enzymes are composed of two parts namely; apoenzyme and coenzyme. Apoenzyme is the protein portion of the enzyme whereas coenzyme is the nonprotein fraction. If the nonprotein portion of the enzyme is an organic substance, it is called as coenzyme; if it is a metal ion, it is named as cofactor. In order to be an enzyme completely active, these two parts must exist. This total active enzyme system is called as the haloenzyme or whole enzyme.

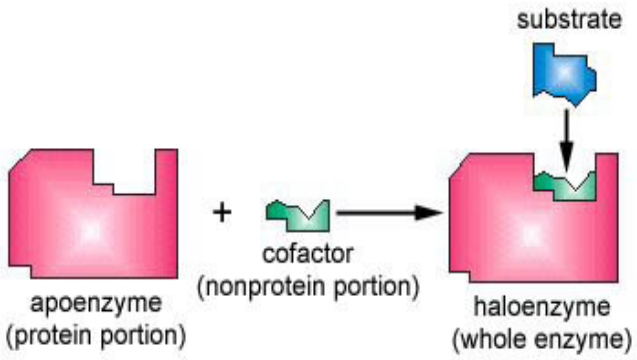


Figure1.8 Active enzyme system

Being specific to its substrate, each enzyme can act upon one particular component or sometimes on specific bonds in the compounds. The specifity of the enzyme was explained with *lock-and-key analogy* of Fischer [36] as shown in figure 1.9.

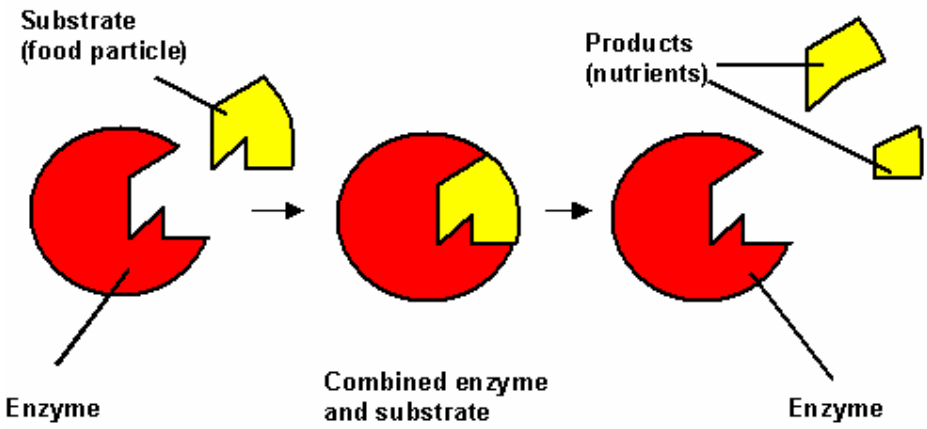


Figure1.9 Enzyme specifity

According to this analogy, enzyme can be considered as a lock and the substrate as a key. If the key is correctly fit the lock, the lock will be opened. In other words, a substrate which fits the active site of an enzyme will be converted into products.

1.5.1 Enzyme Classification

Various enzymes were discovered after the purification of urease by Berzelius. Moreover, same enzyme that is obtained from different sources may have different catalytic and physical properties. For instance alcohol dehydrogenase which is obtained from yeast, containing four subunits and four Zn^{2+} atoms per molecule, has a molecular weight of 150,000. Same enzyme obtained from horse liver is 70,000 in molecular weight, contains two subunits and four Zn^{2+} atoms [37]. In order to identify each enzyme easily and correctly, they are classified.

Enzymes were classified in six main classes by the Enzyme Commission (EC). These six main classes are:

1. Oxidoreductases
2. Transferases
3. Hydrolases
4. Lyases
5. Isomerases
6. Ligases

The enzymes which belong to the oxireductase group catalyze the oxidation reduction reactions. Enzyme oxidizes or reduces its substrate by a transfer of hydrogen or oxygen. Transferases catalyze the transfer of functional groups from substrate to acceptor molecules. Hydrolases are responsible for

splitting of a molecule into two or more smaller molecules (hydrolysis reaction) with the help of water. Lyases are the enzymes which remove groups from their substrates means other than hydrolysis. Isomerases are the enzymes that facilitate the interconversion of isomers. Ligases catalyze the combination reaction of two molecules with the expense of ATP.

The Enzyme Commission gave a code (EC number) which contains four numbers. The first number determines the main class the enzyme belongs. The second and the third numbers indicate the type of reaction that is catalyzed and the fourth number gives an identity to the enzyme by defining the substrate of the enzyme.

1.5.2 Single Substrate Enzyme Kinetics

By measuring the effect of an enzyme on the rate of chemical reaction, catalytic activity of an enzyme can be determined. A. J. Brown found that the rate of hydrolysis of sucrose by invertase increased with increasing substrate concentration up to a point and then leveled off. He proposed that before the catalytic activity of the enzyme, enzyme and substrate must come together in order to form a complex. Michaelis and Menten also studied this catalytic hydrolysis reaction in 1913 [38].

The mechanism below is the representation of single substrate enzyme catalyzed reaction:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

where E represents free enzyme, S is the substrate, ES stands for enzyme-substrate complex and P denotes the product. By the addition of substrate, enzyme and substrate combine to form ES complex. The concentration of this complex will increase until all free enzyme molecules are consumed. Later on, further addition of substrate increases the concentration of ES and the reaction rate in negligible amounts [39].

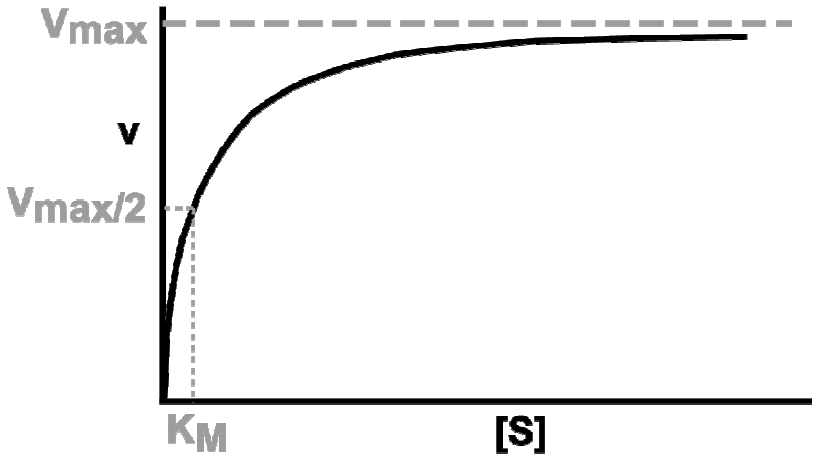


Figure 1.10 Change in reaction rate via addition of substrate

The rate of formation of ES is:

$$v_1 = k_1 [E] [S]$$

The rate of breakdown of ES at any time to form either products or reactants can be expressed as:

$$v_2 = k_{-1}[ES] + k_2[ES]$$

The rate of formation of ES complex is equal to the its breakdown rate (steady state assumption). Then,

$$v_1 = v_2$$

$$k_1 [E] [S] = k_{-1}[ES] + k_2[ES]$$

$$k_1 [E] [S] = [ES] (k_{-1} + k_2)$$

Further rearrangements yield:

$$\frac{[E] [S]}{[ES]} = \frac{k_{-1} + k_2}{k_1} = K_m$$

where K_m denotes Michaelis constant. If $[E_0]$ is the initial free enzyme concentration, the concentration of the free enzyme at a time will be:

$$[E] = [E_0] - [ES]$$

Substituting this into the previous equation of K_m yields:

$$K_m = \frac{([E_0] - [ES])[S]}{[ES]}$$

Rearranging the equation yields:

$$[ES] = \frac{[E_0]\,[S]}{[S] + Km}$$

The overall rate of reaction is:

$$v_0 = k_2\,[ES]$$

and v_0 / k_2 is equal to [ES]. Substitution of this into the previous equation yields:

$$v_0 = \frac{k_2\,[E_0]\,[S]}{[S] + Km}$$

Maximum reaction rate is obtained when all free enzymes is used to form enzyme substrate complex. This is achieved in the case of high substrate concentrations. When this condition is obtained, the maximum reaction rate (v_{max}) will be:

$$v_{max} = k_2\,[E_0]$$

Substituting v_{max} into previous equation will produce the Michaelis-Menten equation:

$$v_0 = \frac{v_{max}\,[S]}{[S] + K_m}$$

K_m is *the Michaelis constant* which defines the affinity of enzyme to its substrate. There exists an inverse relationship such as high K_m means low

affinity of enzyme to its substrate and vice versa.V_{max} is the maximum reaction rate that is attained when all enzyme molecules are in the form of enzyme-substrate complex.

As seen in figure1.10, at low substrate concentrations reaction rate is directly proportional to substrate concentration. This means that the reaction is first order with respect to substrate concentration. On the other hand, when maximum reaction rate is attained the rate becomes independent to substrate concentration. Thus, the reaction is said to be zero order with respect to substrate concentration. At this condition the enzyme is saturated with its substrate.

1.5.2.1 Lineweaver-Burk Equation

The graph of Michealis-Menten equation is not quite suitable for the determination of V_{max} and K_m. Since this graph is a curve, it cannot be extrapolated upwards from substrate concentrations which are away from saturating. Thus, a linear graph is needed for the determination of V_{max} and K_m.

Lineweaver and Burk [40] obtain a linear graph by taking the reciprocal of Michaelis-Menten relation:

$$\frac{1}{v_0} = \frac{[S] + Km}{v_{max}\,[S]} = \frac{[S]}{v_{max}\,[S]} + \frac{K_m}{v_{max}\,[S]}$$

V_{max} and K_m parameters can be determined easily by plotting $1/v_0$ versus $1/[S]$ as given in figure 1.11. The slope of this graph is equal to K_m/V_{max},

intercept on abscissa gives $-1/K_m$ and intercept on ordinate is equal to $1/v_{max}$.

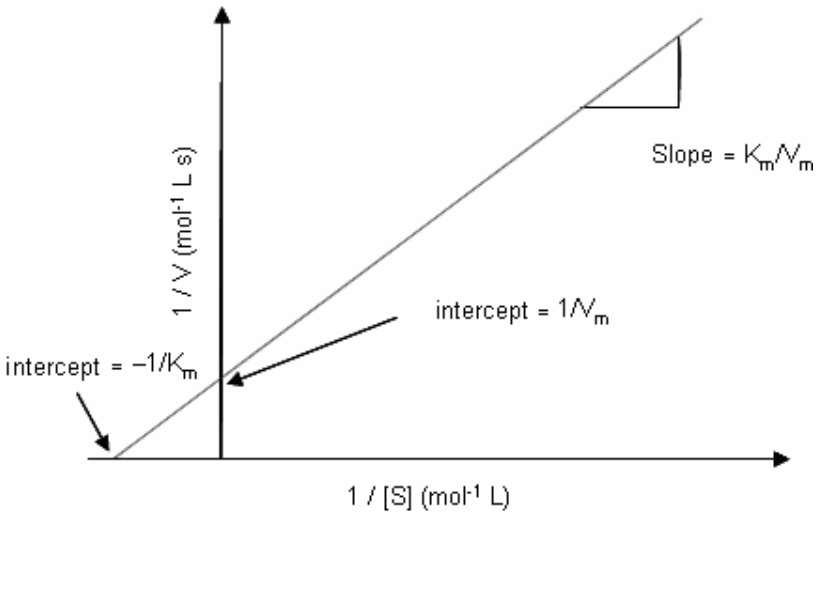


Figure1.11 Lineweaver-Burk plot

1.6 Enzyme Immobilization

Since enzymes are proteins, the factors that denature proteins can also reduce the activity of enzymes or inactivate them. These factors are temperature, pH and inhibitors. Free enzymes are easily affected from these factors and mostly irreversible denaturation of enzymes occurs. In order to reduce or eliminate the effects of these factors enzymes are immobilized.

Immobilization of an enzyme is achieved by restricting its mobility via chemical or physical methods. While the enzyme that is immobilized unable to move its catalytic activity is still present.

1.6.1 Advantages of Immobilization

Immobilization of enzymes provides several advantages over free enzymes. Immobilization provides heterogeneous catalysis instead of homogeneous catalysis where enzyme and substrate coexists in a homogeneous solution. Removing the enzyme from a reaction where the enzyme is used in its free form is not easy. On the other hand, immobilization provides easy separation of the catalyst from the reaction mixture.

Moreover, easy separation of the enzyme from the products prevents the contamination of product by enzyme and reduces the cost by eliminating the necessary purification steps.

Immobilization also provides repetitive use of enzymes. Generally, enzymes are expensive substances and using free enzymes for reactions increase the cost. Free enzymes can easily be denatured irreversibly due to temperature, pH and inhibitor effects. In the case of immobilization, the environment that confines the enzyme will protect the enzyme against such effects and enzyme will be active for reuse.

To be able to remove the enzyme from the reaction mixture whenever necessary is yet another advantage of enzyme immobilization. Immobilized enzymes also have long half-lives and predictable decay rates.

1.6.2 Immobilization Methods

Enzymes can be immobilized mainly by two methods; via binding or physical retention [41,42]. In the binding type of immobilization, a bond forms between enzyme and the matrice. On the other hand, there is no such a bond formation in immobilization by physical retention.

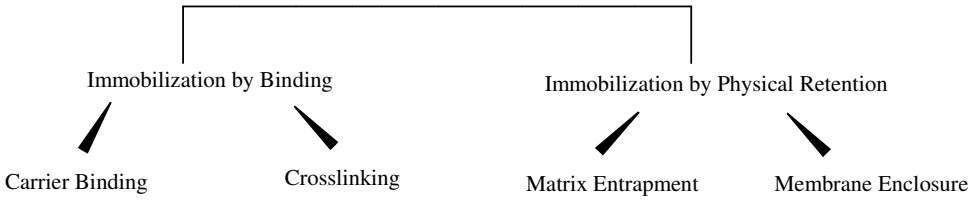


Figure 1.12 Immobilization methods

1.6.2.1 Binding to Carriers

In this type of immobilization, enzyme binds to a water insoluble carrier [43]. The carriers used in this method are dextran [44], polyacrylamide gel [45] and cellulose [46]. Adsorption, ionic binding and covalent binding are the subclasses for this method.

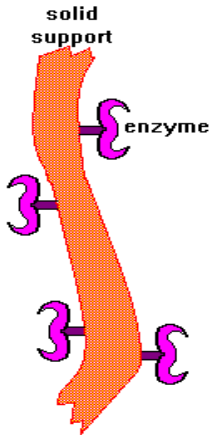


Figure 1.13 Carrier binding

Adsorption of an enzyme onto a water insoluble carrier by physical means is a very simple method. Hydrophobic interactions and ionic bonds are some other factors.

In this immobilization method, there is little or no destruction on the active site of the enzyme, hence the activity is little affected [42]. Nelson and Griffin studied the immobilization of invertase on active charcoal in 1916 [47].

The main disadvantage of this method is the desorption of the enzyme due to the weak adsorptive forces.

Ionic binding is achieved by the electrostatic attraction of oppositely charged groups of the carrier and the enzyme. The carriers usually used are synthetic polymers and polysaccharides which have ion-exchange centers. This immobilization method was first used by Mitz [48] in 1956. He immobilized catalase in cellulose.

Covalent binding is an immobilization technique based on the formation of covalent bonds between the support matrix and the enzyme [49,50]. The binding reaction must be performed under conditions that do not cause loss of enzymatic activity and the active site of the enzyme must not be affected by the reagents used. Conformational structure and the active site of the enzyme may be affected by less suitable reaction conditions. On the other hand, enzyme leakage does not occur because of the strong binding between the enzyme and carrier.

1.6.2.2 Crosslinking

Crosslinking is a method of immobilization achieved by intermolecular crosslinking of the protein, either to functional groups on an insoluble support matrix or to other protein molecules. This method is an expensive method of

immobilization since some protein material will be acting as a support when crosslinking the enzyme to itself. The main advantage of this method is the prevention of leakage or desorption of the enzyme since the enzyme is covalently linked to the support matrix or to itself.

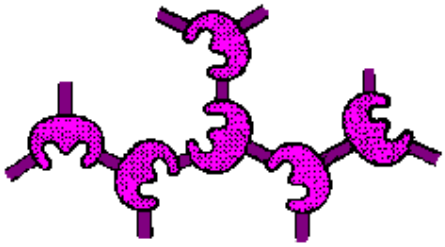


Figure 1.14 Crosslinking

1.6.2.3 Entrapment

This method is the physical retention type of immobilization. Thus, there is no change in the active site of the enzyme and enzyme activity is not lost. The enzyme being immobilized is localized within the lattice of a polymer matrix [51-54] or membrane. In this method substrate penetration is allowed whereas enzyme is not. Since most of the polymerization reactions require severe reaction conditions, careful selection of the suitable conditions for enzyme immobilization is required in order to prevent activity loss.

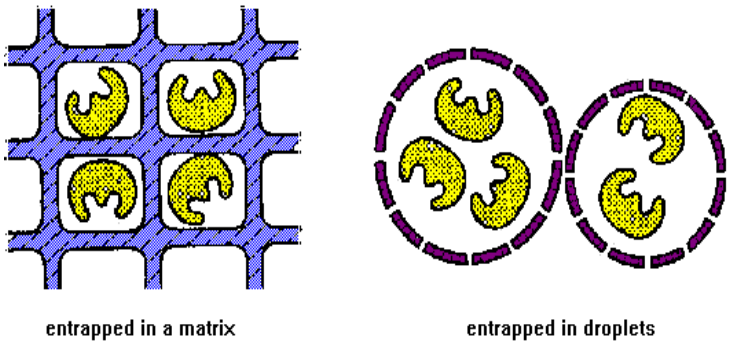


Figure 1.15 Entrapment

Gel entrapment and fiber entrapment are examples for matrix entrapment. In the former, enzymes are immobilized in a gel which is a crosslinked water insoluble polymer. Enzyme molecules are physically entrapped within the highly crosslinked polymer matrix and cannot permeate out of the gel, whereas favorable sized substrate and product molecules can transfer across to ensure a continuous transformation.

Morever, enzymes can be entrapped in fibers. High surface area for enzyme binding is one of the advantages of fiber entrapment over gel entrapment. Since fibers are generally resistant to weak acids and alkalis some organic solvents with high ionic strengths can be used.

1.6.2.4 Membrane Enclosure

In this immobilization method enzyme is retained within a defined space by a semipermeable membrane. This membrane is permeable to substrate and product, but impermeable to the enzyme. There is no chemical change on the enzyme hence, enzyme molecules are free in solution. The contact area of the

enzyme with its substrate is high in this case compared to classical entrapment method.

1.7 Glucose Oxidase Immobilization

Glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC.1.3.4) was discovered by Müller [55]. It catalyzes oxidation of β-D-glucose to D-glucono-1,5-lactone (which spontaneously hydrolyses non-enzymatically to gluconic acid) in aerobic conditions. Figure 1.17 shows the catalysis reaction of the enzyme.

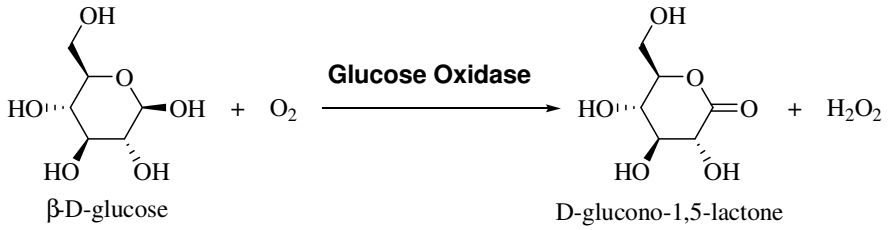


Figure 1.16 Glucose oxidase catalysis reaction

Aspergillus niger and *Penicillium glaucum* are the main sources of the enzyme. The other fungi which contain glucose oxidase enzyme are *Aspergillus oryzae, Penicillium amagaskiense,* and *Penicillum notatum.* Higher plants other than fungi and animals do not possess this enzyme. The enzyme has a molecular weight of 150,000 Dalton and contains 2 mol of flavin adenine dinucleotide (FAD) per mole of enzyme. FAD is the coenzyme of glucose oxidase enzyme (GOD). Isoelectric point of the enzyme is pH 4.2.

Glucose oxidase is a highly specific enzyme. L-Glucose, D-Mannose and β-D-glucose are some of the substrates of this enzyme. The activity of the enzyme towards β-D-glucose is higher than the others. Molecular oxygen is required for complete catalytic reaction. Under anaerobic conditions, the enzyme does not catalyze the complete reaction.

The enzyme finds uses in the removal of either glucose or oxygen from foodstuffs in order to improve their storage capability.

In literature, various types of analysis were made for glucose detection. Enzymatic chemiluminescence assay of glucose by means of a hybrid flow-injection/sequential injection method with soluble enzyme was reported [56].

Some other methods were also reported fluorescence-based glucose sensors [57] and flow injection analysis [58].

Detection of glucose via glucose biosensors offers some advantages such as high sensitivity, rapid detection, high specifity besides saving time, having simple procedure, reducing the cost of analysis. In literature, several applications were done in order to prepare biosensors or enzyme electrodes. These involve immobilization of glucose oxidase in different matrices via different immobilization methods including entrapment within a polymer matrix, encapsulation [59], crosslinking, covalent binding [60-62], gel entrapment [63,64], membrane enclosure [65] and fiber entrapment [66].

Immobilization of glucose oxidase into conducting polymer matrices during electropolymerization has been studied due to the advantages of procedure. It is a simple and low-cost process to produce a biosensor. Moreover, this technique is rapid one-step procedure where film thickness can also be controlled easily. This control provides to obtain reproducible biosensors. Polypyrrole [62,67] and

their derivatives [53,54] are the mostly used host matrices for the immobilization of the enzymes.

1.8 Polyphenol Oxidase Immobilization

Polyphenol oxidase (PPO, also known as tyrosinase; EC 1.14.18.1) is the enzyme that was discovered by Schoenbein in 1856 in mushrooms. Bananas, apples, potatoes, peaches, avocados, tea leaves and grapes are the other sources of this enzyme. It is also found in some higher animals including insects. The enzyme protects the plant from the attack of the microorganisms and insects since it forms an impervious scab of melanin pigment when the plant is wounded. The enzyme is responsible from the pigmentation of skin, hair and eye in humans.

Polyphenol oxidase is a copper containing enzyme with a molecular weight of 130,000 Daltons. This enzyme catalyzes two different types of reactions (figure 1.18). Both reactions involve phenolic substances.

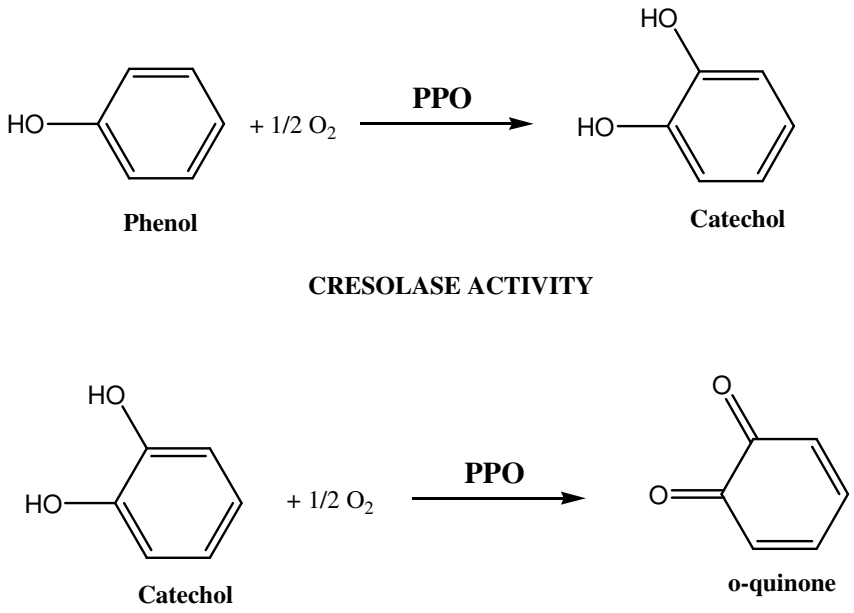


Figure 1.17 Polyphenol oxidase catalysis reactions

The cresolase activity of PPO enzyme is the orthohydroxylation of monophenols. Moreover, this enzyme catalyzes the oxidation of orthodiphenols to orthoquinones which is referred as the catecholase activity.

Melanin is the final product of the tyrosine oxidation by polyphenol oxidase enzyme [68]. Firstly, PPO enzyme catalyzes the hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA) and DOPA is oxidized to dopaquinone. This product is unstable and polymerizes into melanin. Since DOPA is the precursor of dopamine which is an important neural message transmitter, the cresolase activity of the enzyme is important for human health. The deficiency of dopamine which is found in the substantia nigra of the brain results in Parkinson's disease [69].

PPO enzyme has wide variety of applications. One of the applications is the determination of phenolics in waste waters. Phenol is a toxic substance which possesses high oxygen demand [70]. Due to this oxygen demand, they can easily deplete the oxygen present in water. Thus, discharge of phenols into water may affect the ecosystem of water. Besides the detection of phenols [71] in waste waters, water purification can be achieved by using this enzyme [72,73].

Polyphenol oxidase enzyme is also used to determine the quality of vegetable and fruit storage. Bruises, wounds and other mechanical damages result in browning in fruits and vegetables due to O_2 penetration.Browning reaction, in general, leads to undesirable results with respect to texture, sweetness, and overall flavor [74].

In literature, determination of phenolics were done by liquid chromatography [75,76], HPLC and/or spectrometry [77,78] and biosensors [79-82].

PPO enzyme is an instable enzyme and inactivated rapidly. Enzyme immobilization provides good long-term stability and operational stability [53].

Wine is one of the sources of phenolic compounds. Especially, they present in large amounts in red wine. Phenolics contribute to red wine sensory characteristics such as color, flavor and astringency [83]. They also have strong antioxidant and antimicrobial properties due to their ability acting as free-radical scavenging [84-87]. These phenolics are found in the seeds and skin of the grapes.

Chemical composition of a wine can change by soil type, atmospheric conditions, wine making process and climatic conditions. Thus, the amounts of phenolics can change from one brand of wine to another.

1.9 Aim of the Study

i. To achieve the electrochemical synthesis of conducting copolymer of pyrrole functionalized polystyrene with pyrrole P(PStPy-co-Py) and their characterization.

ii. To construct enzyme electrodes by immobilization of GOD and PPO enzymes in polypyrrole (PPy) and P(PStPy-co-Py) conducting polymer matrices.

iii. To characterize enzyme electrodes by determining kinetic parameters, pH, temperature and storage profiles.

iv. To use GOD and PPO enzyme electrodes for the analysis of glucose in orange juices and polyphenols in wines respectively.

CHAPTER II

EXPERIMENTAL

2.1 Chemicals

Glucose oxidase, Type II-S, (GOD, EC: 1.1.3.4), 47,200 units/g solid, peroxidase, Type II, (POD, EC: 1.11.1.7), o-dianisidine, polyphenol oxidase (Tyrosinase, PPO, EC: 1.14.18.1), 1,530 units/mg solid were purchased from Sigma. The substrates, glucose and catechol were also obtained from Sigma.

Pyrrole was obtained from Merck and purified before use and stored at 4 °C. Dichloromethane was purchased from Merck and used as received. The supporting electrolyte, sodium dodecyl sulfate (Sigma) was used as received. Sulfuric acid was obtained from Merck.

Acetate buffer (pH=5.1) was prepared from sodium acetate (Sigma) and acetic acid. Citrate buffer (pH=6.5) was prepared from citric acid and sodium citrate, both of which were purchased from Sigma. 3-methyl-2-benzothiazoline hydrazone (MBTH) was obtained from Sigma.

Folin & Ciocalteu's Phenol Reagent and Bovine Serum Albumin (BSA) were also obtained from Sigma.

Sodium potassium tartrate (Baker & Adamson), pancreac sodium chloride (Montplet & Estaban SL), sodium carbonate (Delta), copper(II) penta hydrate (Merck) were used in the preparation of Lowry's reagent. Sodium hydroxide was obtained from Sigma.

2.1.1 Preparation of Lowry Reagent

Lowry's protein determination method [88] was used for the specific activity measurement of glucose oxidase and polyphenol oxidase enzymes. Lowry's reagent was prepared using three different solutions, A, B and C. Solution A was 2% (w/v) sodium carbonate solution prepared in 100mM sodium hydroxide solution with a total of 1 liter. Solution B was 2 % (w/v) sodium potassium tartrate prepared in 10 ml deionized water. 1% (w/v) cupric sulfate solution was prepared in 10 ml deionized water and coded as solution C. Lowry Reagent was prepared by pipetting (in mililiters) the following reagents:

Solution A	100.0 ml
Solution B	1.0 ml
Solution C	1.0 ml

2.2 Apparatus

2.2.1 Electrolysis Cell

The synthesis of conducting copolymer of pyrrole functionalized polystyrene with pyrrole P(PStPy-co-Py) was performed in a three electrode cell via constant potential electrolysis. Working and counter electrodes were platinum (Pt). A silver wire was used as the reference electrode.

2.2.2 Potentiosat

Wenking POS-73 model potentiosat was used in the synthesis of P(PStPy-co-Py).

A potentiostat is a device which compensates for the IR drop in the solution and keeps the voltage difference between reference electrode and working electrode at a constant initially determined value despite the changes in the current passing through the electrolytic cell. It eliminates the IR drop in the electrolysis medium by continuous comparison of working electrode potential to reference electrode potential.

2.2.3 Four Probe Conductivity Measurements

Conductivities of polypyrrole and P(PStPy-co-Py) films were measured by four probe technique. Conductivity of a conducting polymer film can be measured also with a two probe technique but errors can occur in these measurements due to contact resistance. Four equally spaced tips were placed onto a head. These tips make electrical contact with a polymer film which lies on an insulating surface. From the outer two tips, a constant current is applied and voltage drop across the inner two tips is measured (Figure 2.1).

The conductivities of the conducting polymer films were measured by using the following equation:

$$\sigma = \ln 2 / (\pi R t)$$

where σ is the conductivity, R is the resistance of the sample and t is the thickness of the film.

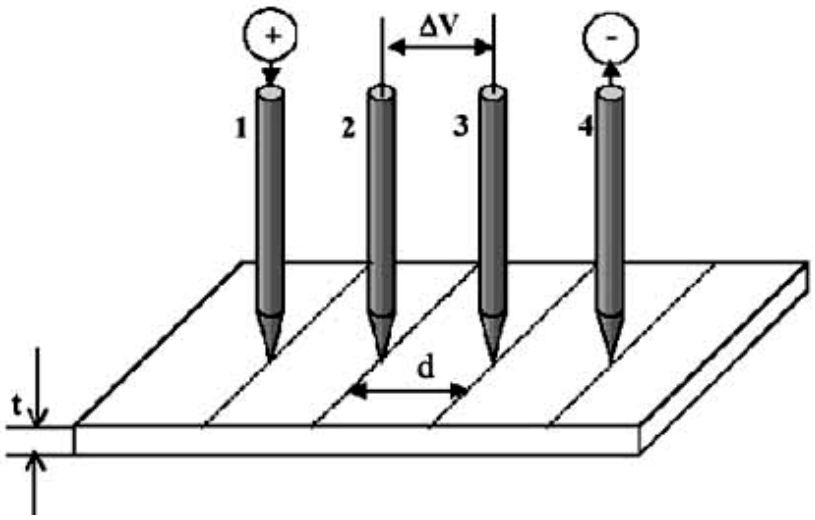


Figure 2.1 Four probe conductivity measurement

2.2.4 Scanning Electron Microscopy (SEM)

Surface morphologies of polymers were inspected by a JEOL model JSM-6400 scanning electron microscope.

2.2.5 UV-Visible Spectrophotometer

Enzyme activities were determined via spectrophotometric methods. A Shimadzu UV-1601 model spectrophotometer was used to measure absorbance of the products formed by the enzymatic reactions.

2.2.6 Fourier Transform Infrared Spectrometry (FTIR)

In this work, FTIR spectra of the polymers were recorded on a Varian 1000 FT-IR spectrometer.

2.3 Experimental Procedures

2.3.1 Synthesis of Conducting Copolymer of Pyrrole with Pyrrole Fuctionalized Polystyrene

Pyrrole functionalized polystyrene was synthesized previously [89]. Number average molecular mass (M_n) of pyrrole functionalized polystyrene calculated from gel permeation chromatography and from H NMR were 2550 and 2500 respectively. Synthesis of conducting copolymer of pyrrole with pyrrole functionalized polystyrene was achieved by constant potential electrolysis in a three electrode cell, a working electrode, a counter electrode and a Ag wire as reference electrode. A platinum electrode (1cm x 1cm) coated with PStPy (0.25% w/v in dichloromethane) was used as the working electrode in the electrolysis. Sodium dodecyl sulfate (SDS) was used as the supporting electrolyte. produced film was left in the solvent of PStPy for a day.

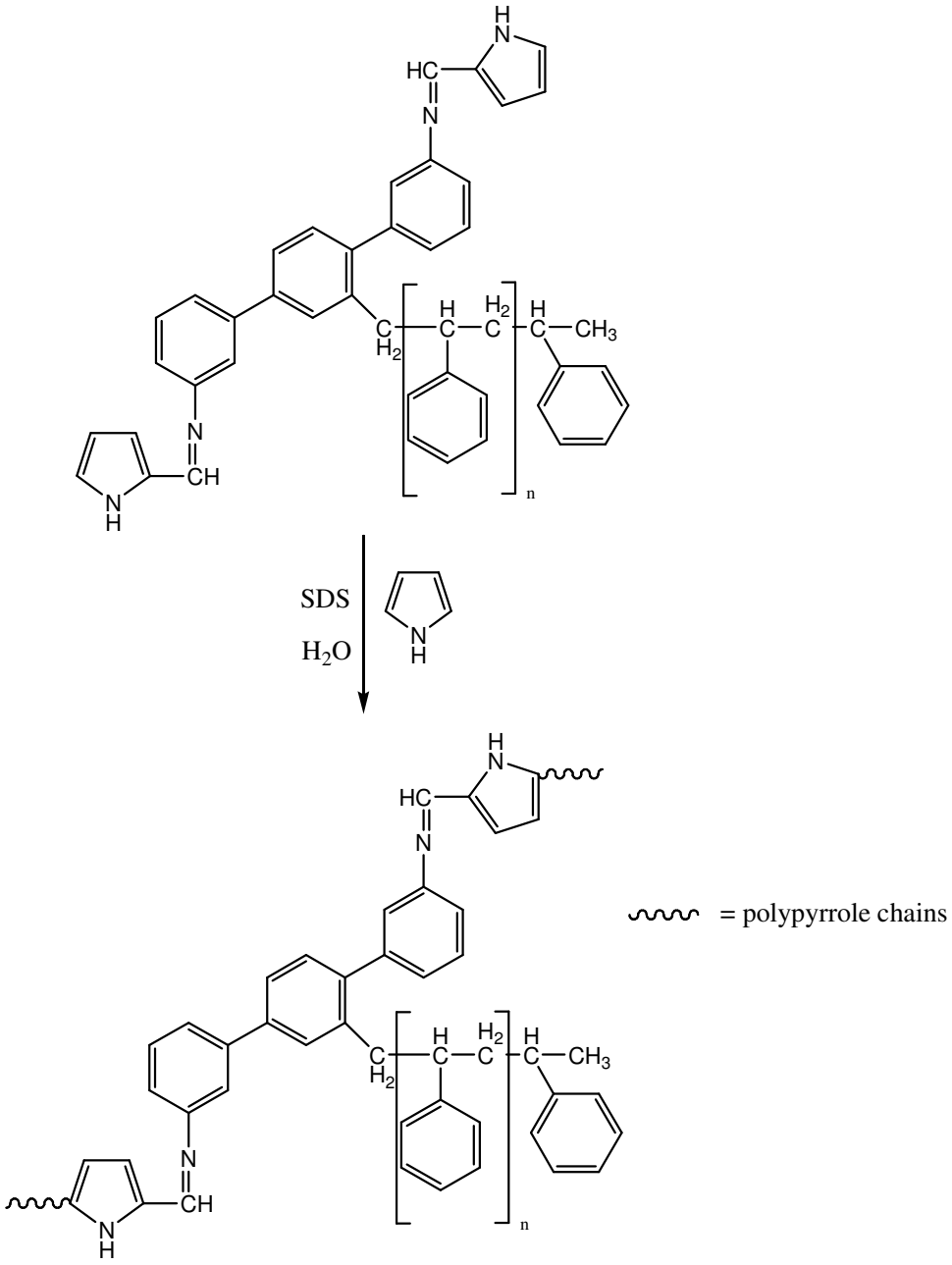


Figure 2.2 Electrochemical synthesis of P(PStPy-co-Py)

2.3.2 Immobilization of Enzymes in PPy and P(PStPy-co-Py) Matrices

Glucose oxidase was immobilized by electropolymerization of pyrrole on either bare or P(PStPy-co-Py) (0.25% w/v in dichloromethane) coated platinum electrodes. The electrolysis solution consists of 2mg/ml GOD (47,200 units/g), 0.6 mg/ml sodiumdodecyl sulfate (SDS) as the supporting electrolyte, 0.144 M pyrrole and 10 ml acetate buffer (pH=5.1). Electrochemical polymerization reactions were performed under constant potential of +1.0 V for 30 minutes at room temperature. After the polymerization, the enzyme electrodes were washed with distilled water and stored in acetate buffer at 4 ^{0}C.

Immobilization of polyphenol oxidase was achieved with the same procedure for glucose oxidase. In PPO immobilization, the electrolysis solution consisted of 0.4 mg/ml PPO (1,530 units/mg), 1mg/ml SDS, 0.072 M pyrrole and 10 ml citrate buffer (pH= 6.5). The rest was the same as in the case of glucose oxidase immobilization.

2.3.3 Determination of Glucose Oxidase Activity

A modified version of Sigma Bulletin was used for the activity determination of native and immobilized glucose oxidase [90]. For the determination of free glucose oxidase activity, different concentrations of glucose solutions (0.1mM-3mM) were prepared in acetate buffer and preincubated in water bath for 10 minutes at 25 ^{0}C. Then, 0.1 ml glucose oxidase enzyme (2 mg/ml) were added and shaken for 1 minute in water bath. Then, 0.5 ml aliquots were drawn and 0.1 ml peroxidase (POD, 60 U/ml) were added to this aliquots to catalyze the reaction of hydrogen peroxide. Later on, 2.4 ml o-dianisidine (0.21mM) were added as the reducing agent. Finally, the reaction was stopped by the addition of 0.5 ml sulfuric acid (2.5M). Absorbances of solutions were measured at 530 nm after mixing.

For the immobilized glucose oxidase activity measurements, glucose solutions at different concentrations (0.5 mM-80 mM) were prepared in acetate buffer. These solutions were placed in water bath at 25 ^{0}C for 10 minutes. Then, enzyme electrode was placed in glucose solution and shaken for specific time intervals. The enzyme electrode was then removed and 0.5 ml of aliquots were drawn and same reagents in same amounts were added as in the case of free glucose oxidase activity measurements.

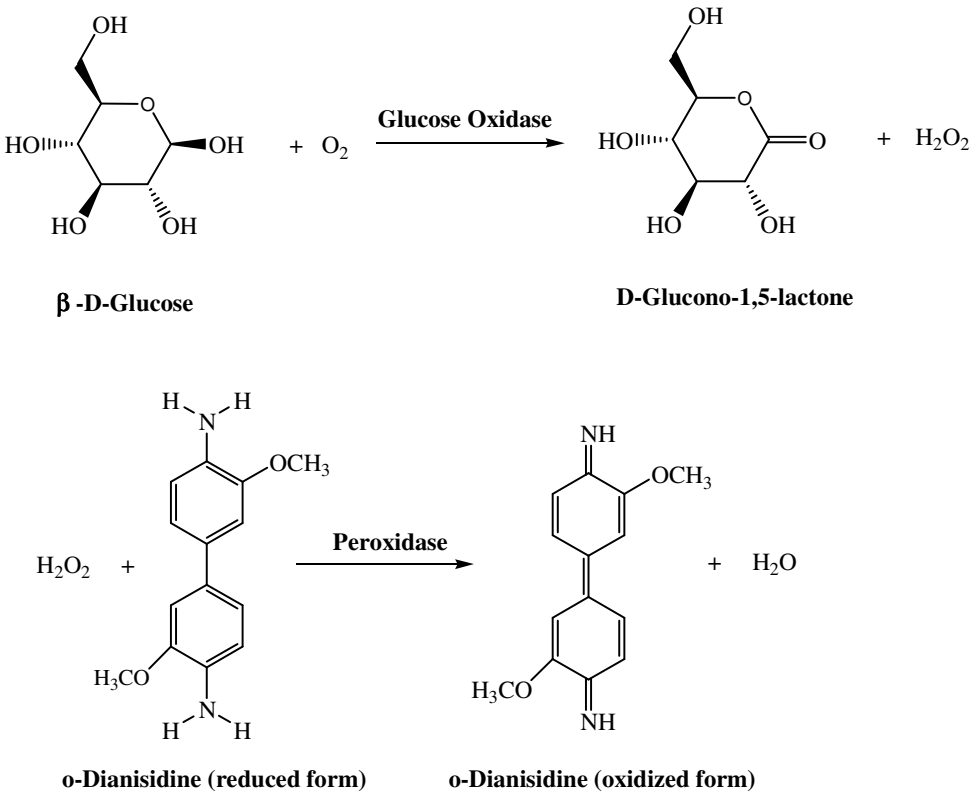


Figure 2.3 Assay reactions of glucose oxidase

Amount of enzyme required to produce 1 µmol of D-gluconic acid and H_2O_2 per minute at pH 5.1 at 25 °C is defined as 1 unit of glucose oxidase activity.

2.3.4 Determination of Polyphenol Oxidase activity

Activities of free and immobilized polyphenol oxidase enzyme were determined by using Besthorn's Hydrazone Method [91]. In this method quinones produced by the enzymatic reaction of polyphenol oxidase interact with 3-methyl-2-benzothiozoline hydrazone (MBTH). As a result red products formed instead of brown colored pigments which is the case in the absence of coloring reagent [92,93].

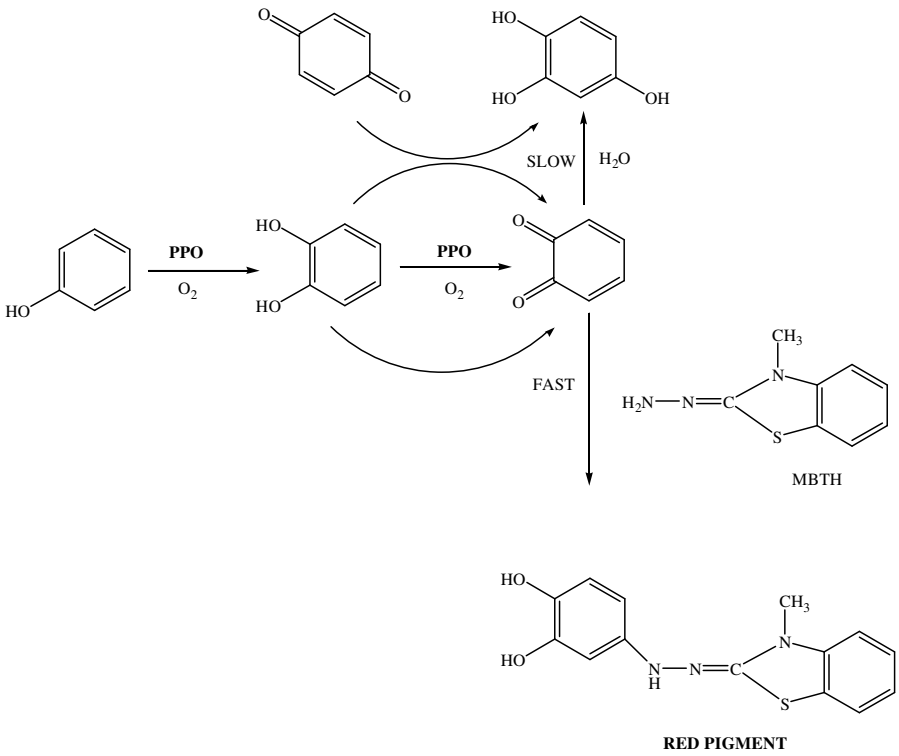


Figure 2.4 Assay reactions of polyphenol oxidase

In order to determine free PPO activity, different concentrations of catechol solutions (0.1 mM- 2.5 mM) were prepared in citrate buffer and 1.5 ml of these solutions were placed test tubes. 0.015 mg PPO/ml enzyme solution were prepared in citrate buffer. 0.5 ml MBTH solutions were added and 1 minute of reaction time was given before the addition of enzyme solution. Then, 0.2 ml enzyme solution were added. After shaken for 1 minute, 0.8 ml of 5% sulfuric acid and 1 ml acetone were added for a total volume of 4 ml. After mixing, absorbances were measured at 495 nm.

Immobilized PPO activities were also performed according to the same assay. Instead of free enzyme, enzyme electrodes were used. Different concentrations

of catechol solutions (0.1 mM- 500 mM) were prepared in citrate buffer and placed into water bath at 25 ^{0}C for 10 minutes for preincubation. 1 ml of 3% MBTH solution prepared in ethanol was added to the test tube containing 3 ml of catechol solution. Then, 1 minute of reaction time was given before inserting the enzyme electrode. After enzyme electrode was immersed in the test tube, it was shaken for 5 minutes and then the enzyme electrode was taken out of the solution. 1ml of 5% sulfuric acid and 1 ml acetone were added for a total volume of 6 ml. After mixing, absorbances were measured at 495 nm.

2.3.5 Determination of Kinetic Parameters

The kinetic parameters (V_{max} and K_m) for glucose oxidase and polyphenol oxidase enzyme were determined from Lineweaver-Burk plots which were drawn by the reaction rate data obtained from the kinetic studies of the reaction performed at varying concentrations of glucose and catechol. Both of these kinetic studies were done at a constant temperature of 25^{0}C and at a constant pH (pH 5.1 for glucose oxidase and pH 6.5 for polyphenol oxidase).

2.3.6 Determination of Temperature Stability

The optimum temperature for immobilized glucose oxidase and polyphenol oxidase were determined by changing the incubation temperature between 10 ^{0}C and 80 ^{0}C. The procedures for activity measurements were the same for glucose oxidase and polyphenol oxidase activity measurements.

2.3.7 Determination of pH Stability

pH stability studies of immobilized enzymes were done at 25 ^{0}C in the pH range of 2.0-12.0 for glucose oxidase and 2.0-11.0 for polyphenol oxidase. Activities were determined as described above for each enzyme.

2.3.8 Operational Stability of Enzyme Electrodes

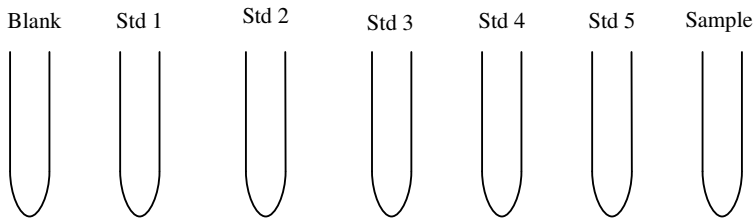
Stabilities of enzyme electrodes were investigated with repetitive use. For this purpose, the activities of immobilized enzymes were measured for 40 successive meaurements.

2.3.9 Shelf-life Determination

Shelf-life studies of enzyme electrodes were investigated for a 50 day period. During this time interval enzyme electrodes were stored in their buffers at 4 ^{0}C when they are not in use.

2.3.10 Protein Determination

The amount of enzyme entrapped in enzyme electrodes was determined by Lowry's protein determination method [88]. Standard solutions of Bovine Serum Albumin (BSA) were prepared at different concentrations. These standard solutions were used to obtain a calibration curve. 0.1 ml of aliquots were taken from the electrolysis cell before and after the electrolysis.



LR	5.0 ml	5.0 ml	5.0 ml	5.0 ml	5.0 ml	5.0 ml	5.0 ml
A	1.0 ml	0.9 ml	0.8 ml	0.6 ml	0.4 ml	0.2 ml	1.0 ml
B	0.0 ml	0.1 ml	0.2 ml	0.4 ml	0.6 ml	0.8 ml	0.0 ml
ES	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml

LR: Lowry Reagent, A: 0.85% w/v NaCl solution in deionized water,
B: 0.01% Protein Standard (0.1 mg/ml in 0.85% NaCl solution,
ES: Electrolysis solution as described in section 2.3.2.

The test tubes were prepared as described above and left for 10 minutes at 25 ^{0}C for incubation after mixing. Then, 0.5 ml Folin-Chiocalteau's reagent (50% v/v in deinozied water) were added to each tube and mixed. They were incubated at 25 ^{0}C and left for 30 minutes for maximum color formation at 750 nm [88]. Finally, absorbances were measured at 750 nm.

2.3.11 Determination of Glucose in Orange Juices

Glucose oxidase electrodes were used in the determination of glucose amount in two brands of Turkish orange juices (Brand D and Brand M). The juices were

filtered and diluted in a ratio of 1:50 with acetate buffer. They were used as the substrates and activity assay described for glucose oxidase was applied.

2.3.12 Determination of Phenolic Compounds in Red Wines

Two brands of Turkish red wines (Brand K and Brand D) were used to determine phenolic compounds with polyphenol oxidase entrapped enzyme electrodes. Total phenolic compounds in wines produced in Turkey were reported as 2000-3000 mg/L [90-92]. Red wines were diluted to a 1:3 volume with citrate buffer and polyphenol oxidase activity assay was applied.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Characterization of P(PStPy-co-Py)

Characterization of P(PStPy-co-Py) was done via FTIR, four probe conductivity measurements and scanning electron microscopy.

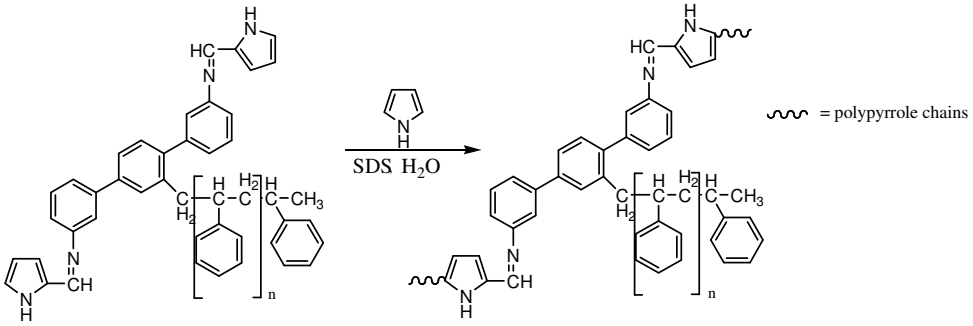


Figure 3.1 Electrochemical synthesis of P(PStPy-co-Py)

3.1.1 FTIR Studies

In order to prove whether the copolymer was achieved via constant potential electrolysis, infrared analysis were done on both pyrrole functionalized polystyrene (PStPy) and the conducting copolymer of pyrrole with pyrrole functionalized polystyrene P(PStPy-co-Py).

In the FTIR spectrum for PStPy, the characteristic peaks of the polymer were observed (Figure 3.2). Azomethine peak was observed at 1598 cm^{-1}. Benzene peaks were observed at fingerprint region. Pyrrole peak was observed at 3438cm^{-1}.

As seen in figure 3.3 all the characteristic peaks of PStPy were also observed in this spectrum besides the peaks at 1036 cm^{-1}, 1120 cm^{-1} and 1168 cm^{-1} belonging to SDS dopant anion. Hence, it was concluded that copolymerization was achieved.

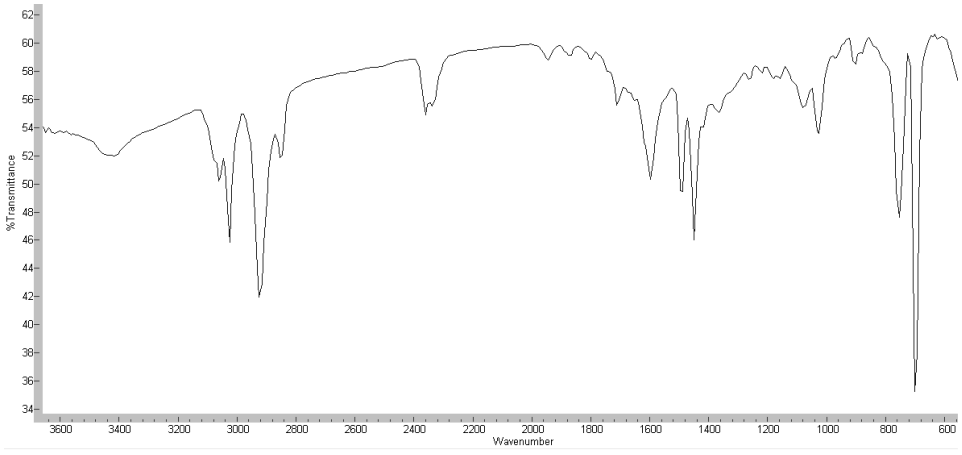


Figure 3.2 Infrared spectrum of PStPy

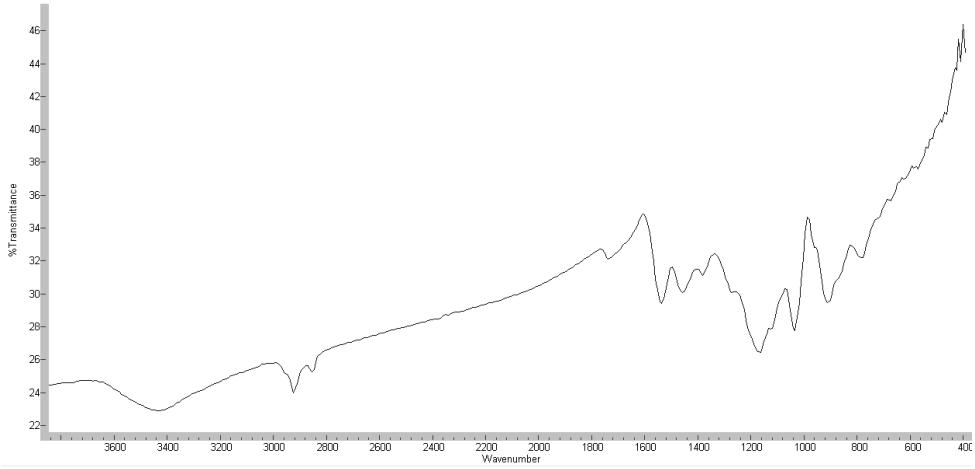


Figure3.3 Infared spectrum of P(PStPy-co-Py)

3.1.2 Conductivities of the Films

Films of PPy and P(PStPy-co-Py) were obtained by electropolymerization of pyrrole on bare and PStPy coated platinum electrodes in SDS-water media as described in section 2.3.1. In order to obtain free standing films, electrolysis were performed for one hour. Conductivities of the resulting films were measured by four probe technique and given in table 3.1. Since an insulating polymer was copolymerized into a conducting copolymer, the conductivity of the resulting material is lower than the conductivity of PPy.

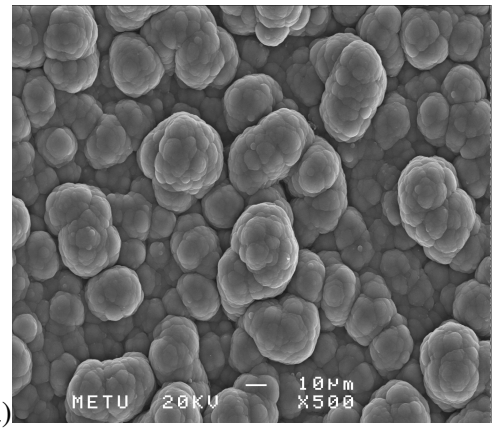
Table 3.1 Conductivities of the films

Matrice	**Conductivity (S/cm)**
PPy	7.4×10^{-3}
P(PStPy-co-Py)	9.0×10^{-6}

3.1.3 Morphologies of the Polymer Films

The morphologies of the PPy and P(PStPy-coPy) films were investigated by Scanning Electron Microscopy (SEM) (JEOL JSM-6400). Morphological differences among the polymer films were seen in Figure 3.4. Cauliflower structure of SDS doped PPy film was destroyed upon copolymerization with pyrrole functionalized polystyrene (PStPy). The structure of P(PStPy-co-Py) looks like a popcorn.

(a)



(b)

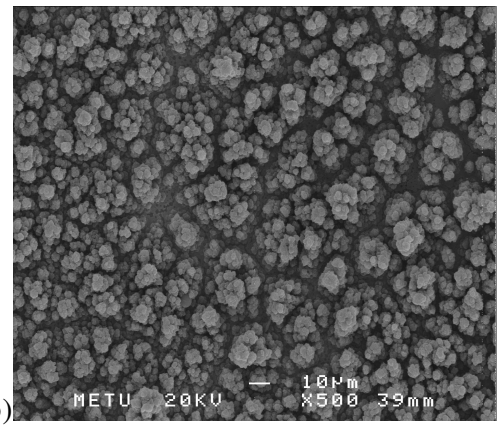


Figure 3.4 SEM micrographs of (a) Ppy film, (b) P(PStPy-co-Py) film

3.2 Immobilization of Enzymes

3.2.1 Kinetic Parameters of Immobilized Enzymes

Maximum reaction rate (V_{max}) and Michaelis-Menten constant (K_m) for free and immobilized glucose oxidase and polyphenol oxidase were found from the Lineweaver-Burk plots [40]. Kinetic parameters for free and immobilized glucose oxidase were given in Table 3.2.

Table 3.2 Kinetic parameters of glucose oxidase

	V_{max}		K_m (mM)
Free	0.68	(μmol/min.ml)	3.0
Ppy	0.04	(μmol/min.electrode)	11.8
P(PStPy-co-Py)	0.11	(μmol/min.electrode)	5.1

As seen from the table, immobilization affects the kinetic parameters of the enzyme. The reason behind may be the new microenvironment provided by the matrix and the structural changes on enzyme. As expected, V_{max} value of free enzyme is higher than that of immobilized ones. Due to presence of a conducting polymer wall, enzyme-substrate complex formation becomes more difficult. Substrates cannot easily pass through the pores of the conducting polymers and this result in the decrease of reaction rate. P(PStPy-co-Py) matrix provides better environment for glucose oxidase compare to PPy matrix because of the higher reaction rate and higher substrate affinity.

The results of the kinetic studies of free and immobilized polyphenol oxidase enzyme were tabulated in Table 3.3.

Table 3.3 Kinetic parameters of polyphenol oxidase

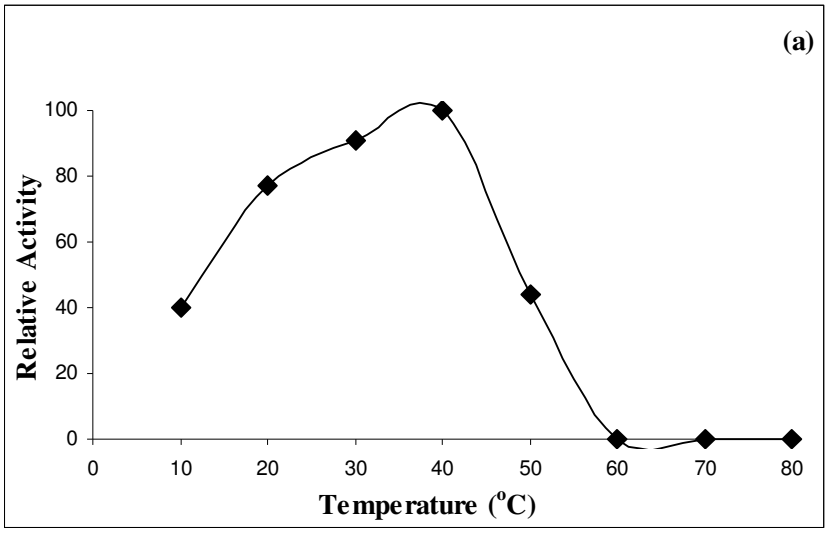
	V_{max}	K_m (mM)
Free	0.10 (μmol/min.ml)	0.14
Ppy	0.0064 (μmol/min.electrode)	17.0
P(PStPy-co-Py)	0.0064 (μmol/min.electrode)	23.0

The effect of immobilization on the kinetic parameters of the enzyme was also seen in polyphenol oxidase compared to the free enzyme. K_m parameters are different in these matrices although V_{max} values are approximately the same. In P(PStPy-co-Py) matrix, the affinity of the enzyme towards catechol is lower than that of in PPy matrix. This means that enzyme and substrate leave each other easily. However, when they come together product formation occurs more frequently compared to PPy matrix.

3.2.1 Effect of Temperature on the Activity of Enzymes

Temperature effect on the activity of enzyme electrodes was investigated by changing the incubation temperature. The optimum temperature for free glucose oxidase was reported as 30 ^{0}C [51]. As seen in Figure 3.5 (a and b) the temperature of maximum activity for PPy/GOD electrode is 40 ^{0}C and for P(PStPy-co-Py)/GOD electrode are 30 ^{0}C and 40 ^{0}C.

The effect of temperature on polyphenol oxidase enzyme electrodes was also investigated and given in Figure 3.6 (c and d). PPy/PPO electrode showed maximum activity at 60 ^{0}C and P(PStPy-co-Py)/PPO electrode at 70 ^{0}C. Immobilized enzyme had higher stability against temperature compared to free enzyme (40 ^{0}C) [53].



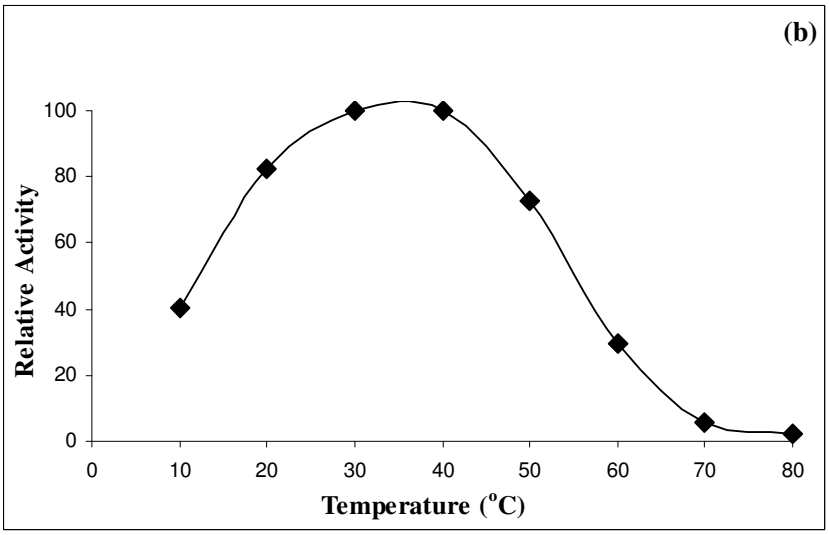
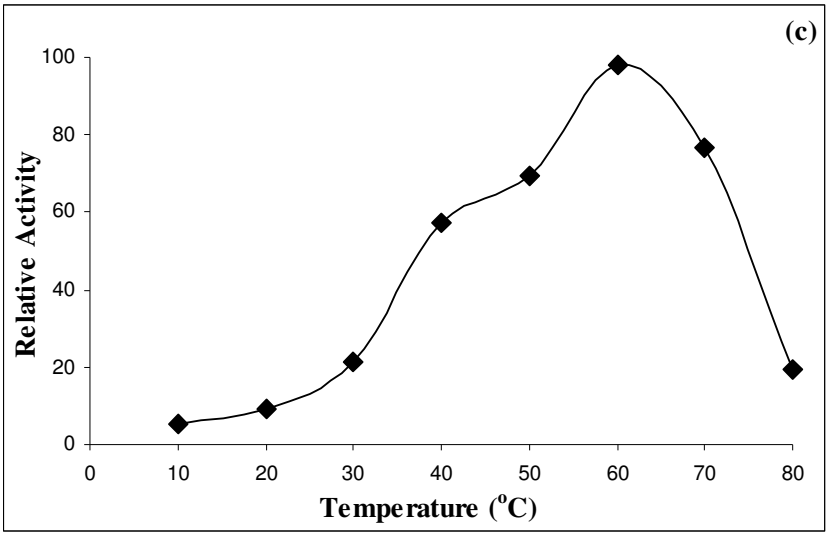


Figure 3.5 Temperature optimization of glucose oxidase in (a) PPy matrix, (b) P(PStPy-co-Py)matrix



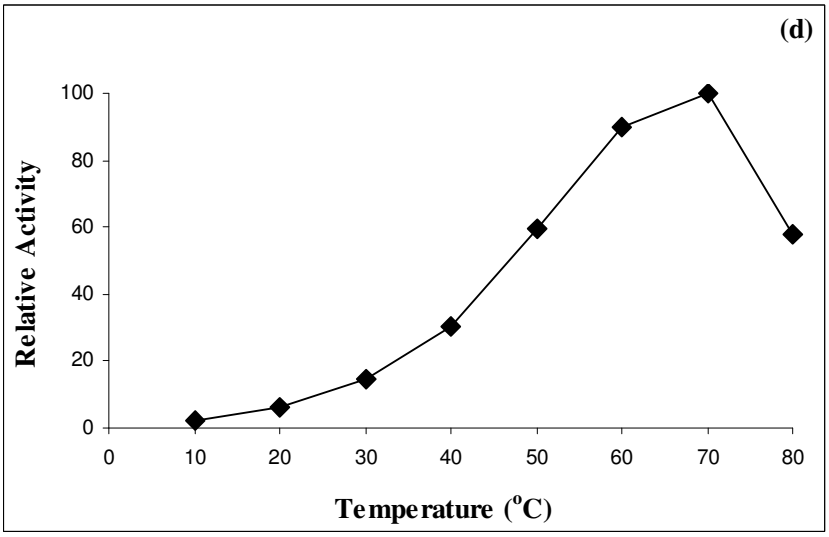
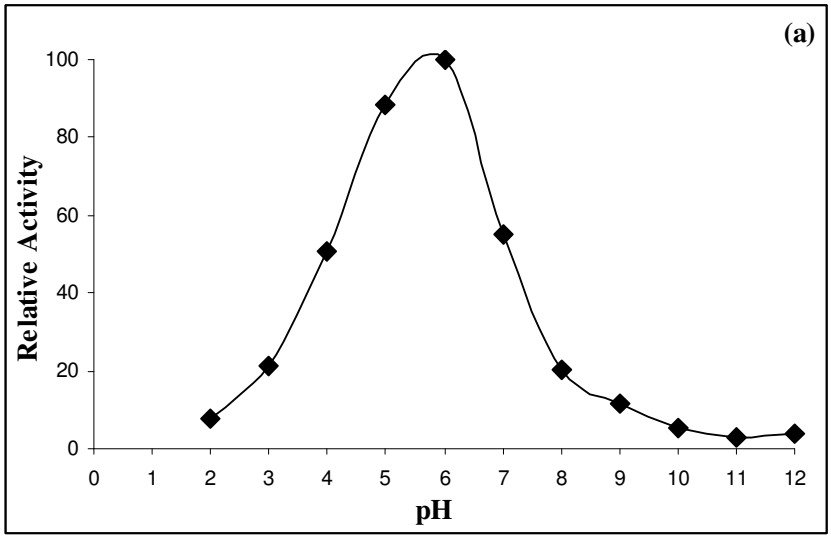


Figure 3.6 Temperature optimization of polyphenol oxidase in (c) PPy matrix, (d) P(PStPy-co-Py)matrix

3.2.2 Effect of pH on the Activity of Enzymes

The change in activities of both immobilized glucose oxidase and polyphenol oxidase were investigated at different pH values. PPy/GOD enzyme electrode reveals maximum activity at pH 6 (figure 3.7 (a)). After pH 8 enzyme electrode lost almost all of its activity. On the other hand, P(PStPy-co-Py)/GOD enzyme electrode showed higher activities over a broad range of pH values in the alkaline side (figure 3.7 (b)). This shift towards the alkaline side may be due to the attraction of H-ions in solution by negatively charged groups of matrice. Hence, the environment around the enzyme has lower pH value than the surrounding solution where pH is actually high.

PPy/PPO enzyme electrode had higher activities between pH 8 and pH 11 (Figure 3.8 (c)). P(PStPy-co-Py) enzyme electrode had higher activities in a broader pH range (pH 6-pH 11) compared with the former (Figure 3.8 (d)). Polyphenol oxidase enzyme was protected well from the effect of pH upon immobilization in P(PStPy-co-Py) matrix. As a result, this enzyme electrode can be used effectively in wide range of pH.



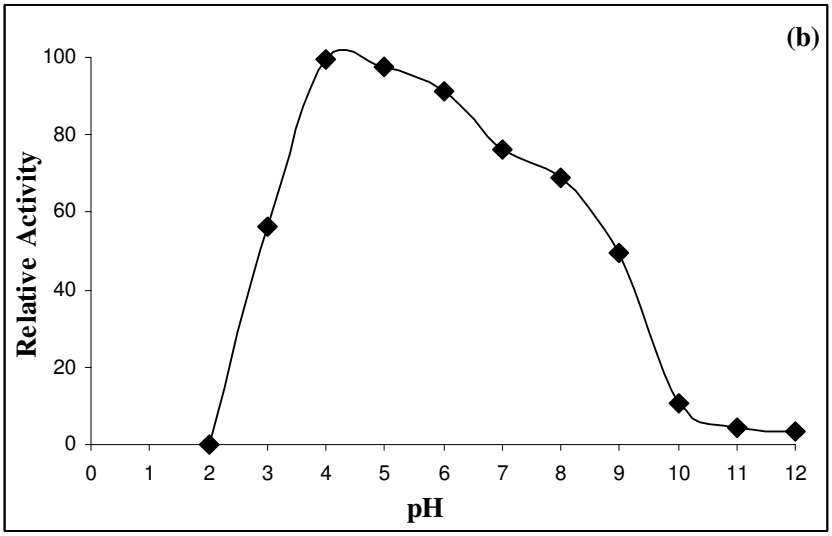
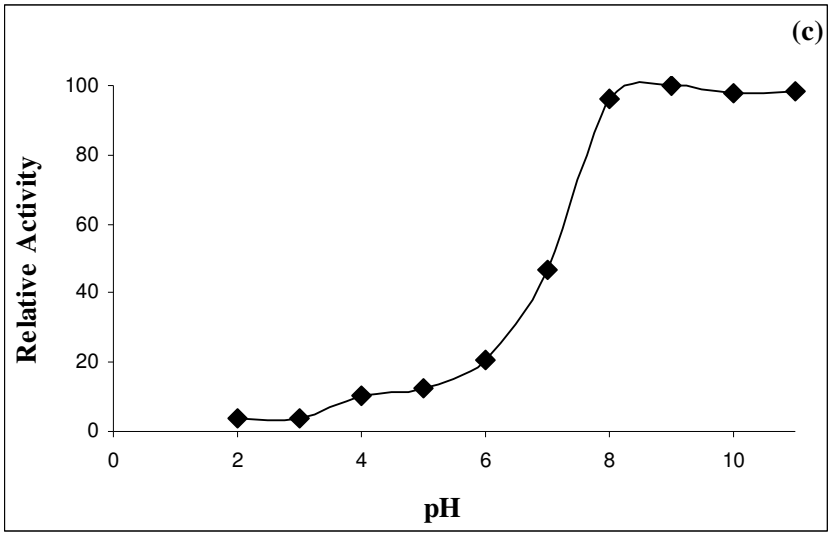


Figure 3.7 pH Optimization of glucose oxidase in (a) PPy matrix,
(b) P(PStPy-co-Py) matrix



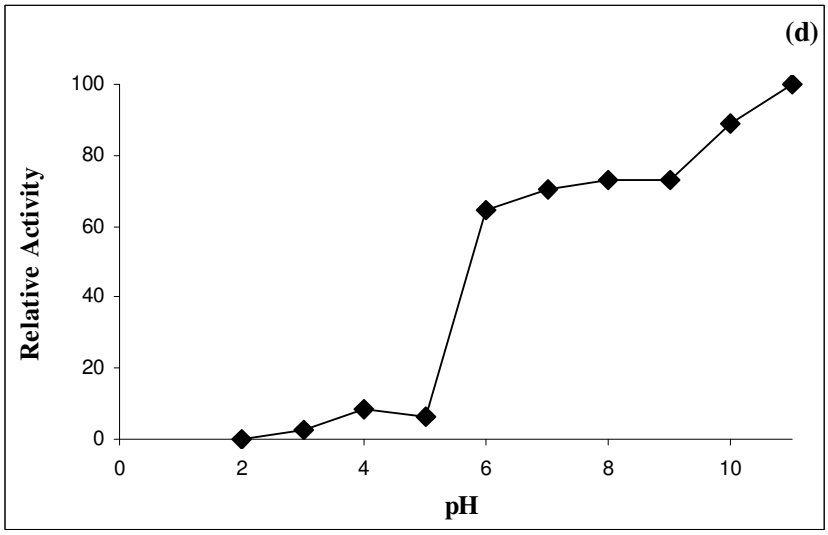
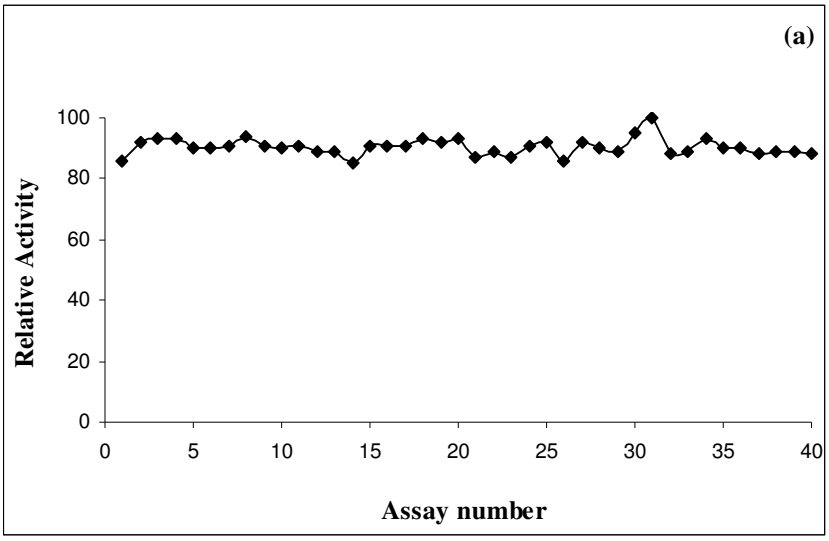


Figure 3.8 pH Optimization of polyphenol oxidase in (c) PPy matrix, (d) P(PStPy-co-Py) matrix

3.2.3 Operational Stabilities of Enzyme Electrodes

In order to investigate the stability of enzyme electrodes in terms of repetitive uses, 40 successive measurements were done at 25 ^{0}C during one day. Glucose oxidase enzyme immobilized in either PPy matrix or P(PStPy-co-Py) matrix gave constant responses for every uses. Almost no change in the activity of enzyme was observed (figures 3.9 (a) and (b)).

P(PStPy-co-Py)/PPO enzyme electrode had a good operational stability upon repetitive uses compare to PPy/PPO enzyme electrode. The former lost its 40% activity after 40 repetitive uses whereas the latter lost 60% (figures 3.10 (c) and (d)).



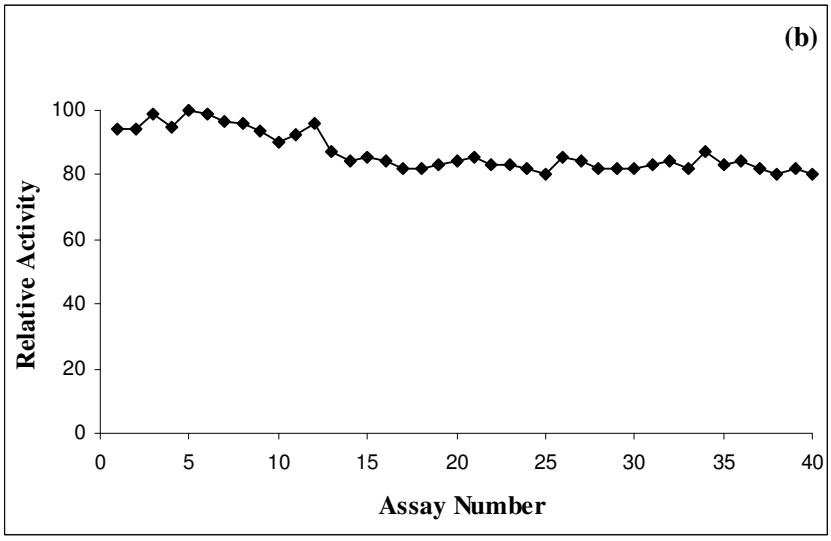
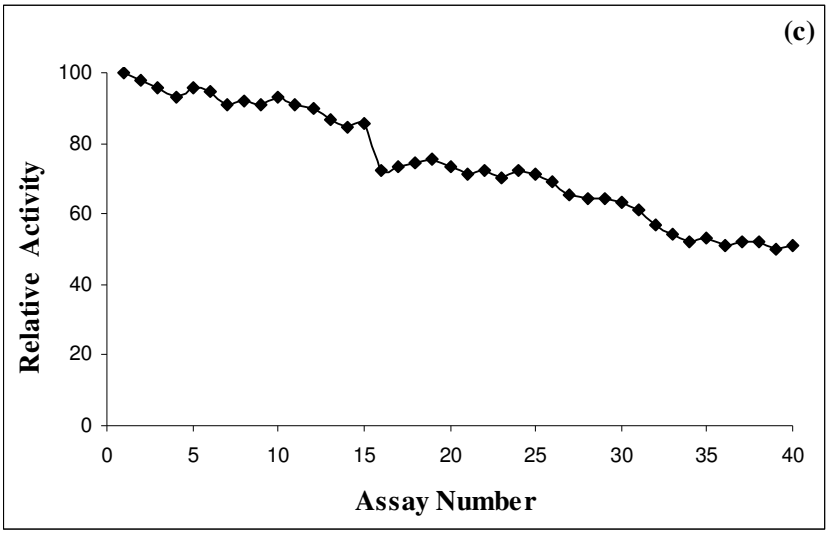


Figure 3.9 Operational stability of (a) PPy/GOD enzyme electrode,
(b) P(PStPy-co-Py)/GOD enzyme electrode



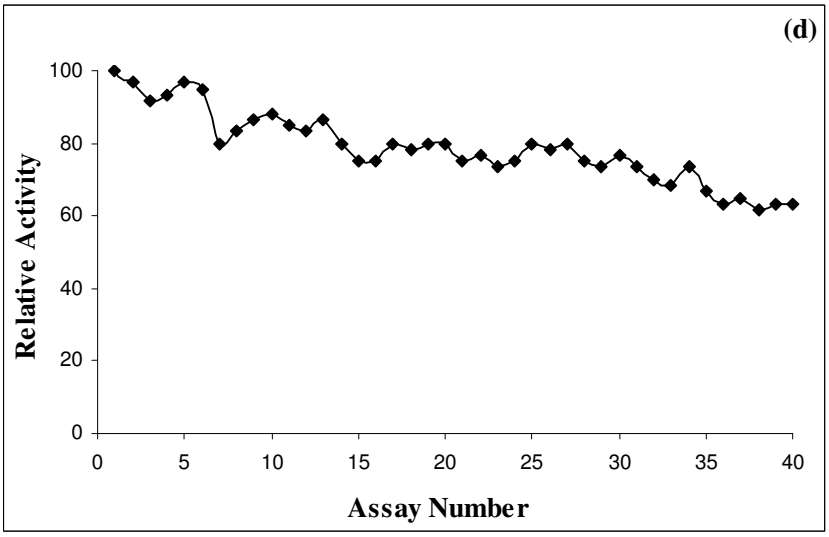


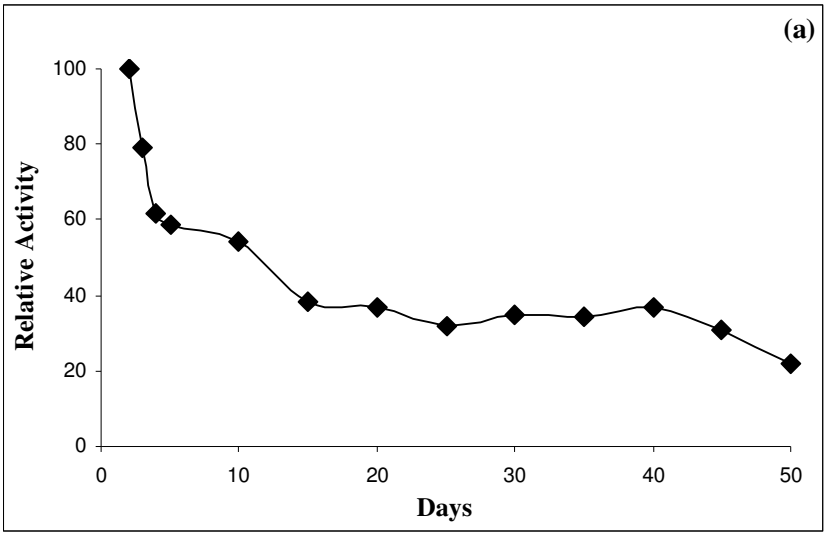
Figure 3.10 Operational stability of (c) PPy/PPO enzyme electrode,
(d) P(PStPy-co-Py)/PPO enzyme electrode

3.2.4 Storage Stability

The storage of enzymes is critical due to easy denaturation because of extreme temperatures and pH. In order to determine the shelf-life of enzyme electrodes, activities of each electrode were checked over a 50 days period. Enzyme electrodes were stored in their buffers at 4 ^{0}C when they were not in use.

PPy/GOD enzyme electrode lost 70% of its activity at the 25th day whereas P(PStPy-co-Py) enzyme electrode save 90% of original activity during that time (Figures 3.11 (a) and (b)).

PPO enzyme immobilized in either PPy or P(PStPy-co-Py) matrices lost its 50% of activity at the 10th day (Figures 3.12 (c) and (d)).



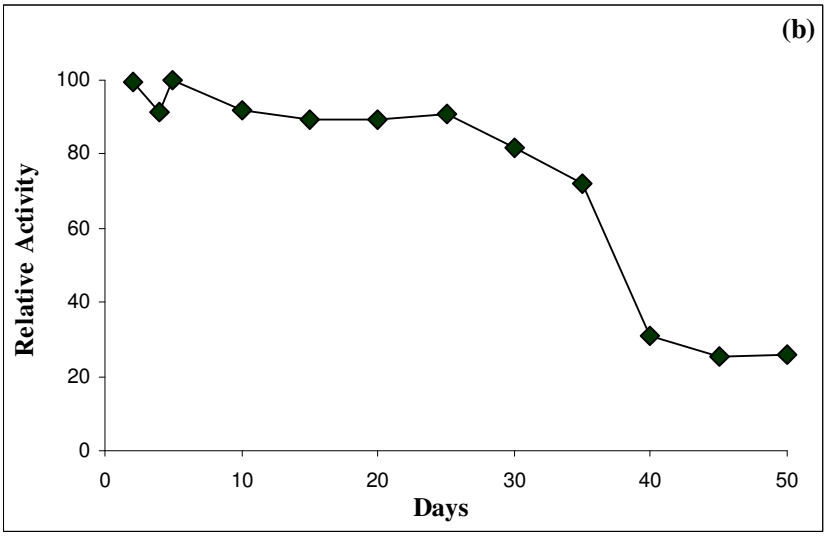
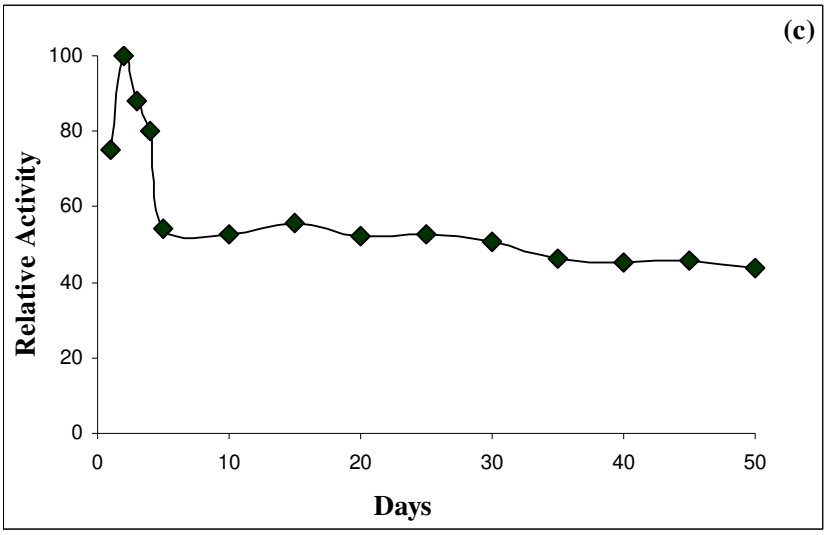


Figure 3.11 Storage stability of (a) PPy/GOD enzyme electrode,
(b) P(PStPy-co-Py)/GOD enzyme electrode



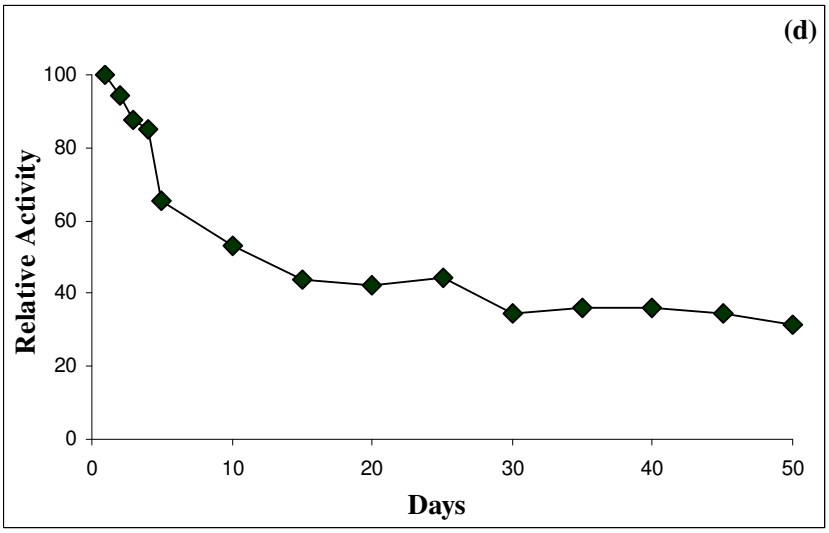


Figure 3.12 Storage stability of (c) PPy/PPO enzyme electrode,
(d) P(PStPy-co-Py)/PPO enzyme electrode

3.2.5 Protein Determination

The amount of GOD and PPO enzyme entrapped in PPy and P(PStPy-co-Py) matrices were determined by Lowry's method [88]. Standard calibration curve of Bovine Serum Albumin was used for that matter. Results were given in table 3.4. The amount of GOD enzyme entrapped in P(PStPy-co-Py) matrix is 2.5 times higher than that of in PPy matrix. This result is consistent with the higher reaction rate of GOD enzyme in P(PStPy-co-Py).

The amount of entrapped polyphenol oxidase enzyme was the same in both matrices. This result is also consistent with the maximum reaction rates obtained for both matrices.

Table 3.4 Amount of entrapped enzyme

	GOD (mg)	**PPO (mg)**
PPy	9.5×10^{-5}	2.4×10^{-4}
P(PStPy-co-Py)	2.5×10^{-4}	2.4×10^{-4}

3.2.6 Sample Analysis

3.2.6.1 Determination of Glucose in Orange Juices

GOD enzyme electrodes were used for the determination of glucose amount in two brands of orange juices of Turkey (Brand D and M). Lane-Eynon analyses of glucose amount for both brands were performed before [51]. Glucose amounts detected are given in table 3.5. Amount of glucose detected with P(PStPy-co-Py)/GOD electrode in Brand M was not even

close to the amount detected with Lane-Eynon method. This enzyme electrode is not good enough to detect glucose in Brand D.

Table 3.5 Glucose amount in two brands of orange juices.

	Brand D (g/100ml)	**Brand M (g/100ml)**
Lane-Eynon Method*	1.50	2.15
PPy*	1.45	1.44
P(PStPy-co-Py)	ND	1.30

* [51], ND: not detected

3.2.6.2 Determination of Phenolic Compounds in Red Wines

PPO enzyme electrodes were used for the determination of phenolic compounds in two brands of Turkish wines (Brand K and D). Total amounts of phenolics were determined by activity determination of enzyme electrodes (as described in section 2.3.4) by using wines as the substrate.

Determination of phenolics in these two brands of wine did not give accurate results due to the inhibitors present. However, as given in table 3.6 immobilization protects the enzyme from the action of inhibitors.

Amount of phenolics present in Brand K is twice as much as the one in brand D. This was confirmed by enzyme electrodes. However, results of P(PStPy-co-Py)/PPO electrode was lower than for PPy/PPO electrode. It may be concluded that copolymer matrix does not protect enzyme from inhibitors as PPy matrix does.

Table 3.6 Phenolic compounds in Turkish red wines.

	Brand K	**Brand D**
Free PPO*	220mg/L	270mg/L
PPy/PPO*	4000mg/L	2200mg/L
P(PStPy-co-Py)/PPO	1954mg/L	1026mg/L

***** [53]

CHAPTER IV

CONCLUSIONS

Electrochemical synthesis of conducting copolymer of pyrrole with pyrrole functionalized polystyrene (P(PStPy-co-Py)) was performed and characterized via FTIR, Four Probe Conductivity Measurements and Scanning Electron Microscopy (SEM).

Glucose oxidase and polyphenol oxidase enzymes were immobilized both in PPy and P(PStPy-co-Py) matrices. Maximum reaction rates and Michaelis-Menten constants were determined (Table 3.7). Glucose oxidase immobilized in P(PStPy-co-Py) matrix had maximum reaction rate compared to that of immobilized in PPy matrix. These results are consistent with the amount of enzymes immobilized in both matrices. Moreover, amount of entrapped enzyme was determined by Lowry's protein determination method [88]. Since more enzyme was immobilized in P(PStPy-co-Py) matrix compared to PPy matrix, enzyme had high reaction rate in this matrix. Polyphenol oxidase immobilized in both matrices had the same reaction rates which may be due to the same amount of enzyme immobilized in both matrices.

Temperature, pH, storage and operational stabilities of enzyme electrodes were investigated. Glucose oxidase enzyme had maximum activity at 30 ^{0}C in PPy matrix, 30 ^{0}C and 40 ^{0}C in P(PStPy-co-Py) matrix. Polyphenol oxidase enzyme had maximum activity at 60 ^{0}C in PPy matrix and 70 ^{0}C in P(PStPy-co-Py) matrix.

pH optimization studies showed that glucose oxidase enzyme immobilized in PPy matrix had maximum activity at pH 6 whereas it had higher activities in a broad pH range when immobilized in P(PStPy-co-Py) matrix. Polyphenol oxidase immobilized in PPy matrix had higher activities between pH 8 and pH 11, this pH range is broader (pH6-pH11) when immobilized in P(PStPy-co-Py) matrix.

GOD enzyme electrodes were used for the determination of glucose in orange juices and PPO enzyme electrodes were used for the determination of phenolics in two Brands of Turkish wines.

Table 3.7 Overall results of experiments

	Temperature (^{0}C) at which maximum activity was observed	pH at which maximum activity was observed	Amount of entrapped enzyme (mg)	V_{max} (μmol/min.electrode)	K_m (mM)
PPy/GOD	40	6	$9.5x10^{-5}$	0.04	11.8
P(PStPy-co-Py)/GOD	30 and 40	4 and 5	$2.5x10^{-4}$	0.11	5.1
PPy/PPO	60	8-11	$2.4x10^{-4}$	0.0064	17.0
P(PStPy-co-Py)/PPO	70	11	$2.4x10^{-4}$	0.0064	23.0

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