ACTIVITY ANALYSIS OF IMMOBILIZED TYROSINASE ENZYME IN THE PRESENCE OF DIFFERENT INHIBITORS

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ABSTRACT

ACTIVITY ANALYSIS OF IMMOBILIZED TYROSINASE ENZYME IN THE PRESENCE OF DIFFERENT INHIBITORS

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Immobilization of tyrosinase enzyme was performed in the matrices obtained via copolymerization of terephthalic acid bis-(2-thiophen-3-yl ethyl) ester (TATE) with pyrrole. During electrochemical polymerization of pyrrole, enzyme molecules were entrapped in the copolymer matrice. Activity measurements were performed by using Besthorn's Hydrazone method which includes spectrophotometric analysis of quinones produced by the enzyme. Enzyme electrodes were characterized in terms of maximum reaction rate (Vmax) and Michaelis-Menten constant (Km). In addition to kinetic parameters, stability of enzyme electrodes towards environmental conditions such as pH and temperature was investigated. Usage stability and shelf-life analysis were also examined.

Wines, especially red wines, contain numerous biologically active compounds,

the most important of which are polyphenols, whose nutritional importance is attributed to their antioxidant power. The amounts of phenolic compounds in different red wines were analyzed by using obtained enzyme electrodes. The phenolic compound determination using free enzyme cannot reflect the actual values since there are also naturally found inhibitors in red wines. Benzoic acid, cinnamic acid and sorbic acid were utilized to understand the behavior of immobilized tyrosinase in the conducting polymer matrices toward inhibition.

Keywords: Electrochemical polymerization, tyrosinase, immobilization, inhibition, wine

ÖZ

TUTUKLANMIŞ TİROSİNAZ ENZİMİNİN FARKLI İNHİBİTÖRLER VARLIĞINDA AKTİVİTE TAYİNİ

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Tez Yöneticisi: Prof. Dr. Levent Toppare

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Tereftalik asit bis-(2-tiyofen–3-il-etil) ester (TATE) kullanılarak pirolle iletken kopolimerler elde edildi. Bu elektrokimyasal kopolimerleştirme sırasında tirosinaz enzimi bu matris içerisinde tutuklandı. Aktivite tayinleri, tirosinaz tarafından oluşturulan kuinon molekülünün spektrofotometric analizini içeren Besthorn's Hydrazone metodu ile yapıldı. Enzim elektrotları, maksimum reaksiyon hızı (Vmax) ve Michaelis-Menten sabiti bakımından karakterize edildi. Bunun yanı sıra, enzim elektrotlarının pH ve sıcaklık gibi çevresel etkenlere karşı kararlılığı araştırıldı. Kullanım kararlılıkları ve raf ömürleri tayin edildi.

Şaraplar, özellikle de kırmızı şaraplar, çok sayıda biyolojik olarak etkin madde içerirler. Bunlardan en önemlisi polifenollerdir. Polifenollerin önemi güçlü antioksidan olma özelliklerinden kaynaklanır. Değişik markaların kırmızı şaraplarında fenolik maddelerin miktarı oluşturulan enzim elektrotları kullanılarak

tayin edildi. Kırmızı şaraplarda bulunan çok sayıdaki inhibitörlerden dolayı, serbest halde enzim kullanılarak yapılan fenolik madde tayini gerçek değerleri yansıtamaz. Bu yüzden, tutuklanan tirosinaz enziminin inhibisyona karşı tavrını anlayabilmek için, benzoik asit, sinnamik asit ve sorbik asit inhibitörleri kullanılarak aktivite tayini yapıldı.

Anahtar Kelimeler: Elektrokimyasal polimerleşme, tirosinaz, enzim tutuklaması, inhibisyon, şarap

To My Family

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LIST OF ABBREVIATION

PPO	Polyphenol oxidase
L-DOPA	L-dihydroxyphenylalanine
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
LDL	Low density lipoproteins
TBAFB	Tetrabutylammonium tetrafluoroborate
SDS	Sodium dodecyl sulfate
MBTH	3-methyl-2-benzothiozoline
CCE	Constant potential electrolysis
CCP	Constant current electrolysis
FTIR	Fourier Transform Infrared Spectrophotometer
NMR	Nuclear Magnetic Resonance Spectrometer
SEM	Scanning electron Microscope
TATE	Terephthalic acid bis-(2-thiophen-3-yl ethyl) ester
PTATE	Homopolymer of terephthalic acid bis-(2-thiophen-3-yl ethyl) ester
PPy	Polypyrrole
PTATE/PPy	Copolymer of homopolymer of terephthalic acid bis-(2-thiophen-3-yl ethyl) ester with pyrrole
PTATE/PPy/PPO	Immobilized polyphenol oxidase in copolymer matrix of homopolymer of terephthalic acid bis-(2-thiophen-3-yl ethyl) ester with pyrrole
PPy/PPO	Immobilized polyphenol oxidase in the matrix of polypyrrole
BSA	Bovine Serum Albumin

BA	Benzoic acid
CA	Cinnamic acid
SA	Sorbic acid

CHAPTER 1

INTRODUCTION

1.1 Conducting Polymers

1.1.1 Brief History of Conducting Polymers

Conducting polymers are organic materials that exhibit the conducting properties of metals while retaining the mechanical properties and processibility of polymers. They contain π- electron backbone responsible for their unusual electronic properties, and these properties make conducting polymers attract a great deal of attention and become a hot research area for many academic institutions [1].

The first report of high level of conductivity in a polymer was seen in 1977 when it was found that polyacetylene become conducting when oxidized by suitable reagents [2]. Polyacetylene was the first conjugated polymer to show this special electrical conductivity. In 2000, Alan Heeger, Alan MacDiarmid, and Hideki Shirakawa were awarded with the Nobel Prize in chemistry 'for the discovery and development of electrically conductive polymers'. They established that polymer plastics can be made to conduct electricity if alternating single and double bonds link their carbon atoms, and electrons are either removed through oxidation or introduced through reduction. Normally the electrons in the bonds remain localized and cannot carry an electric current. However, when the material is doped with strong electron acceptors such as iodine, the polymer began to conduct nearly as well as a metal, with conductivity 10^{11} times higher than pure polyacetylene [2,3]. Although polyacetylene exhibits a very high conductivity in the doped form, because of its instability in air, lack of tractability, and the tedious processibility, its industrial use is limited. For these reasons, studies in this field have been devoted to synthesizing

soluble and stable polyacetylenes [4,5]. Unfortunately these substituted derivatives exhibit electrical conductivities that are much lower than the parent polymer. New conducting polymers based on aromatic systems and heteroatomics such as aniline, pyrrole and thiophene have been developed. Although they exhibit lower conductivity than polyacetylene, these polymers possess higher environmental stability and structural versatility. Some examples are shown in Figure 1.1 [6].

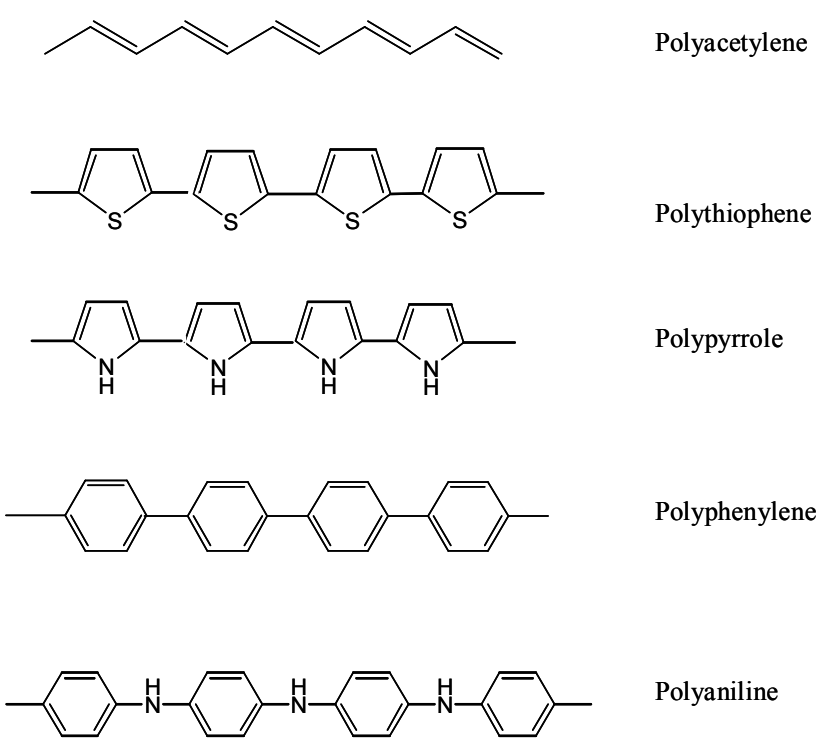


Figure 1.1 Chemical structures of some conducting polymers

Because of the easy synthesis, good environmental stability and long term stability of electrical conductivity, conducting polymers replace metals and

semiconductors in the electrical and electronics industry. There is a wide application range of conducting polymers. Some examples are light emitting diodes [7,8], electrochromic devices [9-11], batteries [12], actuators [4], sensors [13], drug delivery [14], and enzyme immobilization [15-17].

1.1.2 Band Theory

Band theory is a useful tool in the understanding of electrical conductivity. Electrical conductivity (σ) depends on the number of charge carriers (n), the carrier charge (q) and the carrier mobility (μ). The relationship between conductivity and these parameters is expressed by the general equation.

$$\sigma = q\, n\, \mu \qquad (1.1)$$

According to band theory, a solid consists of N atoms that are packed tightly together. Each individual electronic energy state splits in to N levels which are very close together and considered as a continuous energy band. The valence band is the highest occupied energy band and the conduction band is the lowest unoccupied energy band. There is an energy difference between these two bands. This energy difference is called as the band gap, which is the energy required to promote an electron from the valence band to conduction band. In insulators the electrons in the valence band are separated by a large gap (> 3.0 eV) from the conduction band, in conductors like metals the valence band overlaps the conduction band, and in semiconductors there is a small enough gap (0.5 – 3.0 eV) between the valence and conduction bands that thermal or other excitations can bridge the gap. With such a small gap, the presence of a small percentage of a doping material can increase conductivity dramatically. An important parameter in the band theory is the Fermi level, the top of the available electron energy levels at low temperatures. The position of the Fermi level with the relation to the conduction band is a crucial

factor in determining electrical properties [18].

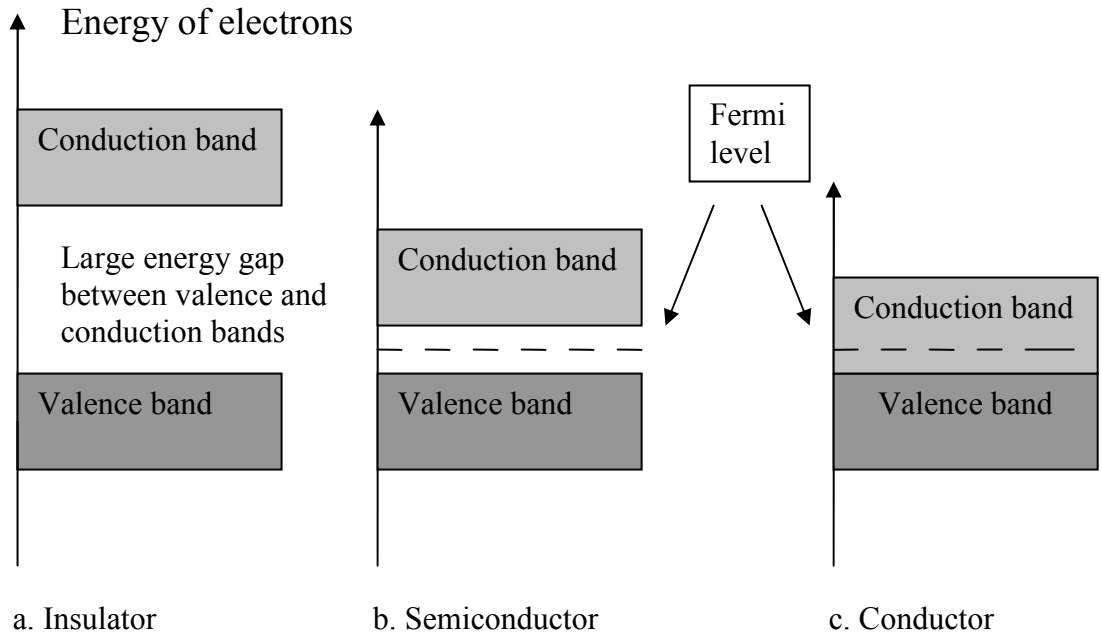


Figure 1.2 Energy bands of solids

1.1.3 Doping Process

Doping is the creation defects in the polymer's structural chain without destroying it. These defects can be radicals, anions, cations or combination of these and are called as charge carriers. In their natural form, conjugated polymers are semiconductors. They may store charge in two ways. In an oxidation process it could either lose an electron from one of the bands or it could localize the charge over a

small section of the chain. Localizing the charge causes a local distortion due a change in geometry, which costs the polymer some energy. However, the generation of this local geometry decreases the ionization energy of the polymer chain and increases its electron affinity making it more able to accommodate the newly formed charges. This method increases the energy of the polymer less than it would if the charge was delocalized and, hence, takes place in preference of charge delocalization.

The oxidative doping of polypyrrole proceeds in the following way. An electron is removed from the Π system of the backbone producing free radical and a spinless positive charge. The radical and cation are coupled to each other via local resonance of the charge and the radical. In this case, a sequence of quinoid-like rings is used. The distortion produced by this is of higher energy than the remaining portion of the chain. The creation and separation of these defects costs a considerable amount of energy. This limits the number of quinoid-like rings that can link these two bound species together. In the case of polypyrrole it is believed that the lattice distortion extends over four pyrrole rings. This combination of a charge site and a radical is called a polaron. This could be either a radical cation or radical anion. This creates a new localized electronic state in the gap, with the lower energy states being occupied by single unpaired electrons. The polaron state of polypyrrole is symmetrically located about 0.5 eV from the band edges. Upon further oxidation the free radical of the polaron is removed, creating a new spinless defect called a bipolaron. This is of lower energy than the creation of two distinct polarons. At higher doping levels it becomes possible that two polarons combine to form a bipolaron. Thus at higher doping levels the polarons are replaced with bipolarons. The bipolarons are located symmetrically with a band gap of 0.75 eV for polypyrrole. This eventually, with continued doping, forms into a continuous bipolaron bands. Their band gap also increases as newly formed bipolarons are made at the expense of the band edges. For a very heavily dope polymer it is conceivable

that the upper and the lower bipolaron bands will merge with the conduction and the valence bands respectively to produce partially filled bands and metallic like conductivity. This is shown below (Figure 1.3).

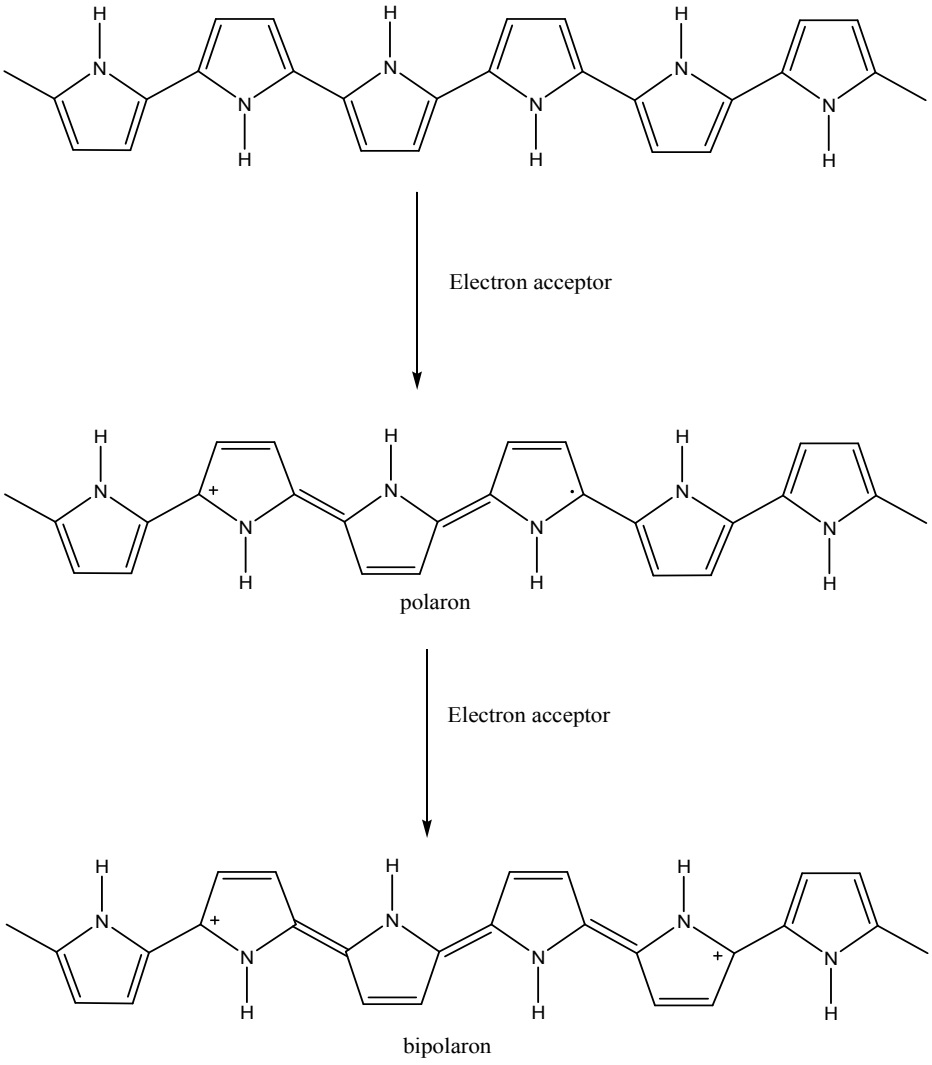


Figure 1.3 The structural representation of oxidative doping of polypyrrole.

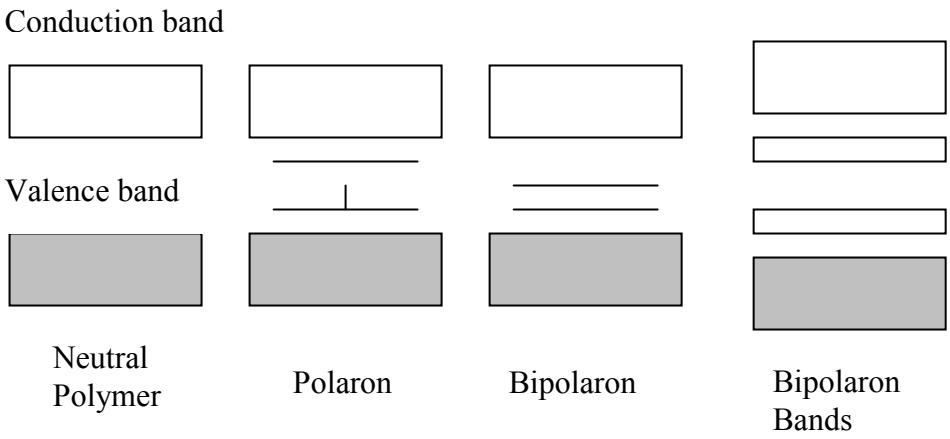


Figure 1.3 (Cont.)

Conjugated polymers with a degenerate ground state have a slightly different mechanism. As with polypyrrole, polarons and bipolarons are produced upon oxidation. However, because the ground state structure of such polymers are two fold degenerate, the charged cations are not bound to each other by a higher energy bonding configuration and can freely separate along the chain. The effect of this is that the charged defects are independent of one another and can form domain walls that separate two phases of opposite orientation and identical energy. These are called solitons and can sometimes be neutral. Solitons produced in polyacetylene are believed to be delocalized over about 12 CH units with the maximum charge density next to the dopant counter ion. The bonds closer to the defect show less amount of bond alternation than the bonds away from the centre. Soliton formation results in the creation of new localized electronic states that appear in the middle of the energy gap. At high doping levels, the charged solitons interact with each other to form a soliton band which can eventually merge with the band edges to create true metallic conductivity.

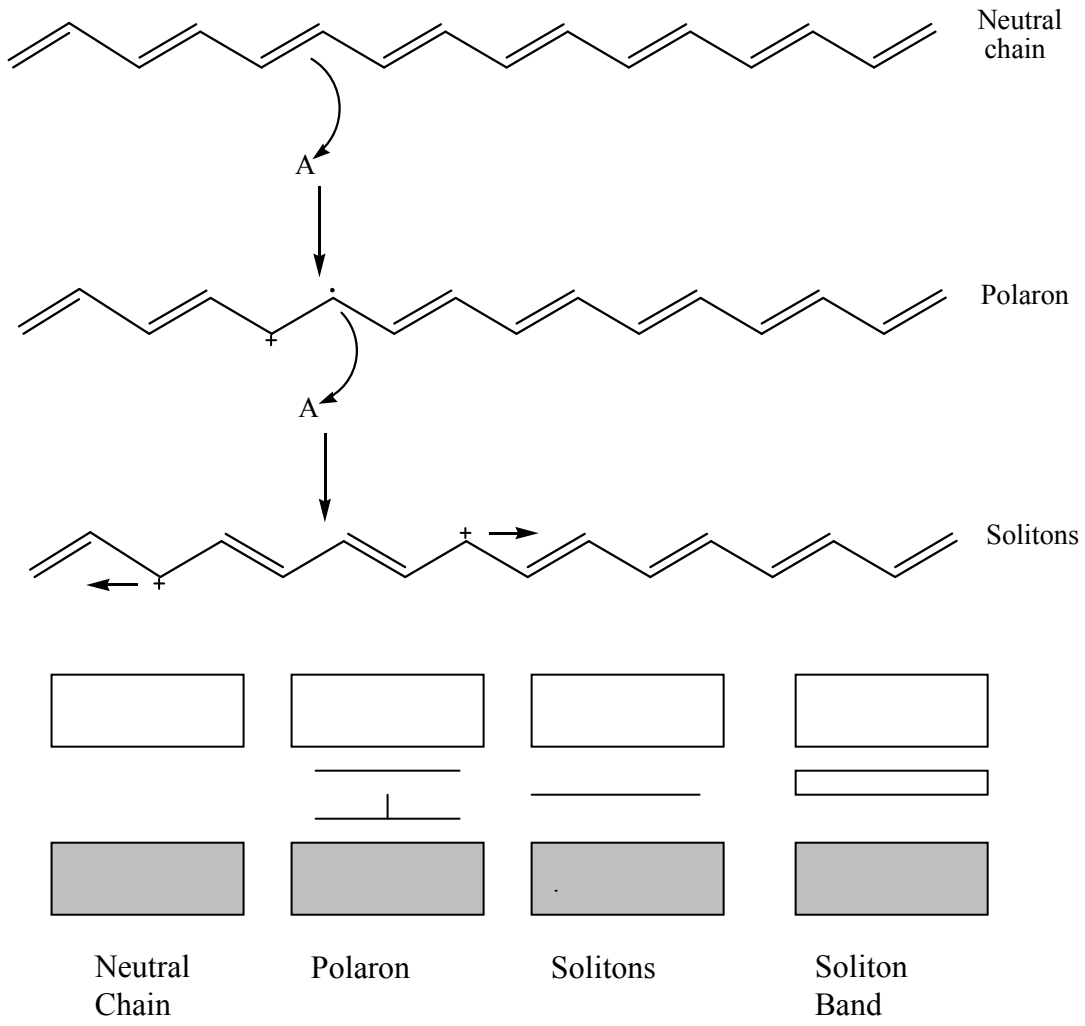


Figure 1.4 Soliton structures of polyacetylene.

1.1.4 Synthesis of Conducting Polymers

Conducting polymers can be synthesized by electrochemical polymerization or chemical polymerization.

1.1.4.1 Electrochemical Polymerization

Electrochemical polymerization occurs by suitable monomers which are electrochemically oxidized to create an active monomeric and dimeric species which react to form a conjugated polymer backbone. This polymerization technique starts with the removal of one electron from a monomer unit to form radical cation (Figure 1.5). Since the electron transfer reaction is much faster than the diffusion of the monomer from the bulk solution, it follows that a high concentration of radicals is continuously maintained near the electrode surface. Then, a chemical reaction involves the spin-pairing of two radical cations to form a dihydro dimer dication, which subsequently undergoes the loss of two protons and rearomatization to form the dimer. Aromatization is the driving force of the chemical step. Coupling occurs primarily through the α-carbon atoms of the heterocyclic ring since these are the positions of highest unpaired electron π-spin density and hence reactivity. At the applied potential, the dimer, which is more easily oxidized than the monomer, exists in a radical cation form and undergoes further coupling reactions with other radical cations. This electropolymerization mechanism continues until the oligomer becomes insoluble in the electrolytic medium and precipitates onto the anode surface [19,20].

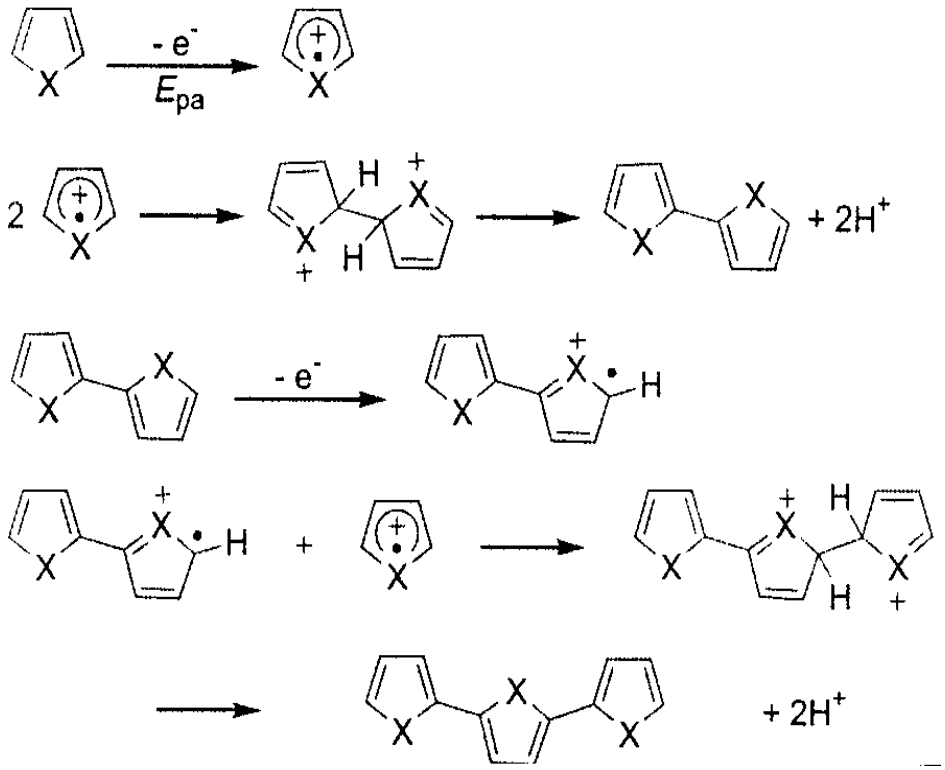


Figure 1.5 Electrochemical polymerization mechanism for aromatic five membered heterocycles.

Electrochemical polymerization offers many advantages. Smooth polymer films on conductive substrates can be synthesized and this method allows for easy probing of electrical and optical properties. The oxidation potential can be easily controlled and the properties of the deposited polymer allows for preparation of a variety of different conducting polymers prepared from the same monomer system by simply varying specific electrochemical parameters such as applied potential, electrolyte, temperature or electrode [21].

The main problem of electrochemical polymerization is conjugated backbone that gives conductivity. This causes such polymers to be intractable, insoluble films or powders that can not melt. To overcome such a problem, the polymer can be modified so that it may be more easily processed or the polymer can be manufactured in its desired shape or form. In order to synthesize, three main methods can be used. The first method is to synthesize a malleable polymer that can be easily converted into a conjugated polymer. This is done when the initial polymer is in the desired form and then, after conversion, is treated so that it becomes a conductor. The treatment used is most often thermal treatment. The precursor polymer used is often made to produce highly aligned polymer chain which is retained upon conversion. These are used for highly orientated thin films and fibres. Such films and fibres are highly anisotropic, with maximum conductivity along the stretch direction. The second method is the synthesis of copolymers or derivatives of a parent conjugated polymer with more desirable properties. This method is the more traditional one for making improvements in properties to a polymer. What is done is to try to modify the structure of the polymer to increase its processibility without compromising its conductivity or its optical properties. All attempts to do this on polyacetylene have failed as they always significantly reduced its conductivity. However, such attempts on polythiophenes and polypyrroles proved more fruitful. The hydrogen on carbon 3 on the thiophene or the pyrrole ring was replaced with an alkyl group with at least four carbon atoms in it. The resulting polymer, when doped, has a comparable conductivity to its parent polymer whilst be able to melt and it is soluble. A water soluble version of these polymers has been produced by placing carboxylic acid group or sulphonic acid group on the alkyl chains. If sulphonic acid groups are used along with built-in ionizable groups, then such system can maintain charge neutrality in its oxidized state and so they effectively dope themselves. Such polymers are referred to as "self-doped" polymers. One of the most highly conductive derivatives of polythiophene is made by replacing the hydrogen on

carbon three with a -CH_2-O-CH_2CH_2-O-CH_2CH_2-O-CH_3. This is soluble and reaches a conductivity of about 1000 S cm^{-1} upon doping. The third method is to grow the polymer into its desired shape and form. An insulating polymer impregnated with a catalyst system is fabricated into its desired form. This is then exposed to the monomer, usually a gas or a vapor. The monomer then polymerizes on the surface of the insulating plastic producing a thin film or a fibre. This is then doped in the usual manner. A variation of this technique is electrochemical polymerization with the conducting polymer being deposited on an electrode either the polymerization stage or before the electrochemical polymerization. This cast may be used for further processing of the conducting polymer. For instance, by stretching aligned bends of polyacetylene/polybutadiene the conductivity increase 10 fold, due to the higher state of order produced by this deformation. The final method is the use of Langmuir-Blodgett trough to manipulate the surface active molecules into a highly ordered thin films whose structure and thickness which are controllable at the molecular layer. Amphiphilic molecules with hydrophilic and hydrophobic groups produces monolayers at the air-water surface interface of a Langmuir-Blodgett trough. This is then transferred to a substrate creating a multilayer structure comprised of molecular stacks which are normal about 2.5 nm thick. The main advantage of this technique is its unique ability to allow control over the molecular architecture of the conducting films produced. It can be used to create complex multilayer structures of functionally different molecular layers as determined by the chemist. By producing alternating layers of conductor and insulator it is possible to produce highly anisotropic film which is conducting within the plane of the film, but insulating across it [22].

1.1.4.2 Chemical Polymerization

Chemical polymerization is very cheap and sample method. Monomers are oxidized by oxidizing agents or catalyst to produce conducting polymers [23].

The advantage of chemical synthesis is that it offers mass production at reasonable cost. However, there are several drawbacks that cause the formation of low quality conducting polymers. Firstly, polymer is formed in its oxidized form. The oxidized polymer chains can precipitate from polymerization medium limiting the degree of polymerization. Secondly, some oxidizing agents may be quite powerful for monomers used. This causes overoxidation of the polymer by decreasing the inherent properties of it. Another drawback is the occurrence of side chain reactions during chemical polymerization resulting in the formation of coupling defects along the backbone [24].

1.2 Enzymes

Enzymes are the catalysts that function to enhance the rates of biochemical reactions without themselves suffering any overall change. The reactants of enzyme-catalyzed reactions are termed as substrates and each enzyme is quite specific in character, acting on a particular substrate or substrates to produce a particular product or products.

The existence of enzymes has been known since nineteenth century. The active agent breaking down the sugar was partially isolated in 1833 and it is given the name of diastase. Then, a substance was extracted from gastric juice and called pepsin. The general name 'ferments' was given to these and other active preparations. It was 1878, the term 'enzyme', comes from Greek *enzumé* meaning in yeast, and was proposed by Kühne. The Büchners showed that sugar fermentation could take place when a yeast cell extract was added even though no living cells were present. In 1926, Sumner was able to isolate and crystallize the enzyme urease from Jack-bean extracts. He was awarded with Nobel Prize in 1947 for this work [25].

All enzymes are proteins. As proteins, enzymes have unique sequence of

aminoacids (primary structure) and three-dimensional structure (secondary and tertiary structure). Additionally, some enzymes possess a quaternary structure when they are composed of two or more polypeptide chains. Ribozyme which is a class of RNA modifying catalysts are known as the unique enzyme that are not in the protein structure. Ribozymes are molecules of ribonucleic acid that catalyze reactions on the phosphodiester bond of other RNAs.

Some enzymes are conjugated proteins and contain a cofactor in addition to the amino acid sequence. Cofactor can be either inorganic compounds such as metal ions or organic compounds. If the cofactor is an organic compound, it is commonly referred to as a coenzyme and typically has a structure related to the vitamins. The metal ions typically act as activators of the enzymes and can be found as either an integral structural component of, or loosely bound to the enzyme. Similarly, coenzymes can be either permanently or intermittently bound to the enzyme molecule. The heat-labile, nondialyzable protein portion of the enzyme is known as apoenzyme. Together the apoenzyme and the cofactor or coenzyme form the catalytically active unit called holoenzyme [26].

1.2.1 Enzyme Classification

A systematic way of naming and classifying enzymes was a necessity by increasing the number of known enzymes. The Commission on Enzymes (EC) was established in 1956 under the direction of the International Union of Biochemistry (IUB) to establish a systematic process for enzyme nomenclature. From the work of this original committee and subsequent recommendations, a completely revised version of Enzyme Nomenclature was published in 1972. The enzymes are classified and named according to the reaction they catalyze and the enzyme name has two parts. The first names the substrate, and the second, ending in –ase, indicates the type of reaction catalyzed. Each has an EC code number that characterizes the reaction

type, each subclass and sub-subclass, and specific enzyme [26]. The six classes of enzymes are listed such as;

1. Oxidoreductases: Catalyze oxidation-reduction reactions between two substrates

2. Transferases: Catalyze the transfer of a group other than hydrogen between two subunits.

3. Hydrolases: Catalyze the hydrolytic cleavage of compounds.

4. Lyases: Catalyze the removal of groups from substrates without hydrolysis, leaving double bonds in the product.

5. Isomerases: Catalyze the interconversion of isomers.

6. Ligases: Catalyze the joining of two molecules coupled with the hydrolysis of a pyrophosphate bond in ATP or similar component.

1.2.2 Enzyme Kinetics

In order for a reaction to take place, reactant molecules must have sufficient energy to overcome a potential energy barrier known as the energy of activation. Enzymes function as biochemical catalysts by lowering the energy of activation as shown in Figure 1.6. They accomplish this task by attaching to the reacting substrate molecules, forming an enzyme-substrate (ES) complex. This ES complex brings the substrate molecules into proper alignment with the enzyme so that its catalytic activity can be exerted and product can be formed. Once catalysis has occurred, the enzyme remains unchanged and is free to catalyze other reactions. The general reaction can be written as:

$$E + S \rightarrow ES \rightarrow P + E$$

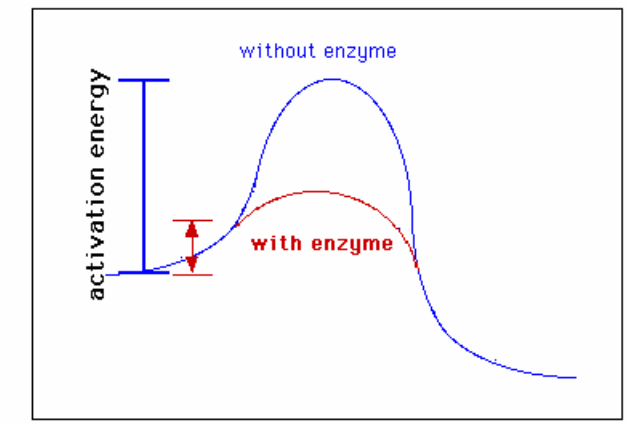


Figure 1.6 The schematic diagram showing the free energy changes of a reaction both uncatalyzed and catalyzed with an enzyme.

1.2.2.1 Michaelis-Menten Equation

The unique role of enzymes makes the kinetic of enzyme-catalyzed reactions significantly different from that of conventional chemical kinetics. This was first realized by L. Michaelis and M.L. Menten in 1913, when they developed a quantitative theory for the enzyme kinetics [25-27]. The simplest general equation for a single-substrate enzyme-catalyzed reaction is:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P$$

Where the terms k_1, k_{-1} and k_2 are rate constants for, respectively, the association of substrate and enzyme, the dissociation of unaltered substrate from the enzyme and the dissociation of product (= altered substrate) from the enzyme. There

is the theoretical possibility of a reverse reaction, with ES complex forming from E and P, but this can be ignored because initial rates of reaction are considered, i.e. when the enzyme is first provided with substrate, so there should not be any product available to combine with enzyme.

The overall rate of the reaction (v) is limited by the step ES to E + P, and this will depend on two factors - the rate of that step (i.e. k_2) and the concentration of enzyme that has substrate bound, i.e. [ES]. This can be written as:

$$v = k_2[ES] \tag{1.2}$$

At this point, two assumptions must be made. The first is the availability of a vast excess of substrate, so that [S]>> [E]. Secondly, it is assumed that the system is in steady-state, i.e. that the ES complex is being formed and broken down at the same rate, so that overall [ES] is constant. The formation of ES will depend on the rate constant k_1 and the availability of enzyme and substrate, i.e. [E] and [S]. The breakdown of [ES] can occur in two ways, either the conversion of substrate to product or the non-reactive dissociation of substrate from the complex [25]. In both instances the [ES] will be significant. Thus, at steady state we can write:

$$k_1[E][S] = k_{-1}[ES] + k_2[ES] \tag{1.3}$$

The next couple of steps are rearrangements of this equation. First of all the rate constants can be collected together on the right-hand side because they are both multiplied by [ES], this gives:

$$k_1[E][S] = (k_{-1} + k_2)[ES] \tag{1.4}$$

Then dividing both sides by (k_{-1} + k_2), this becomes:

$$\frac{k_1[E][S]}{k_{-1}+k_2}=[ES] \tag{1.5}$$

The three rate constants are now on the same side of the equation. As the name implies, these terms are constants, so we can actually combine them into one term. This new constant is termed the Michaelis constant and is written Km [25].

$$Km=\frac{(k_{-1}+k_2)}{k_1} \tag{1.6}$$

Substituting this definition of Km into the previous equation gives:

$$[ES]=\frac{[E][S]}{Km} \tag{1.7}$$

The total amount of enzyme in the system must be the same throughout the experiment, but it can either be free (unbound) E or in complex with substrate, ES. If we term the total enzyme E_0, this relationship can be written out:

$$[E_0]=[E]+[ES] \tag{1.8}$$

This can be rearranged (by subtracting [ES] from each side) to give:

$$[E]=[E_0]-[ES] \tag{1.9}$$

So, the [E] free in solution is equal to the total amount of enzyme minus the amount that has substrate bound. Substituting this definition of [E] back into equation 1.7 gives us:

$$\frac{([E_0]-[ES])[S]}{Km}=[ES] \tag{1.10}$$

This can now be rearranged in several steps. First of all, the bracket is opened so that the terms $[E_0]$ and [ES] are separately multiplied by [S]

$$\frac{([E_0][S]) - ([ES][S])}{Km} = [ES] \tag{1.11}$$

Next, each side is multiplied by Km, this gives:

$$([E_0][S]) - ([ES][S]) = Km[ES] \tag{1.12}$$

Then the two [ES] terms are collected together on the same side. This gives:

$$[E_0][S] = Km[ES] + [ES][S] \tag{1.13}$$

Then because both terms on the right-hand side are multiplied by [ES], they can be collected together into a bracket:

$$[E_0][S] = [ES](Km + [S]) \tag{1.14}$$

Dividing both sides by (Km + [S]) now gives:

$$\frac{[E_0][S]}{Km + [S]} = [ES] \tag{1.15}$$

Substituting this left-hand side into equation 1.2 in place of [ES] results in:

$$v = \frac{k_2[E_0][S]}{Km + [S]} \tag{1.16}$$

The maximum rate called Vmax would be achieved when all of the enzyme molecules have substrate bound. Under conditions when [S] is much greater than [E], it is fair to assume that all E will be in the form ES. Therefore $[E_0] = [ES]$. Thinking

again about equation 1.2, the term Vmax can be substituted for v and $[E_0]$ for [ES]. This would give:

$$V\max = k_2[E_0] \tag{1.17}$$

The term $k_2[E_0]$ was present in the previous equation, so it can be replaced with Vmax, giving a final equation:

$$\nu = \frac{V\max[S]}{Km + [S]} \tag{1.18}$$

This final equation is actually called the Michaelis-Menten equation.

The significance becomes clearer when it is considered the case when the rate of reaction (υ) is exactly half of the maximum reaction rate (Vmax). Under those circumstances, the Michaelis-Menten equation could be written:

$$\frac{V\max}{2} = \frac{V\max[S]}{Km + [S]} \tag{1.19}$$

On dividing both sides by Vmax and then multiplying both sides by (Km +[S]) this becomes:

$$Km + [S] = 2[S] \tag{1.20}$$

Thus when the rate of the reaction is half of the maximum rate:

$$Km = [S] \tag{1.21}$$

The Km of an enzyme is therefore the substrate concentration at which the reaction occurs at half of the maximum rate [25]. If a normalized plot of the initial rate against initial substrate concentration for a reaction obeying the Michalies-Menten kinetics is considered:

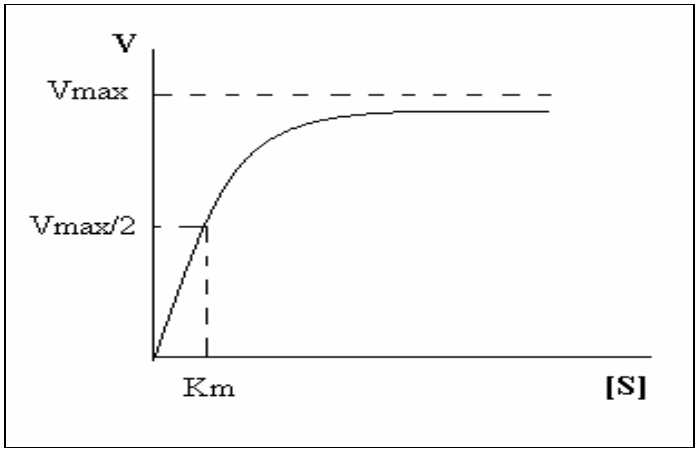


Figure 1.7 A normalized plot of the initial rate (v) against initial substrate concentration [S] for a reaction obeying the Michalies-Menten kinetics

Km is an indicator of the affinity that an enzyme has for a given substrate, and hence the stability of the enzyme-substrate complex.

As shown in the shape of the graph (Figure 1.7), at low [S], it is the availability of substrate that is the limiting factor. Therefore as more substrate is added there is a rapid increase in the initial rate of the reaction - any substrate is rapidly mopped up and converted to product. At the Km, 50% of active sites have substrate bound. At higher [S] a point is reached (at least theoretically) where the entire enzyme has substrate bound and is working flat out. Adding more substrate will not increase the rate of the reaction, hence the leveling out observed in the graph [25].

There are limitations in the quantitative (i.e. numerical) interpretation of this type of graph, known as a Michaelis plot. The Vmax is never really reached and therefore Vmax and hence Km values calculated from this graph are somewhat approximate. A more accurate way to determine Vmax and Km (though still

not perfect) is to convert the data into a linear Lineweaver-Burk plot [25].

1.2.2.2 Lineweaver – Burk Plot

Lineweaver-Burk plot is a plot of the reciprocal of velocity of an enzyme-catalyzed reaction versus the reciprocal of substrate concentration. The plot is used to graphically define the maximum velocity of an enzyme-catalyzed reaction and the Michaelis-Menten constant for the enzyme [25].

The plot is derived from the Michaelis-Menten equation:

$$\frac{1}{v} = \frac{Km + [S]}{V\max[S]} \tag{1.22}$$

If the reciprocals of both sides of the Michaelis-Menten equation, this becomes:

$$\frac{1}{v} = \frac{Km}{V\max}\frac{1}{[S]} + \frac{1}{V\max} \tag{1.23}$$

$$y = m.x + b$$

slope y-intercept

Substituting y for 1/Vmax and x for 1/[S], this is now the standard equation for a straight line, with slope = Km/Vmax, and y intercept = 1/Vmax.

A straight plot is much preferred over a curve, particularly when the data is slightly scattered due to experimental error. Slopes and intercepts are relatively easily obtained from a straight line graph. The result is known as the Lineweaver-Burk plot.

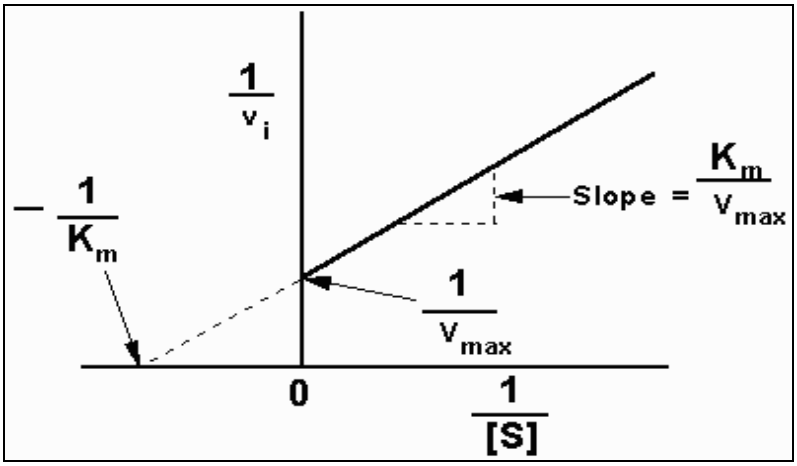


Figure 1.8 Lineweaver-Burk plot

1.2.3 Enzyme Immobilization

Without enzymes, most biochemical reactions would be too slow to even carry out life processes. Enzymes display great specificity and are not permanently modified by their participation in reactions. Since they are not changed during the reactions, it is cost-effective to use them more than once. However, if the enzymes are in solution with the reactants and/or products it is difficult to separate them. Therefore, if they can be attached to the reactor in some way, they can be used again after the products have been removed. The term "immobilized" means unable to move or stationary. And that is exactly what an immobilized enzyme is: an enzyme that is physically attached to a solid support over which a substrate is passed and converted to product. Immobilization of an enzyme provides multiple and repetitive use by increasing stability of enzyme. It prevents contamination and protects enzyme against change in pH, temperature and ionic strength in the bulk solvent.

The support material can have a critical effect on the stability of the enzyme and the efficiency of enzyme immobilization, although it is difficult to predict in

advance which support will be most suitable for a particular enzyme. The most important requirements for a support material are that it must be insoluble in water, have a high capacity to bind enzyme, be chemically inert and be mechanically stable. The enzyme binding capacity is determined by the available surface area, both internal (pore size) and external (bead size or tube diameter), the ease with which the support can be activated and the resultant density of enzyme binding sites. The inertness refers to the degree of non-specific adsorption and the pH, pressure and temperature stability. In addition, the surface charge and hydrophilicity must be considered. The activity of the immobilized enzyme will also depend upon the bulk mass transfer and local diffusion properties of the system [28].

It is important that the method of attachment of an enzyme to a surface must be chosen carefully in order to prevent loss of enzyme activity by not changing the chemical nature or reactive groups in the binding site of the enzyme. It is desired to avoid reaction with the essential binding site group of the enzyme. Alternatively, an active site can be protected during attachment as long as the protective groups can be removed later on without loss of enzyme activity.

1.2.3.1 Enzyme Immobilization Techniques

a) Carrier-Binding:

Immobilization by carrier-binding is the oldest technique. The nature of the carrier affects the amount of enzyme bound to the carrier and the activity of enzyme after immobilization.

The nature of enzyme, particle size, surface area, molar ratio of hydrophilic and hydrophobic groups and chemical composition determine the chosen of carrier.

The enzyme can bind to the carrier by physical adsorption, covalent binding, or ionic binding.

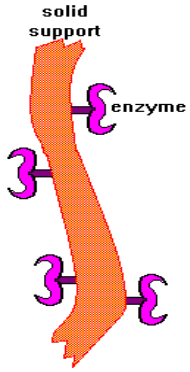


Figure 1.9 Schematic representation of carrier-binding type of enzyme immobilization

Physical Adsorption:

Physical adsorption of an enzyme onto a solid is probably the simplest way of preparing immobilized enzymes. The method relies on non-specific physical interaction between the enzyme protein and the surface of the matrix, brought about by mixing a concentrated solution of enzyme with the solid.

A major advantage of adsorption as a general method of insolubilizing enzymes is that usually no reagents and only a minimum of activation steps are required. As a result, adsorption is cheap, easily carried out, and tends to be less disruptive to the enzymic protein than chemical means of attachment, the binding being mainly by hydrogen bonds, multiple salt linkages, and Van der Waal's forces. In this respect, the method bears the greatest similarity to the situation found in biological membranes in vivo and has been used to model such systems.

Because of the weak bonds involved, desorption of the protein resulting from changes in temperature, pH, ionic strength or even the mere presence of substrate, is often observed. Another disadvantage is non-specific further adsorption of other

proteins or other substances as the immobilized enzyme is used. This may alter the properties of the immobilized enzyme or, if the substance adsorbed is a substrate for the enzyme, the rate will probably decrease depending on the surface mobility of enzyme and substrate.

Ionic Binding:

This method depends on the ionic binding of the enzyme to water-insoluble carriers containing ion-exchange residues.

Polysaccharides & synthetic polymers including anion-cation exchange centers are usually serve as carriers. Ionic binding method causes little changes in the conformation and active site of the enzyme.

Covalent Binding:

Covalent binding method occurs by creating a covalent bond between enzyme and carrier. The carriers used must contain functional groups on the surface. These functional groups form a bond with functional groups (carboxyl group, amino group, hydroxyl group, or sulfydryl group) belonging to amino acid residues on the surface of the enzyme [29].

Immobilization by covalent binding method is more complicated than physical adsorption and ionic binding methods.

b) Crosslinking:

In this type of immobilization, enzyme molecules are attached to an insoluble support medium by intermolecular crosslinking of the protein to either other protein molecules or functional groups. It is an expensive and insufficient method. The support medium comprises of the protein itself which results in low enzyme activity. Also, the conditions that crosslinking reactions are carried out are severe. For this

reason, conformational changes on enzyme may occur [30].

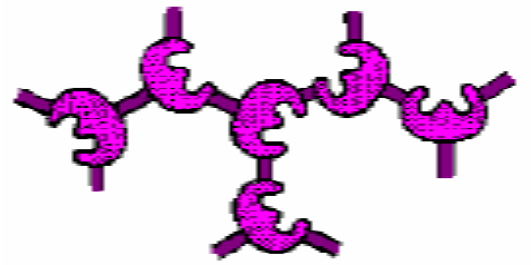


Figure 1.10 Schematic representation of crosslinking type of enzyme immobilization

c) Entrapment:

Enzyme molecules are entrapped in a lattice of polymer matrix or membrane. The molecules of enzyme stay constant inside while substrate molecules penetrate through the matrix. The difference from other two methods is that enzyme itself does not bind to any support [31]. There are two types of entrapment methods:

Lattice-Type: Enzymes are entrapped within the interstitial spaces of a crosslinked water-insoluble polymer. Some synthetic polymers such as polyarylamide, polyvinylalcohol, etc. and natural polymer such as starch can be used to immobilize enzymes.

Microcapsule-Type: Enzymes are enclosed within semi permeable polymer membranes.

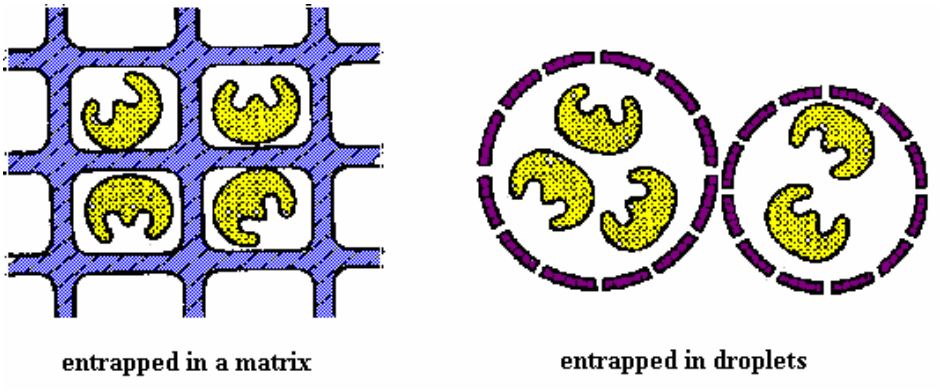


Figure 1.11 Schematic representation of entrapment type of enzyme immobilization

1.2.4 Enzyme Inhibition

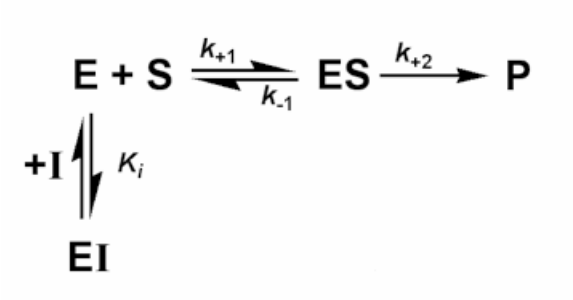
A number of substances may cause a reduction in the rate of an enzyme catalyzed reaction. Some of these (e.g. urea) are non-specific protein denaturants. Others, which generally act in a fairly specific manner, are known as inhibitors. Loss of activity may be either reversible, where activity may be restored by the removal of the inhibitor, or irreversible, where the loss of activity is time dependent and cannot be recovered during the timescale of interest. If the inhibited enzyme is totally inactive, irreversible inhibition behaves as a time-dependent loss of enzyme concentration (i.e. lower Vmax), in other cases, involving incomplete inactivation, there may be time-dependent changes in both Km and Vmax. Heavy metal ions (e.g. mercury and lead) should generally be prevented from coming into contact with enzymes as they usually cause such irreversible inhibition by binding strongly to the amino acid backbone. More important for most enzyme-catalyzed processes is the effect of reversible inhibitors. There are four generally recognized classes of reversible inhibition.

1.2.4.1 Reversible Inhibition

a) Competitive Inhibition

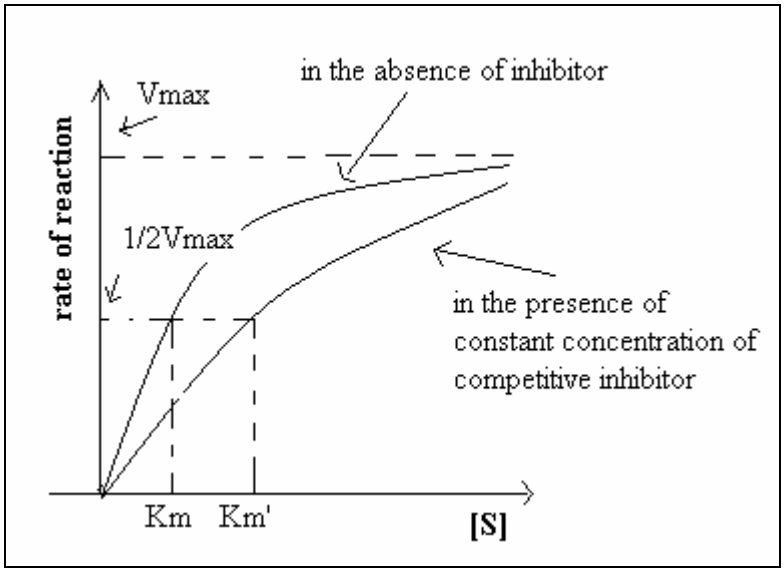
Competitive inhibitors resemble in some respects the substrates whose reactions they inhibit, and because of this structural similarity they may compete for the same binding-site on the enzyme. The enzyme-bound inhibitor then either lacks the appropriate reactive group or it is held in an unsuitable position with respect to the catalytic site of the enzyme or to other potential substrates for a reaction to take place. In either case a dead-end complex is formed, and the inhibitor must dissociate from the enzyme and be replaced by a molecule of substrate before a reaction can take place at that particular enzyme molecule. The effect of a competitive inhibitor depends on the inhibitor concentration, the substrate concentration, and the relative affinities of the substrate and the inhibitor for the enzyme [25].

A competitive inhibitor I can only bind to the unoccupied enzyme E, not to the ES complex. The quantity of complex EI that forms is governed by the equilibrium constant Ki, known as the inhibition constant. If more EI forms, fewer enzymes are available to form productive ES complex. However, since inhibitor I cannot bind to ES, very high substrate concentrations can overcome the inhibitor by forcing the substrate binding equilibrium in the direction of ES, and this brings the enzyme up to its normal Vmax. Hence the term, competitive, describe this inhibition.



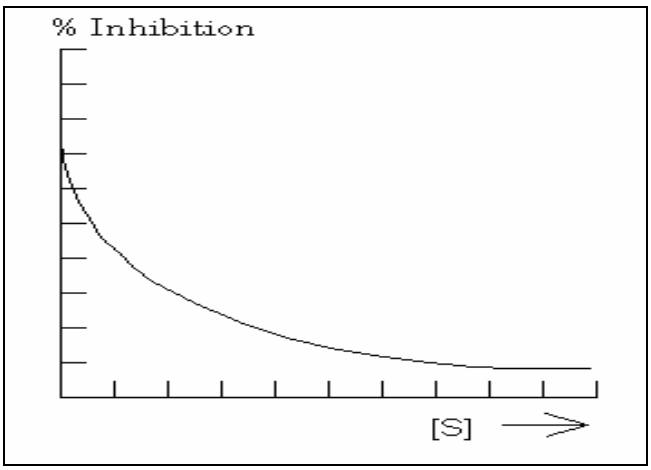
(1.24)

As described in the Michaelis-Menten hyperbolic plot, the rate of the enzyme reaction with a constant concentration [I] of inhibitor as [S] is varied. The rate rises more gradually when inhibitor is present, and levels off at a lower Vmax′ (1/2 Vmax). If inhibitor concentration [I] is set equal to Ki, this causes the Vmax′ observed to be halved relative to uninhibited enzyme. At very high substrate concentrations, substrate molecules will greatly outnumber inhibitor molecules and the effect of the inhibitor will be negligible. Hence Vmax for the reaction is unchanged. However, apparent Km is clearly increased as a result of the inhibition and given the symbol Km′ [25].



(a)

Figure 1.12 (a) Michaelis-Menten plot showing the effect of a competitive inhibitor (b) The plot of the degree of inhibition versus substrate concentration in the presence of a competitive inhibitor.



(b)

Figure 1.12 (Cont.)

In order to illustrate steady-state kinetics of a simple single-substrate single-binding-site single-intermediate enzyme catalyzed reaction in the presence of competitive inhibitor, I, the dissociation constant (Ki) for the reaction between E and I (equation 1.25) must be defined firstly.

$$Ki = \frac{[E][I]}{[EI]} \tag{1.25}$$

Ki also defined as the inhibitor constant. Taking the inhibitor into account, initial enzyme concentration must be equal;

$$[E_0] = [E] + [ES] + [EI] \tag{1.26}$$

$$[E_0] = [E] + [ES] + \frac{[E][I]}{Ki} \tag{1.27}$$

$$= [E](1 + \frac{[I]}{Ki}) + [ES] \tag{1.28}$$

$$[E] = \frac{([E_0] - [ES])}{(1 + {[I]}/{Ki})} \tag{1.29}$$

By substituting this equation in to equation 1.7;

$$\frac{([E_0] - [ES])[S]}{(1 + {[I]}/{Ki})[ES]} = Km \tag{1.30}$$

$$\frac{([E_0] - [ES])[S]}{[ES]} = Km(1 + \frac{[I]}{Ki}) \tag{1.31}$$

The final equation can be obtained as;

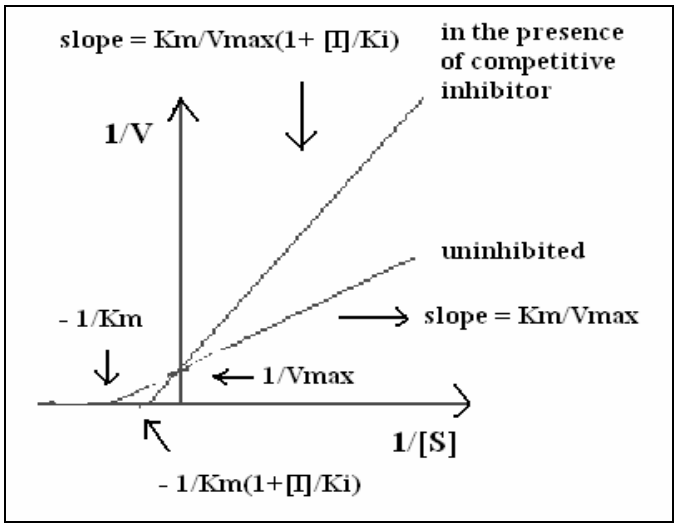
$$V_0 = \frac{V\max[S_0]}{[S_0] + Km(1 + {[I_0]}/{Ki})} \tag{1.32}$$

When analyzing the above equation, it is seen that Km has been increased by a factor [1 + ([I_0]/Ki)]. If the total enzyme concentration is much less than the total inhibitor concentration (i.e. [E_0] << [I_0]), then;

$$\frac{1}{V_0} = \frac{Km'}{V\max[S_0]} + \frac{1}{V\max} \tag{1.33}$$

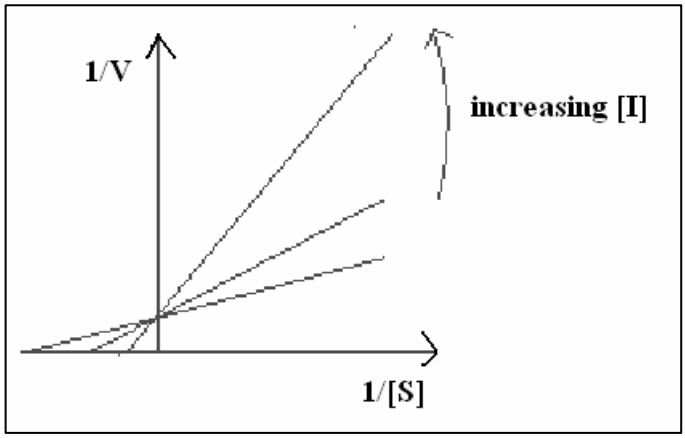
This equation (eqn. 1.33) is called as the Lineweaver-Burk equation. The key kinetic parameters to understand are Vmax and Km. For a simple competitive inhibition, Vmax is unchanged but Km is altered so that Km′ = Km[1 + [I_0]/Ki]. Km′ is the apparent Km value in the presence of a competitive inhibitor. We can use

LaChatelier's principle to understand this. If I binds to E alone, and not ES, it will shift the equilibrium of E + S <==> ES to the left, which would have the effect of increasing the Km′ (i.e. it would appear that the affinity of E and S has decreased). The double reciprocal plot (Lineweaver Burk plot) offers a great way to visualize the inhibition. In the presence of inhibitor, Vmax does not change, but Km appears to increase. Therefore, 1/Km, the x-intercept on the plot will get smaller, and closer to 0. Therefore the plots will consist of a series of lines, with the same y intercept (1/Vmax), and the x intercepts (-1/Km) closer and closer to the 0 as [I] increases. These intersecting plots (Figure 1.13) are the hallmark of competitive inhibition [25].



(a)

Figure 1.13 (a) Lineweaver-Burk plot showing the effect of competitive inhibition (b) the same, showing plots for several inhibitor concentrations at fixed enzyme concentration.



(b)

Figure 1.13 (Cont.)

Once the type of inhibitor has been established, it is desirable to determine the inhibitor constant, Ki. It is obtained from the expression Km′ = Km (1 + [I]/Ki), but a graphical method is preferred to a direct substitution of numbers to allow errors in individual determinations to be averaged out [25]. From the above expression;

$$Km' = (\frac{Km}{Ki})[I] + Km \tag{1.34}$$

So, a slope of Km′ (obtained from Figure 1.13a) against [I] will be linear, with the intercept on the [I] axis giving –Ki (Figure 1.14) [25].

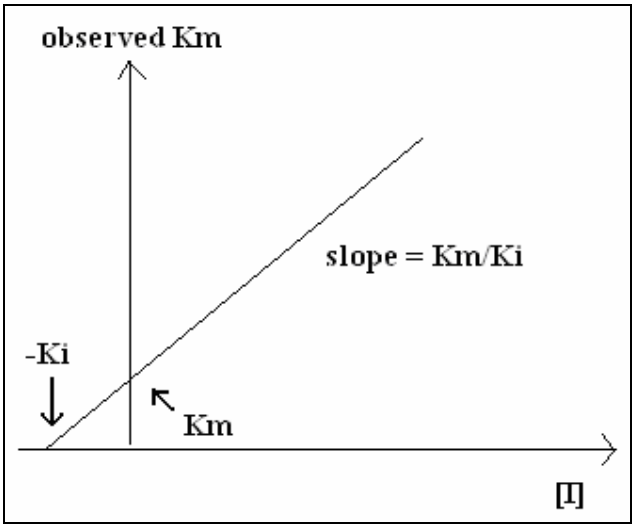
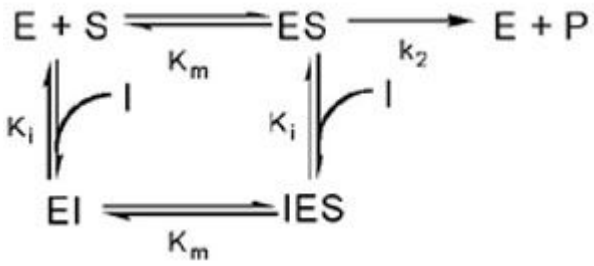


Figure 1.14 Secondary plot for competitive inhibition

b) Non-competitive Inhibition

If a reversible inhibitor can bind to the enzyme at a site that is distinct from the active site, it is described as a "noncompetitive inhibitor." In pure noncompetitive inhibition, the inhibitor binds with equal affinity to the free enzyme and to the enzyme-substrate (ES) complex [25]. The binding is described as shown below:



(1.35)

Even this is a complex situation, for ES can be arrived at by alternative routes, making it impossible for an expression of the same form as the Michaelis-Menten equation to be derived using the general steady-state assumption. In the simplest possible model, simple linear non-competitive inhibition, the substrate does not affect inhibitor binding. Under these conditions the reactions E + I $\rightleftharpoons$ EI and ES + I $\rightleftharpoons$ ESI have an identical dissociation constant Ki and called the inhibitor constant. The total enzyme concentration is effectively reduced by the inhibitor, decreasing the value of Vmax but not altering Km, since neither inhibitor nor substrate affects the binding of the other, as deduced from LaChatelier's principle [25].

As described before,

$$Km = \frac{[E][S]}{[ES]} \tag{1.36}$$

In the presence of a noncompetitive inhibitor which will bind equally well to E or to ES,

$$Ki = \frac{[E][I]}{[EI]} = \frac{[ES][I]}{[ESI]} \tag{1.37}$$

$$[E_0] = [E] + [ES] + [EI] + [ESI] \tag{1.38}$$

$$[E_0] = [E] + [ES] + \frac{[E][I]}{Ki} + \frac{[ES][I]}{Ki} \tag{1.39}$$

$$[E_0] = ([E] + [ES])\left(1 + \frac{[I]}{Ki}\right) \tag{1.40}$$

$$[E]+[ES]=\frac{[E_0]}{\left(1+\frac{[I]}{Ki}\right)} \tag{1.41}$$

$$[E]=\frac{[E_0]}{\left(1+\frac{[I]}{Ki}\right)}-[ES] \tag{1.42}$$

As a conclusion,

$$V_0=\left(\frac{V\max}{1+\frac{[I]}{Ki}}\right)\left(\frac{[S_0]}{[S_0]+Km}\right) \tag{1.43}$$

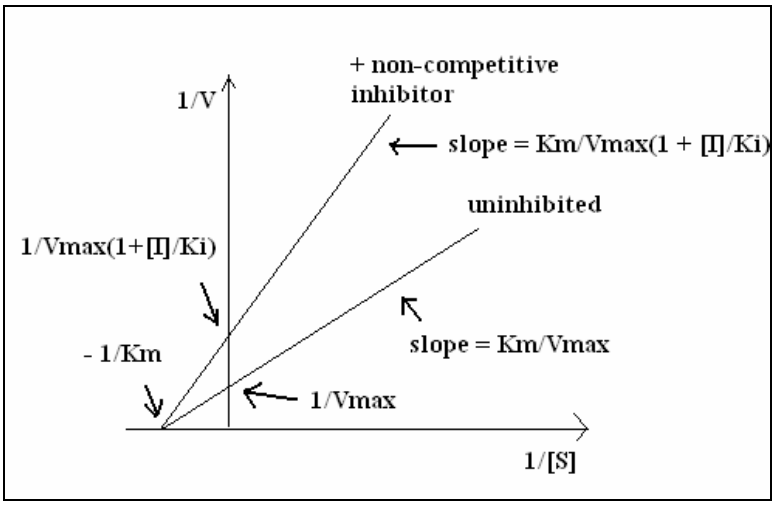
This is the form of the Michaelis-Menten equation. For simple linear non-competitive inhibition, Km is unchanged and Vmax is altered so that,

$$\frac{1}{V\max'}=\frac{1}{V\max}\left(1+\frac{[I]}{Ki}\right) \tag{1.44}$$

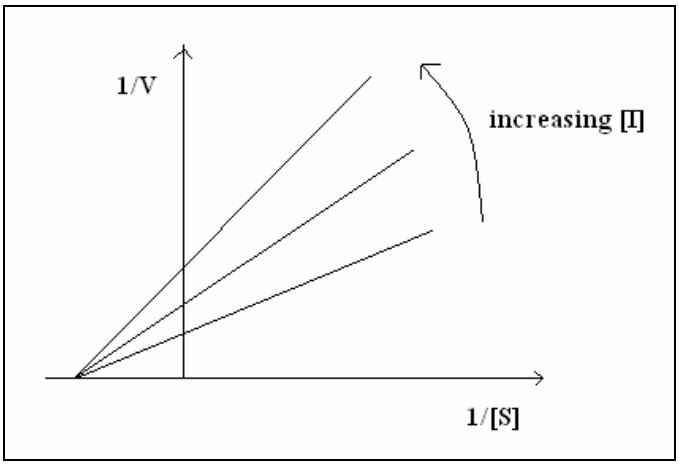
where Vmax′ is the value of Vmax in the presence of non-competitive inhibitor. The Lineweaver-Burk equation for simple non-competitive inhibition is;

$$\frac{1}{V_0}=\frac{Km}{V\max'}\frac{1}{[S]}+\frac{1}{V\max'} \tag{1.45}$$

and Lineweaver-Burk plots showing the effect of such inhibition are shown in Figure 1.15 [25].



(a)



(b)

Figure 1.15 (a) Lineweaver-Burk plot showing the effect of noncompetitive inhibition (b) the same, showing plots for several inhibitor concentrations at fixed enzyme concentration

After establishing the type of inhibition, Ki may be determined from secondary plots.

$$\frac{1}{V\max'} = \left(\frac{1}{V\max Ki}\right)[I] + \frac{1}{V\max} \tag{1.46}$$

Drawing the plots of 1/Vmax′ against [I], the intercept on the [I] axis gives – Ki value (Figure 1.16) [25].

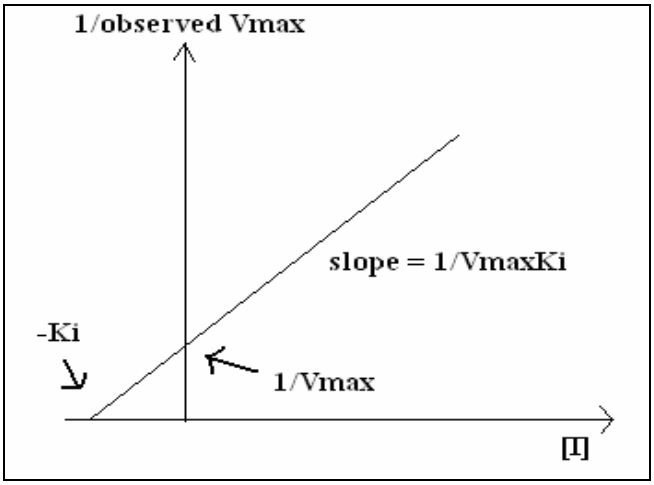
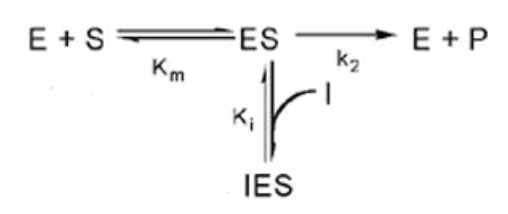


Figure 1.16 Secondary plot for noncompetitive inhibition

c) Uncompetitive Inhibition

Uncompetitive inhibition occurs when I bind only to ES and not free E. One can hypothesize that on binding S, a conformational change in E occurs which

presents a binding site for I. Inhibition occurs since ESI can not form product. It is a dead end complex which has only one fate, to return to ES [25]. This is illustrated in the mechanism below.



(1.47)

Both Km and Vmax are altered, but a distinctive kinetic pattern emerges under steady-state conditions.

$$Ki = \frac{[ES][I]}{[ESI]} \tag{1.48}$$

$$Km = \frac{[E][S]}{[ES]} \tag{1.49}$$

$$[E_0] = [E] + [ES] + [ESI] \tag{1.50}$$

$$[E_0] = [E] + [ES] + \frac{[ES][I]}{Ki} \tag{1.51}$$

$$[E_0] = [E] + [ES]\left(1 + \frac{[I]}{Ki}\right) \tag{1.52}$$

$$[E] = [E_0] - [ES]\left(1 + \frac{[I]}{Ki}\right) \tag{1.53}$$

Substituting for [E] and continuing as in the section 1.2.2.1,

$$V_0 = \frac{V\max[S_0]}{[S_0]\left(1+\frac{[I]}{Ki}\right)+Km} \tag{1.54}$$

This is an equation of the same as the Michaelis-Menten equation. Thus for uncompetitive inhibition,

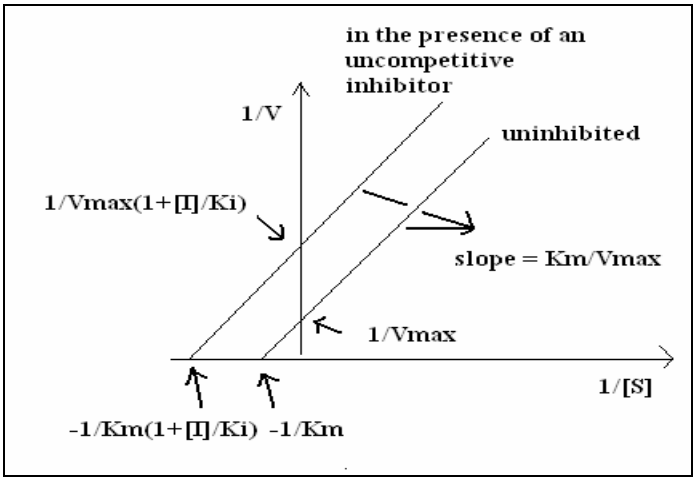
$$V\max' = \frac{V\max}{\left(1+\frac{[I]}{Ki}\right)} \tag{1.55}$$

$$Km' = \frac{Km}{\left(1+\frac{[I]}{Ki}\right)} \tag{1.56}$$

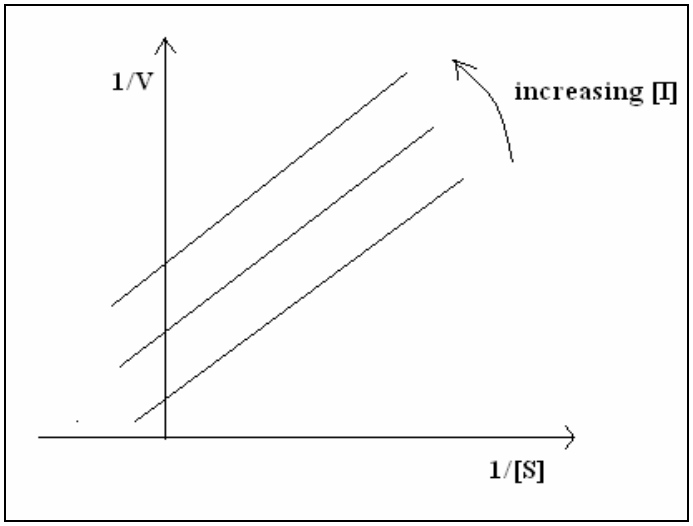
where Vmax′ is the Vmax value in the presence of an uncompetitive inhibitor and Km′ is the apparent Km under the same conditions. The Lineweaver-Burk equation in the presence of an uncompetitive inhibitor is;

$$\frac{1}{V_0} = \frac{Km'}{V\max'}\frac{1}{[S_0]} + \frac{1}{V\max'} \tag{1.57}$$

The slope of a Lineweaver-Burk plot is not altered by the presence of an uncompetitive inhibitor, but both intercepts change (Figure 1.17) [25].



(a)



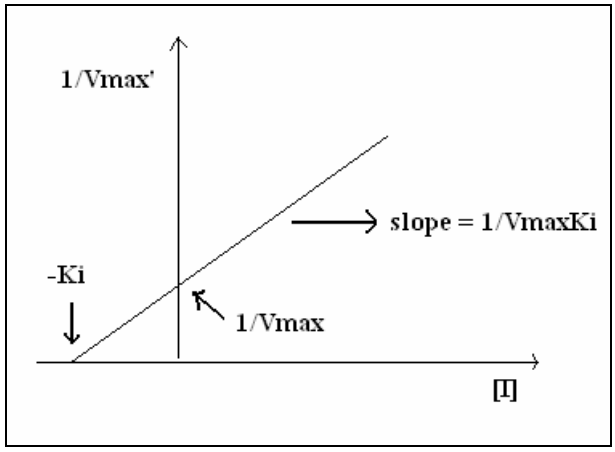
(b)

Figure 1.17 (a) Lineweaver-Burk plot showing the effect of uncompetitive inhibition (b) the same, showing plots for several inhibitor concentrations at fixed enzyme concentration.

As before, the inhibitor concentration Ki can be determined using secondary plots. It can be determined by using either observed Km or observed Vmax values (Figure 1.18).

$$\frac{1}{V\max'} = \frac{1}{V\max}\left(1 + \frac{[I]}{Ki}\right) \tag{1.58}$$

$$\frac{1}{Km'} = \frac{1}{Km}\left(1 + \frac{[I]}{Ki}\right) \tag{1.59}$$



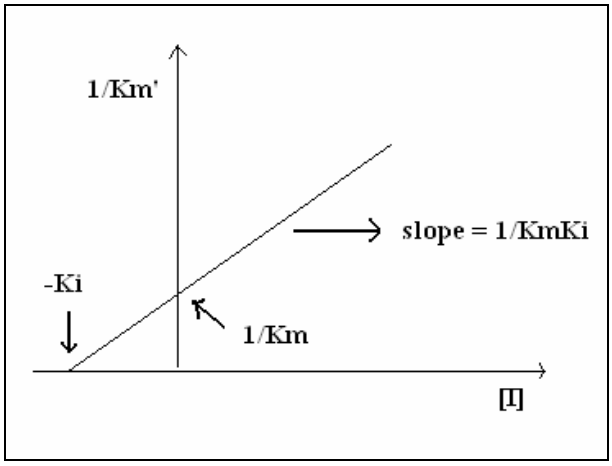


Figure 1.18 Secondary plots for uncompetitive inhibition

1.2.5 Polyphenol Oxidase – Tyrosinase

Polyphenol Oxidases (PPOs) are copper-containing proteins widely distributed through all the phylogenetic scale, from bacteria to mammals. The common feature of all groups is the capacity to catalyze the oxidation of aromatic compounds by oxygen. They are subdivided into two subclasses: tyrosinase and laccases. Both are characterized by the involvement of copper ligands and a role in the biosynthesis of melanins and other polyphenols.

Tyrosinase (EC 1.14.18.1) is a tetramer. The generally accepted weight of the mushroom tyrosinase is 128,000 Dalton. It contains a di-copper centre, strictly resembling that of hemocyanins. This di-copper centre can physiologically exist in three forms: oxy, met, deoxy.

Tyrosinase catalyzes two consecutive reactions: (1) the hydroxylation of monophenols with molecular oxygen to form o-diphenols; (2) the dehydrogenation of o-diphenols with oxygen to form o-quinones. O-quinones are usually highly reactive towards nucleophiles, so only seldom can these species be isolated as stable

products and used, owing to their typical absorption of visible light, for activity measurements. The most obvious nucleophile is water, or more correctly hydroxide ion; a hydroxyquinol then arises, quite prone to autoxidation and potentially again a substrate for the enzyme. The resulting hydroxyquinone is usually unstable, and leads to a complex mixture of unidentified products, most probably oligomers and/or polymers (Figure 1.19).

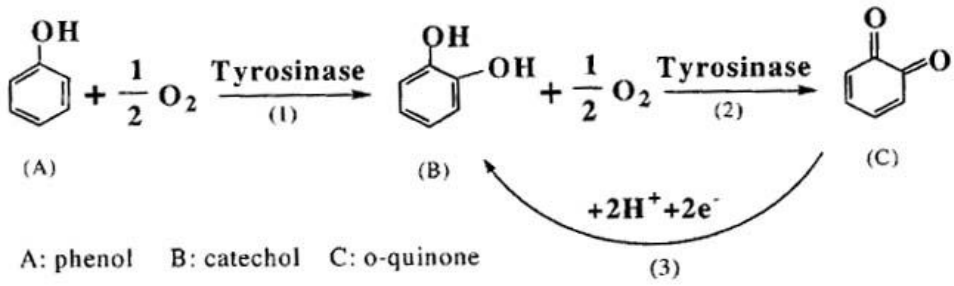


Figure 1.19 Schematic representation of tyrosinase activity.

Tyrosinase catalyzes the synthesis of melanin. The nature of the genoprotective activity of tyrosinase was found to be dependent upon the hydroxylation of tyrosine to L-DOPA and subsequent oxidation of L-DOPA to dopaquinone. This is quite interesting because L-DOPA is normally linked with toxic pro-oxidant properties as it produces highly unstable electrophilic dopa-(semi)quinones. On redox cycling these quinones produce harmful oxy radicals, peroxides, semiquinones, and quinones which are responsible for antitumor activity and neurotoxic damage in Parkinson's disease (based on treatment with L-DOPA). Furthermore, it was also found that the generation of oxygen free radicals, produced

from L-DOPA in the presence of Cu(II), is related with strand breakage of DNA under in vitro conditions [32].

Vitiligo is an autoimmune disease, characterized by hair pigmentation and total melanocyte depletion in the basal layer of the epidermis. Tyrosinase is responsible for the melanin production in the normal melanocytes and melanoma cells and is known to be an autoantigen in various autoimmune disorders. It acts as an autoantigen and serves as a marker for vitiligo [32,33].

Malignant melanoma continues to be a serious clinical problem with a high mortality rate among the human beings and this mortality rate is due to the failure of melanoma cells to respond to cytotoxic treatment in the form of radiation and chemotherapy. Tyrosinase by converting tyrosine into melanin allows selective conversion of inactive prodrugs, modeled on tyrosine, into cytotoxic drugs in melanoma cells [32].

Aromatic compounds, including phenols and aromatic amines, constitute one of major classes of pollutants and are heavily regulated in many countries. They are found in wastewaters of a variety of industries including coal conversion, petroleum refining, resins and plastics, wood preservation, metal coating, dyes and other chemicals, textiles, mining, dressing, and pulp and paper. Most aromatic compounds are toxic and must be removed from wastewaters before they are discharged into the environment [34]. There are many reported successful applications of soluble and immobilized tyrosinase in the cleansing of polluted waters [35-37]. The o-quinones produced by the catalytic activity of tyrosinase undergo spontaneous nonenzymatic polymerization in water. These polymers can be removed from solution through many processes such as adsorption, precipitation, and flotation.

1.2.6. Phenolics in Wine

Wine is composed of numerous chemicals, including alcohols, carbonyls,

esters, nitrogenous compounds, phenolic compounds, organic and non-organic compounds. Especially the phenolics have important functions. Grapes contain naturally occurring phenolic compounds. The phenolic compounds are well known for their sensory properties. These substances give wine its bitterness and astringency and are the foundation of long ageing, since they are effective antioxidants. They are found in the seeds and skins of the grapes.

The general class of phenolic compounds includes two major groups, flavonoids and non-flavonoids.

Flavonoids are a family of compounds that are all derivatives of 2-phenyl-1-benzopyran-4-one. They are lipophilic antioxidants. Flavonols, flavones, catechins, flavanones and anthocyanins are the major categories of the flavonoids [38]. The catechins are monomeric flavan-3-ol units that may combine to form oligomers, known as the proanthocyanidins, and polymers, known as the condensed tannins [39]. Quercetin is one of the major flavonoids in red wine. Flavonoids protect human low density lipoproteins (LDL) from oxidation in vitro. They are free radical scavengers of the reactive oxygen species that cause LDL oxidation. They have also been shown to chelate iron, which acts as a catalyst for these oxidants. Another important antioxidant attribute of flavonoids is their ability to inhibit many enzymes that catalyze LDL oxidation. Red wine flavonoids have been shown to reduce heart disease by decreasing platelet aggregation and LDL oxidation.

The non-flavonoid compounds in wine include hydroxybenzoates, hydroxycinnamates and stilbenes. The most significant compounds in this group in terms of possible health benefits are the stilbene resveratrol and the related compounds piceid and astringin [40,41].

Wine composition is greatly influenced by cultivar's climate, geography, soil type and training system as do phenolic content of grapes [42-44]. For this reason, amount of phenolics vary from one brand and type of wine to another. The content of phenolics may vary quite widely even between different bottles of the same red wine.

Gas and liquid chromatography are the typical methods for the determination of phenolic compounds. However, these methods suffer from complex sample pretreatment procedures. Also, they are unsuitable for on site or field based analyses. This problem can be solved by using biosensors where the bio-component is tyrosinase. Biosensors provide high specificity, high sensitivity and rapid detection mechanism.

1.2.7 Tyrosinase Inhibition

Tyrosinase inhibitors can be divided in to two: copper chelators (competitive with respect to oxygen) and substrate analogues (competitive towards phenol and/or diphenols substrates. However, such a classification is purely indicative, as many inhibitors can not be ascribed to a particular group and many of them behave as mixed-type inhibitors (competitive/non-competitive).

Copper chelators occupy an important position. Their action depends on the redox status of the PPO active site. Tropolone, benzhydroxamic acid, salicylhydroxamic acid, agaritine, barbarine, kojic acid, maltol, mimosine, diethyldithiocarbamate, and phenylthiourea are most popular copper chelators.

Simple catechols generally behave as both substrates and enzyme activators. However, when their quinonisation is chemically difficult or even impossible, they could behave as competitive inhibitors. The most interesting compound of this type is 2,3-dihydroxynaphthalene. Resveratrol family lacking any structural analogy and therefore forming a rather heterogeneous group is another group. Flavonoids' structure is in principle compatible with roles of both substrates and inhibitors (quercetin) [45].

A wide range of aromatic carboxylic acids were inhibitors for the activity of mushroom tyrosinase. All types of inhibitions were obtained depending on the nature of the inhibitor and the method used for the determination of the enzyme activity

[46]. Not only cinnamic, but also benzoic acids have been shown to be more or less effective PPO inhibitors. Benzoic acid behavior towards various PPOs is somewhat complicated. The type of inhibition is usually competitive with respect to catechols substrates and mixed with respect to oxygen. Interestingly, inhibition is strictly pH-dependent, but regardless of the particular pK value of the considered acid. It rather depends on the protonated vs. deprotonated status of a HIS residue, close to the di-copper center and possibly involved in metal binding [45].

Mushroom tyrosinase inhibitors have a wide range of applications in food and cosmetic industry.

Browning in fruits and vegetables is of great concern in food industry. Enzymatic browning affects the appearance, flavor, texture and nutritional value of the foods. The rate of enzymatic browning depends on the concentration of active tyrosinase and phenolic compounds, oxygen availability, pH, and temperature conditions in the tissue [32,47]. Thus, it is necessary to identify the methods to stop enzymatic browning caused by tyrosinase. Enzymatic browning can be controlled by tyrosinase inhibitors. The use of browning inhibitors in food processing is restricted by considerations of relevant to toxicity, wholesomeness, and effect on taste, flavor, texture, and cost.

Use of tyrosinase inhibitors is becoming increasingly important in the cosmetic industry due to their skin-whitening effects. A number of tyrosinase inhibitors are reported from both natural and synthetic sources, but only a few of them are used as skin-whitening agents, primarily due to various safety concerns. Currently, arbutin and aloesin are used in the cosmetic industry as whitening agents [32].

Benzoates and cinnamates are naturally found in red wines with high concentrations. Thus, during the analysis performed for the determination of amount of phenolic compounds, the effect of inhibitors on the measurements should be considered.

CHAPTER 2

EXPERIMENTAL

2.1 Chemicals

Pyrrole was purchased from Aldrich and sodium dodecyl sulfate (SDS) from Sigma. Pyrrole was distilled before use. 2-Tiophen-3-yl-ethanol (Aldrich), terepthaloyl chloride (Aldrich), triethylamine (Merck) and tetrabutylammonium tetrafluoroborate (TBAFB) (Aldrich) were used without further purification.

Tyrosinase (PPO) [E.C 1.14.18.1] was purchased from Sigma. Substrate for tyrosinase, catechol, was obtained from Sigma and all catechol solutions were prepared in citrate buffer. For preparation of citrate buffer, tri-sodium citrate-2 hydrate and citric acid were also used as received. 3-Methyl-2-benzothiozolinone (MBTH), acetone and sulfuric acid used in spectrophotometric activity determination of PPO were also obtained from Sigma. Benzoic acid, cinnamic acid and sorbic acid were also obtained from Sigma.

2.2 Instrumentation

2.2.1 Electrolysis

Constant current (galvanostatic) and/or constant potential (potentiometric) electrolysis were performed for electrochemical synthesis of conducting polymers.

Constant current electrolysis (CCE) is carried out in a cell containing two electrodes which are working and counter electrodes. The current is kept constant

during the electrolysis and the applied potential is allowed to vary. By inspection of the polymerization time, polymer film thickness can be easily controlled. In spite of being a simple method, it has some disadvantages. The nature of the generated species may be unknown because of the variable potential. The involvement of species present in the system in addition to the monomer is generally inevitable; thus complications may arise in the initiation and propagation steps [48]. The working and counter electrodes were platinum foils with an area of 2 cm^2.

Constant potential electrolysis (CPE) is carried out in a cell containing three electrodes which are working, counter and reference electrodes. The potential is maintained on the working electrode and the excessive current is prevented to flow through the reference electrode. The potential of the working electrode is adjusted to a desired value and kept constant while current is allowed to vary. By keeping the potential constant, unwanted species can be eliminated. The voltage between the working and counter electrodes is called as the polymerization potential (Epot) and the applied potential is determined by cyclic voltammetry [48]. The working and counter electrodes were platinum foils with an area of 2 cm^2. The reference electrode was Ag^0/Ag^+.

2.2.2 Fourier Transform Infrared Spectrophotometer (FTIR)

The FTIR spectra of conducting polymer blends were measured as dispersed in KBr pellets, using Varian 1000 FTIR spectrometer.

2.2.3 Nuclear Magnetic Resonance (NMR)

1H-NMR spectrum of the monomer was investigated on a Bruker-NMR Spectrometer (DPX-400) instrument. $CDCl_3$ was used as solvent.

2.2.4 Scanning Electron Microscope (SEM)

The SEM analyses of conducting polymer films were performed by using JEOL Scanning Microscope Model JSM-6400.

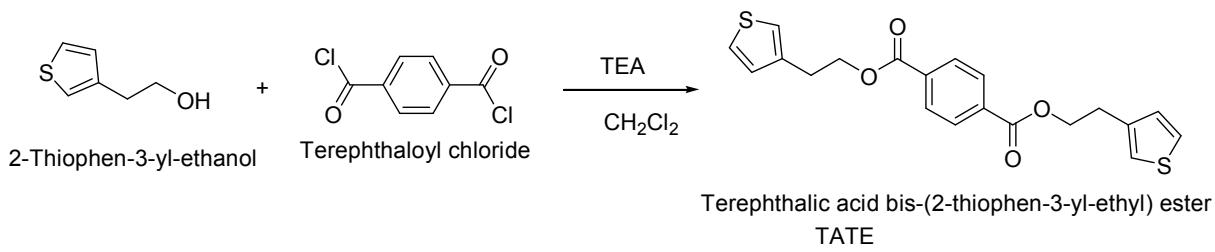
2.2.5 UV-VIS Spectrophotometry

A Shimadzu UV-1601 model spectrophotometer was used in the determination of activities of uninhibited free enzyme immobilized enzyme and inhibited free and immobilized enzyme.

2.3 Experimental Procedure

2.3.1 Synthesis of Terephthalic Acid Bis-(2-Thiophen-3-yl Ethyl) Ester (TATE)

As explained in the previous study [49], 2-thiophen-3-yl-ethanol was dissolved in dichloromethane containing triethylamine. To this solution, terepthaloyl chloride in dichloromethane was added drop wise in half an hour, by cooling in ice bath and under nitrogen atmosphere. The esterification was carried out for overnight at 0°C. Then the solution was washed with 1% HCl solution and water. The organic layer was dried over Na_2SO_4 and the solvent was removed via rotaevaporatory. Twice recrystallization form ethanol provided white crystals of TATE (Figure 2.1).



2-Thiophen-3-yl-ethanol Terephthaloyl chloride

Terephthalic acid bis-(2-thiophen-3-yl-ethyl) ester
TATE

Figure 2.1 Synthesis route of TATE

2.3.2 Homopolymerization of TATE (PTATE)

Homopolymerization of TATE was achieved via constant current electrolysis in a one compartment cell. 0.01 M TATE was dissolved in dichloromethane/TBATFB solvent-supporting electrolyte couple. Electrolysis was performed at 20 mA constant current for 10 minutes (Figure 2.2) [49].

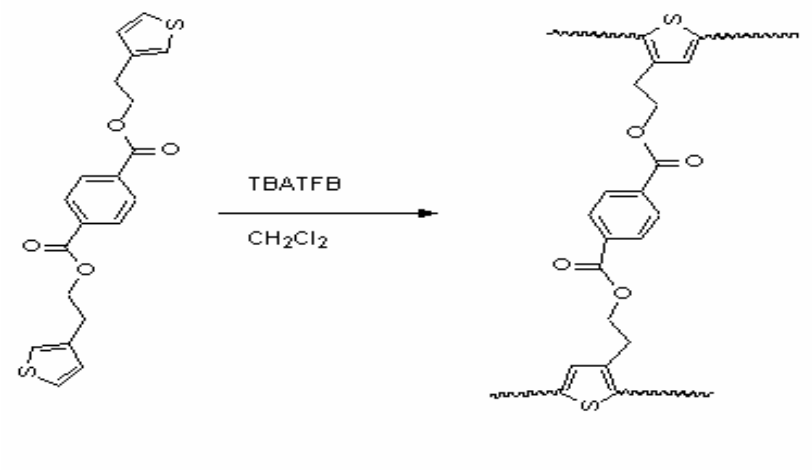


Figure 2.2 Synthesis route of homopolymer of TATE

2.3.3 Copolymerization of PTATE

Copolymerization of PTATE was achieved via constant potential electrolysis in a typical three electrode cell, consisting of PTATE coated Pt electrode as a working electrode, Pt as a counter electrode and Ag/Ag^{+} as a reference electrode. Electrolysis was performed at constant potential of +1.0 V for 20 min at room temperature (Figure 2.3) [50].

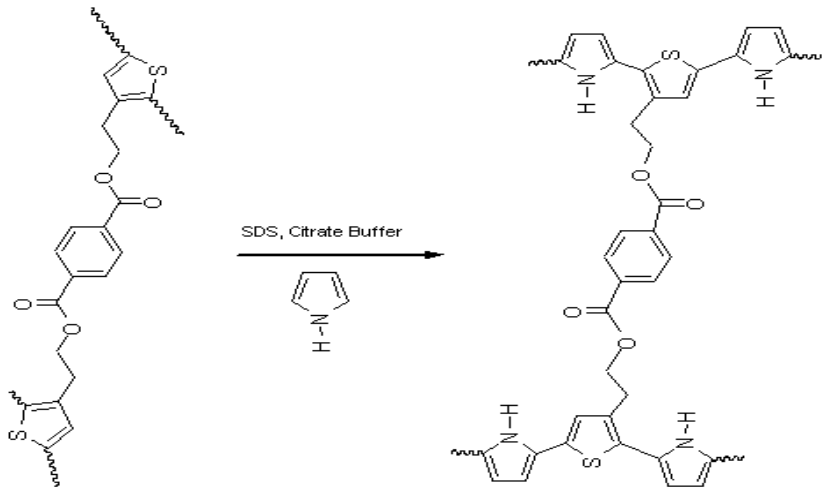


Figure 2.3 Copolymerization route of PTATE

2.3.4 Immobilization of Tyrosinase

2.3.4.1 Preparation of Enzyme Electrodes

Immobilization of tyrosinase was achieved during electrochemical polymerization of pyrrole on previously PTATE coated platinum electrode. Experiment was carried as described in section 2.3.3. The solution consists of 0.3 mg/L tyrosinase, 0.6 mg/L supporting electrolyte (sodium dodecyl sulfate), 0.01M pyrrole and 10 ml citrate buffer (pH 6.5). Enzyme electrodes were kept at 4°C in citrate buffer solution when not in use (Figure 2.4) [50].

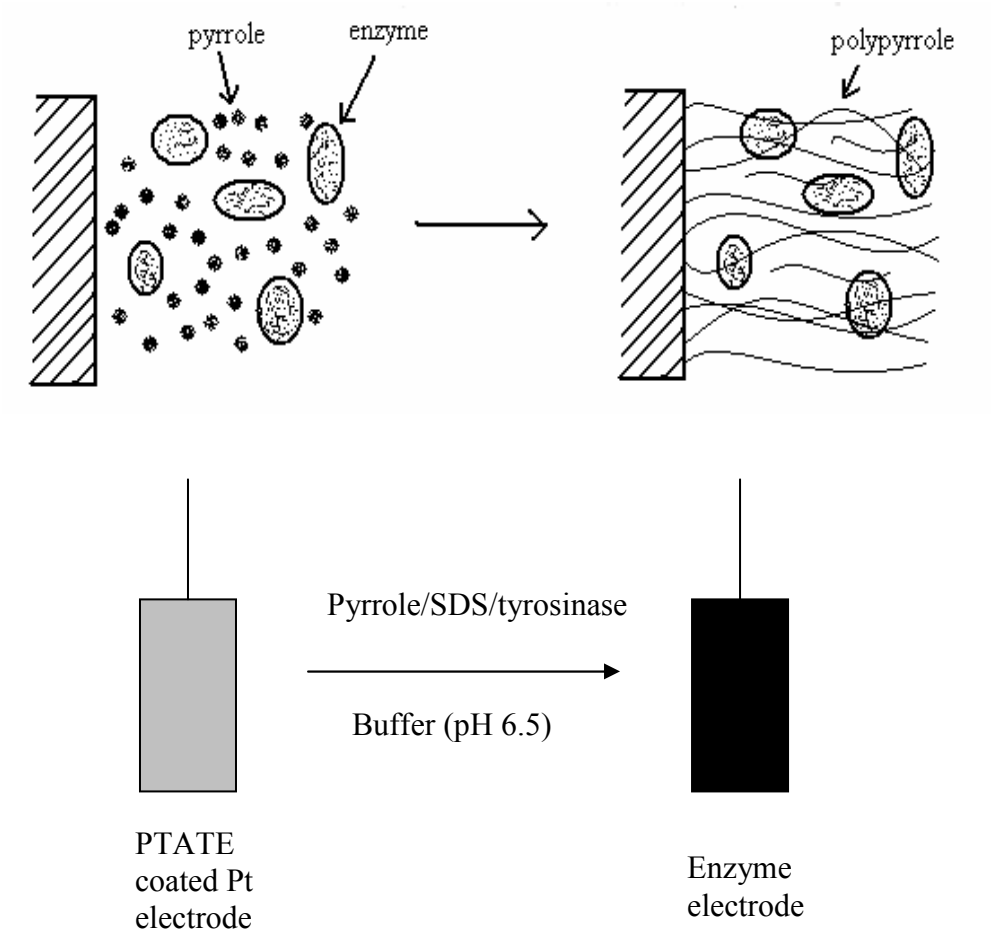


Figure 2.4 Immobilization of tyrosinase

2.3.4.2 Determination of Tyrosinase Activity

Determination of phenolic groups was achieved by Besthorn's Hydrazone Method [51] which includes spectrophotometric measurements. In this method, 3-methyl-2-benzothiozolinone (MBTH) reacts with the quinones produced by the enzyme to yield red products instead of brown colored pigments in the absence of the

color reagent [52]. The pathway proposed by Rodriguez et al is shown in Figure 2.5 [53].

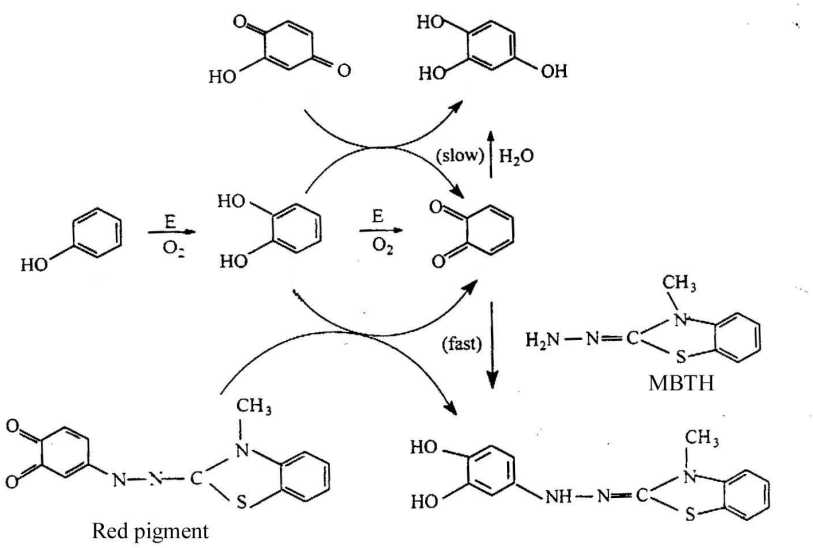


Figure 2.5 Schematic representation of Besthorn's Hydrazone Method

For the determination of free PPO activity, different concentrations of catechol were prepared. Solutions contain 1.0 ml of citrate buffer, 0.5 ml of MBTH (0.3 % in ethanol) and 0.5 ml of catechol. 0.5 ml of enzyme solution (0.1 mg/ml) was added and 1 min, 2 min and 3 min reaction times were given, respectively. After the reaction time, 0.5 ml of sulfuric acid (%5 v/v) was added to stop the enzymatic reaction. Quinone produced reacts with MBTH to form a red color complex and this complex was dissolved by adding 3.0 ml of acetone. After mixing, absorbances were measured at 495 nm by using UV-VIS spectrophotometer.

For immobilized PPO, different concentrations of catechol were prepared (3.0 ml) and put in water bath at 25°C. 1 ml of MBTH solution was added. Enzyme electrode was immersed in the solution and shaken for 5, 10 and 15 minutes. After enzymatic reaction, 1 ml of sulfuric acid and 1 ml of acetone were added for a total volume of 6 ml. After mixing, absorbances were measured at 495 nm by using UV-VIS spectrophotometer.

2.3.4.3 Kinetic Parameters for Free and Immobilized PPO

Kinetic studies of the enzymatic reaction for both free and immobilized PPO were performed at varying concentrations of catechol. Activity assay performed according to section 2.3.4.2.

2.3.4.4 Determination of Optimum Temperature and pH of Immobilized PPO

Optimum temperature and pH determinations were carried out by changing incubation temperature and pH between 10°C - 90°C and 2.5 - 12, respectively. The rest of the procedure was the same as the determination of PPO activity.

2.3.4.5 Determination of Operational Stability and Storage Stability of Immobilized PPO

Enzymes can easily lose their catalytic activity and denatured, so careful storage and handling are essential. To determine the stability against repetitive use and shelf-life of enzyme electrodes, the activity of electrode was checked. 20 measurements on the same day were done to perform the operational stability. For storage stability, measurements were performed for 30 days.

2.3.4.6 Protein Determination

Protein determination measurements were performed by Bradford's Method. Bradford reagent was prepared by mixing 25 ml of phosphoric acid, 12.5 ml of ethanol and 25 mg of Coomassie Brillant Blue (G-Dye). The mixture was diluted to 50 ml with distilled water. During measurements, a solution of Bradford reagent was prepared by mixing 1 volume stock solution with 4 volumes of distilled water.

For the preparation of protein calibration curve, bovine serum albumin (BSA) was used. Different concentrations of BSA were prepared as 1 ml and 2 ml of diluted Bradford reagent was added. The absorbances of these solutions were measured at 595 nm.

2.3.4.7 Determination of Amount of Phenolics in Red Wines with Enzyme Electrodes

Two Turkish red wines (Brand K and Brand D) were analyzed by using free and immobilized enzymes. Red wines were diluted with citrate buffer (pH 6.5) to a 1:3 volume and activity analysis was performed as the analysis mentioned in the 'Determination of PPO Activity' section.

2.3.4.8 Determination of Amount of Phenolics in Red Wines by Folin-Ciocalteau Method

Gallic Acid Stock Solution:

0.5 g of dry gallic acid was dissolved in 10 ml of ethanol and diluted to 100 ml with distilled water. It is stable up to two weeks in refrigerator.

Sodium Carbonate Solution:

200 g of anhydrous sodium carbonate was dissolved in 800 ml of water and brought to boiling. After cooling, a few crystals of sodium carbonate were added and after 24 hr, filtered and completed to 1 liter. To prepare a calibration curve, 0, 1, 2, 3, 5, and 10 ml of the above phenol stock solution were added in to 100 ml volumetric flask and diluted to 100 ml with distilled water. From each calibration solution, 20 µl of sample or blank were pippetted into separate cuvettes, and to each, 1.58 ml of distilled water and then 100 µl of Folin-Ciocalteau reagent were added and mixed. After 5 min, 300 µl of the sodium carbonate solution were added, and mixed. The solutions were kept at 20°C for 2 hr and the absorbance for each solution was determined at 765 nm.

2.3.4.9 Determination of Inhibitory Effects of Benzoic Acid, Cinnamic Acid and Sorbic Acid

Determination of inhibitory effects of these inhibitors was also achieved by Besthorn's Hydrazone Method. For determination of free and immobilized PPO activity in the presence of inhibitors, all steps described in the section for determination of enzyme activity were performed.

For the determination of free PPO activity, different concentrations of catechol were prepared. Solutions contain 0.5 ml of citrate buffer, 0.5 ml of inhibitor solutions at different concentrations, 0.5 ml of MBTH (0.3 % in ethanol) and 0.5 ml of catechol. 0.5 ml of enzyme solution (0.1 mg/ml) was added and 1 min, 2 min and 3 min reaction times were given, respectively. After the reaction time, 0.5 ml of sulfuric acid (%5 v/v) was added to stop the enzymatic reaction. Quinone produced reacts with MBTH to form a red color complex and this complex was dissolved by

adding 3.0 ml of acetone. After mixing, absorbances were measured at 495 nm by using UV-VIS spectrophotometer.

For immobilized PPO, different concentrations of catechol were prepared (2.5 ml) and 0.5 ml of inhibitor solution at different concentrations were added to this solution, then put in water bath at 25°C. 1 ml of MBTH solution was added. Enzyme electrode was immersed in the solution and shaken for 5, 10 and 15 minutes. After enzymatic reaction, 1 ml of sulfuric acid and 1 ml of acetone were added for a total volume of 6 ml. After mixing, absorbances were measured at 495 nm by using UV-VIS spectrophotometer.

CHAPTER 3

RESULTS and DISCUSSION

3.1 Synthesis and Characterization of Terephthalic Acid Bis-(2-Thiophen-3-yl-Ethyl) Ester (TATE)

Synthesis of TATE is a typical esterification reaction of 2-thiophen-3-yl-ethanol with terepthaloly chloride. The ^{1}H-NMR spectrum of TATE is shown in Figure 3.1 [49].

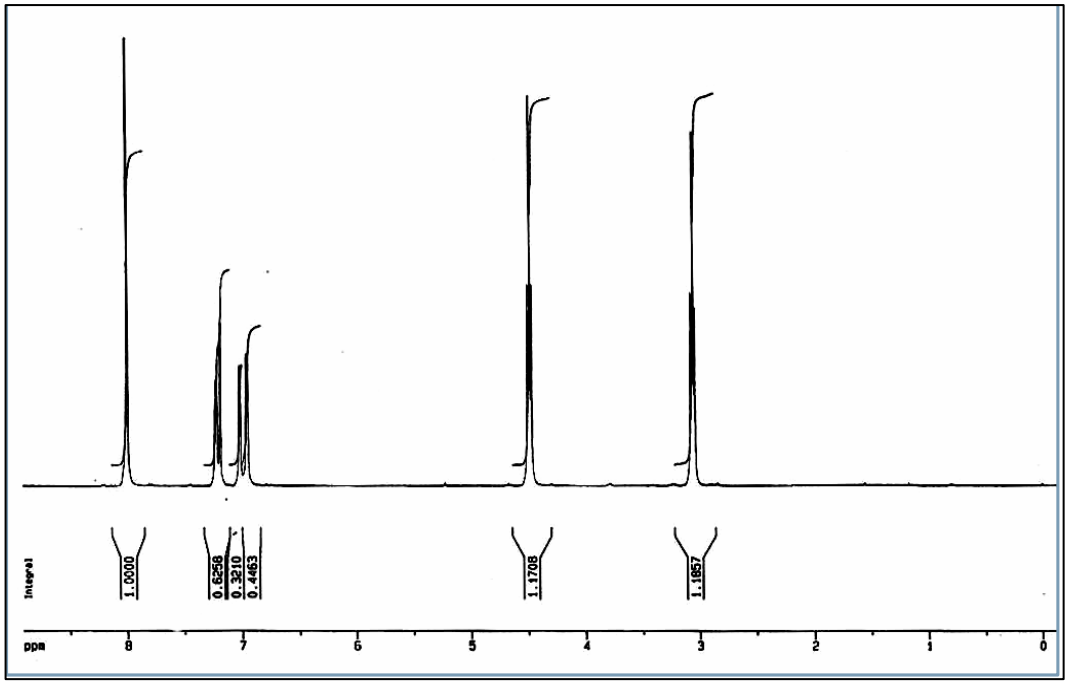


Figure 3.1 ^{1}H-NMR spectrum of TATE

^{1}H-NMR spectrum of the monomer was taken by using $CDCl_3$ as the solvent and chemical shifts (δ) were given relative to tetramethylsilane as the internal standard. ^{1}H-NMR spectrum of the monomer (δ, ppm) shows that 8 (s, 4H) from benzene ring, 6.95-7.3 (m, 6H) from 2,4,5 positions of thiophene ring, 4.5 (t, 4H) from $COOCH_2$, 3.1 (t, 4H) from thiophene-CH_2.

The FTIR spectrum of monomer also confirms the ^{1}H-NMR spectrum (Figure 3.2).

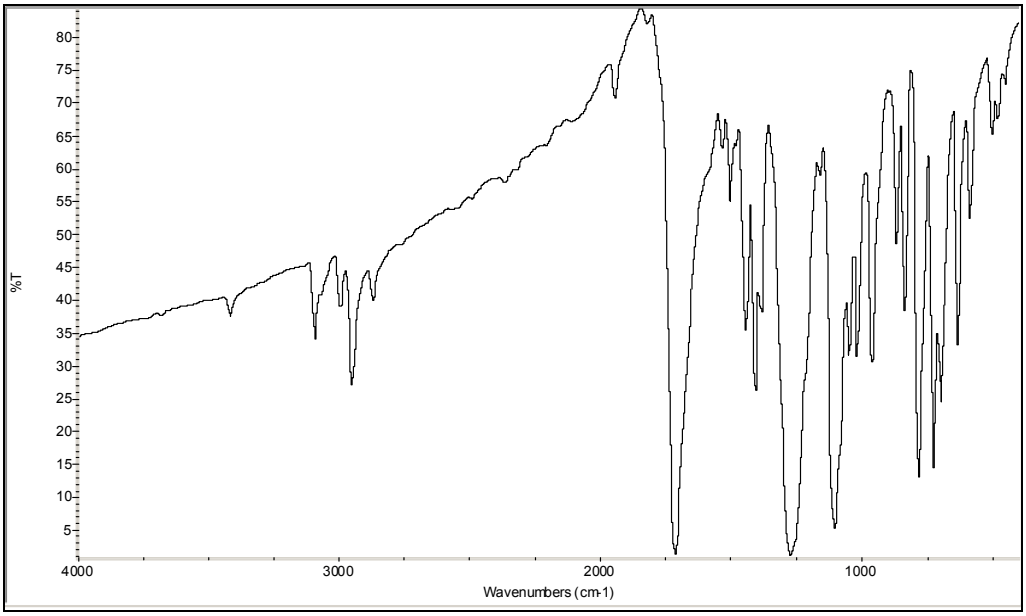


Figure 3.2 FTIR spectrum of TATE

As shown in Figure 3.2, C-H_α stretching modes which arise from thienylene are observed at 780 cm^{-1} and 3093 cm^{-1}. The bands related to the carbonyl group and

C-O-C are present at about 1713 cm^{-1} and 1271 cm^{-1}, respectively. Also, the peak at 833 cm^{-1} is related to β-hydrogen in the thiophene ring. The peaks at 2952 cm^{-1} and 2870 cm^{-1} belong to aliphatic C-H group [49].

3.2 Synthesis and Characterization of Homopolymer of TATE (PTATE)

For the synthesis of PTATE, constant current electrolysis was carried by using TBAFB as supporting electrolyte. As shown in Figure 3.3, the peak at 3093 cm^{-1} disappeared and the intensity of peak at 780 cm^{-1} decreased. These evidences indicate that the polymerization occurs from 2, 5 position of thiophene ring. The shoulder at around 1643 cm^{-1} shows conjugation and is a proof of polymerization. The sharp peaks in FTIR spectrum of the monomer between 1410 cm^{-1} and 1460cm^{-1} became broader, which is also an evidence of conjugation. The presence of dopant ion (BF_4^-) can be seen at 1078 cm^{-1}. The peaks at 1270 cm^{-1} and 1719 cm^{-1} appeared in spectrum reveal the existence of carbonyl group [49].

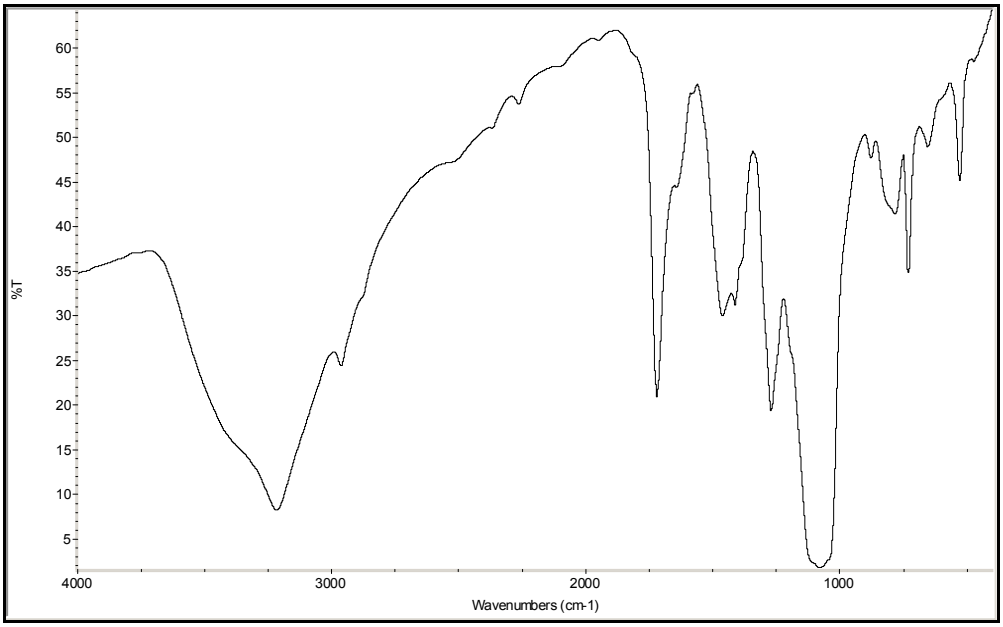


Figure 3.3 FTIR spectrum of PTATE

3.3 Synthesis and Characterization of Copolymer

As described in section 2.3.3, copolymerization of PTATE with PPy was achieved by constant potential electrolysis using SDS (Sodium Dodecyl Sulfate) as the supporting electrolyte. As shown in Figure 3.4, the peaks at 1717 cm^{-1} and 1267 cm^{-1} belong to the carbonyl group and C-O-C moiety, respectively. The peaks appeared at 1041 cm^{-1} and 1122 cm^{-1} show the presence of dopant ion. The peaks generated from PPy appear at 3450 cm^{-1}, 1551 cm^{-1} and 2350 cm^{-1}.

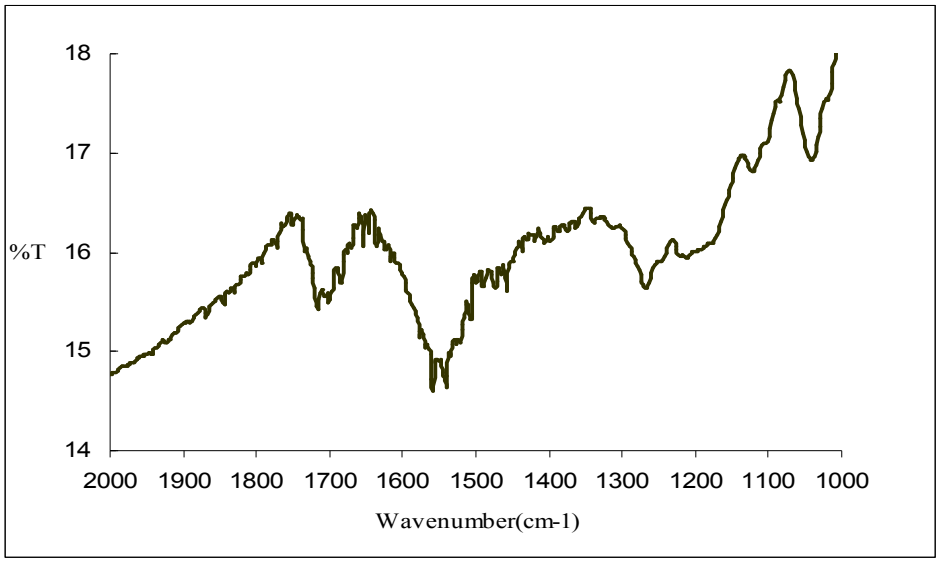


Figure 3.4 FTIR spectrum of the PTATE/PPy

3.4 Immobilization of Tyrosinase (PPO)

3.4.1 Tyrosinase Activity

Activity determinations of tyrosinase was achieved by the spectrophotometric measurement of absorbance o-quinone generated in the enzymatic reaction as described in the section 2.3.4.2. The calibration curve for Besthorn's Hydrazone method is shown in Figure 3.5.

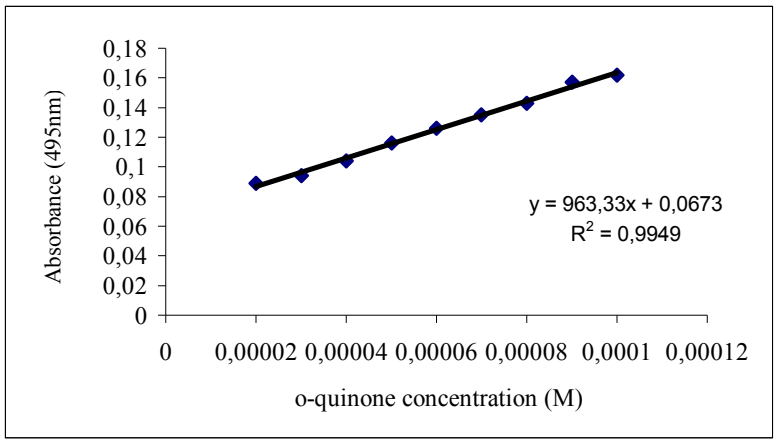


Figure 3.5 The calibration curve for Besthorn's Hydrazone Method

3.4.2 Protein Determination

As described in section 2.3.4.6, the amount of immobilized protein was determined by using Bradford's Method. Bovine serum albumin (BSA) was used for the preparation of protein calibration curve (Figure 3.6).

Results show that 9.3×10^{-3} mg protein and 6.0×10^{-3} mg protein entrapped in the PPy and PTATE/PPy matrices, respectively (Table 3.1) [50].

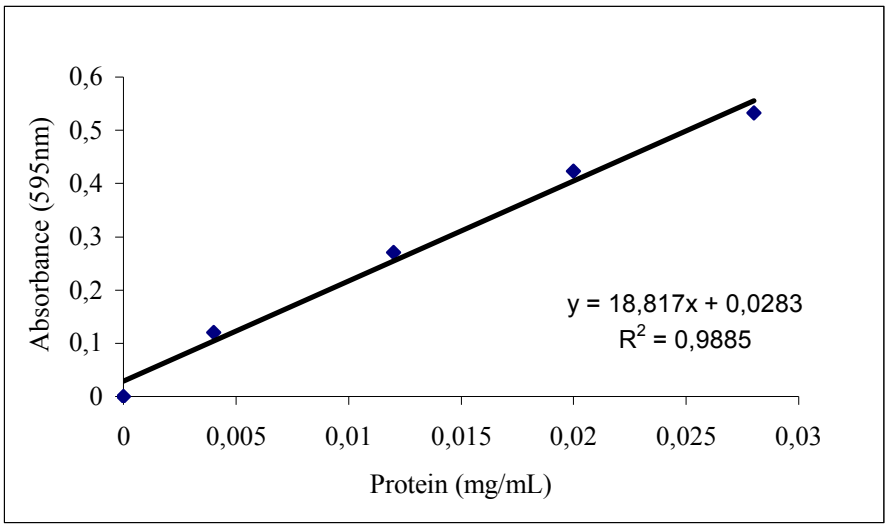


Figure 3.6 Calibration curve for protein determination

Table 3.1 Amounts of protein for enzyme electrodes

Free PPO	0.46 mg protein / mg enzyme powder
PPy/PPO	0.0096 mg protein / electrode
PTATE/PPy/PPO	0.0060 mg protein / electrode

3.4.3 Kinetic Parameters

Kinetic parameters obtained from Lineweaver-Burk plot are maximum

reaction rate (Vmax) and Michaelis-Menten constant (Km).

Free tyrosinase has a maximum reaction rate of 0.45 μmol/min.ml and Km of 0.6 mM (Table 3.2).

Vmax for tyrosinase immobilized in PPy matrix was found as 0.027 μmol/min.ml. For PTATE/PPy/PPO electrode, it was found as 0.017 μmol/min.ml. It is seen that when tyrosinase is immobilized, there is a decrease in reaction rate. However, it is an expected result upon immobilization and the previous studies also confirm the results. When Vmax of immobilized enzyme in PTATE/PPy matrix was compared with that of immobilized enzyme in PPy, it is seen that immobilized enzyme in PTATE/PPy matrix has a much lower Vmax value. These results were also confirmed by the protein amount entrapped in the electrodes which were 9.3×10^{-3} mg protein for PPy/PPO electrode and 6.0×10^{-3} mg protein for PTATE/PPy/PPO electrode.

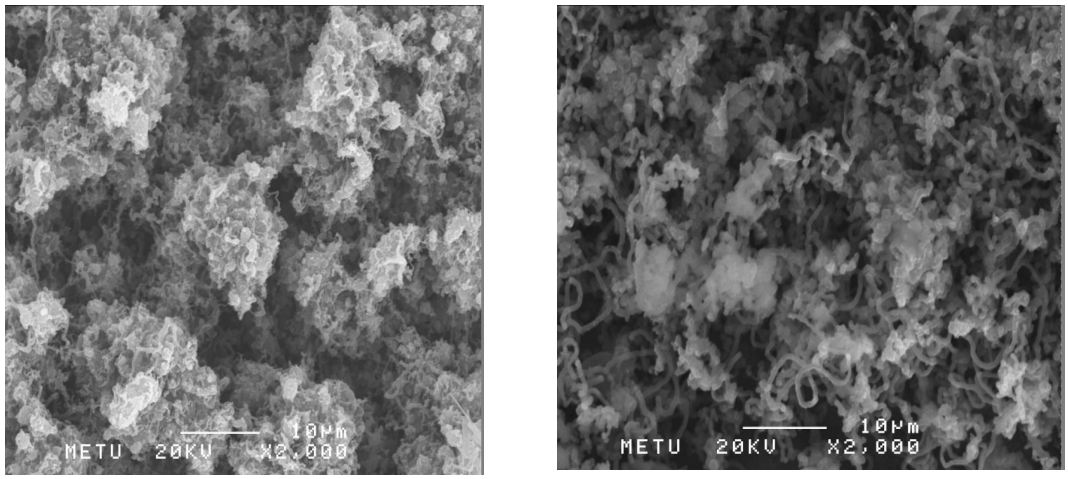
Km is a parameter that shows the affinity of enzyme toward substrate. Lower Km value means high affinity of enzyme toward substrate. While free tyrosinase had a Km value of 0.6 mM, Km value for tyrosinase immobilized in the PTATE/PPy matrix was 22 mM, and Km value for tyrosinase immobilized in PPy matrix was 100 mM. As expected, affinity of enzyme to substrate decreases when it is entrapped in a polymer matrix. However, PTATE/PPy matrix provides a better environment for tyrosinase when compared to PPy matrix (Table 3.2) [50].

Table 3.2 Kinetic parameters for free and immobilized tyrosinase

	Vmax (μmol/(min ml))	Km (mM)
Free PPO	0.45	0.6
PPy/PPO	0.027	100
PTATE/PPy/PPO	0.017	22

3.4.4 Morphologies of Films

The kinetic parameters can also be explained by interpreting the morphologies of the SEM micrographs of the enzyme entrapped films. As shown in Figure 3.7, PTATE/PPy matrix has large pores that give permission to substrate to diffuse through the enzyme; hence a low Km is obtained. However, PPy matrix has more compact environment and substrate cannot reach enzyme so easily, yielding high Km value.



(a) (b)

Figure 3.7 Scanning electron micrographs of (a) PPy/PPO (b) PTATE/PPy/PPO enzyme electrodes.

3.4.5 Stability Measurements of the Enzyme Electrodes

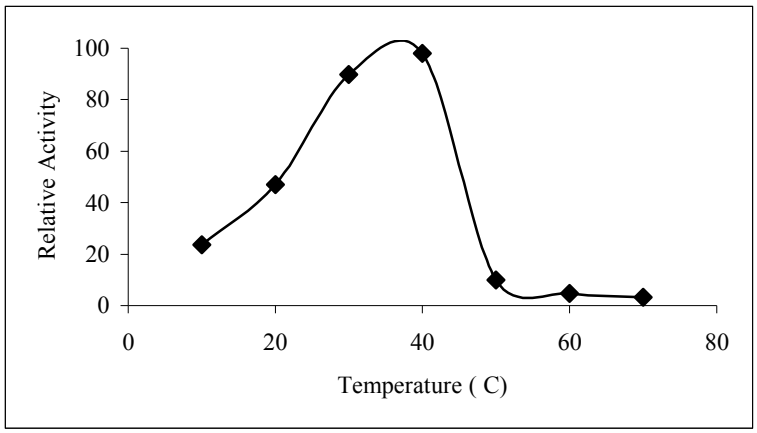
Enzymes are proteins which are peptide-water systems. The active conformation of the protein determines the stability of the catalytic activity of an enzyme. The three dimensional structure of an enzyme is formed by relatively complex and highly precise folding of a single polypeptide chain which has 5-10 amino acids arranged in a specific conformation. Since small changes in the conformation of amino acids at the active site of enzymes can cause dramatic changes in enzyme activity and substrate specificity, it follows that factors which influence protein structure and conformation will also influence the stability of the enzymatic action. Denaturation is the process where enzyme activity is lost via largely irreversible disruption and perturbation of the secondary, tertiary and quaternary protein structure.

The major factors that cause denaturation are the changes in pH and temperature. Immobilization is used widely to stabilize proteins and minimize these factors. The functionalized polymers generate a cage around the enzyme and stabilize the protein by protecting the active site of the enzyme.

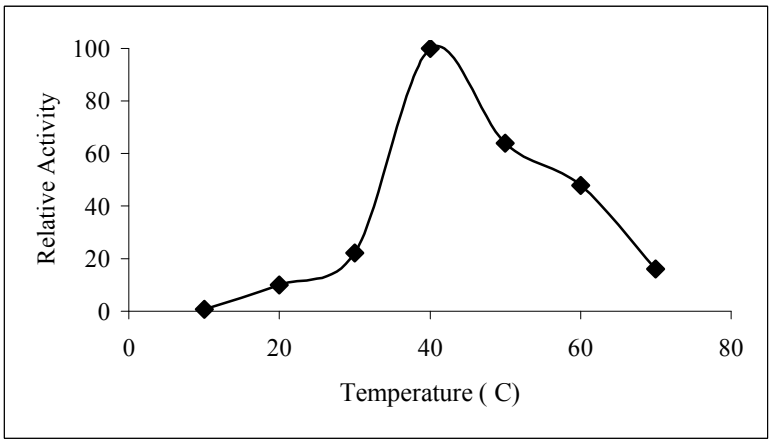
In order to understand the protection degree of the PTATE/PPy matrix, activity of enzyme electrodes in the case of changing pH and temperature was measured. The stability of electrodes in terms of operational stability and shelf life was also investigated.

3.4.5.1 Effect of Temperature on Enzyme Activity

Temperature is a characteristic property that gives information about stability of both free and immobilized enzyme towards environmental conditions. The effect of temperature on the enzyme activity is illustrated in Figure 3.8. For free enzyme, maximum enzyme activity was observed at 40°C and it lost almost all of its activity after 50°C. It would be preferable to use tyrosinase at high temperatures. However, free tyrosinase undergo essentially irreversible denaturation at temperatures above 50°C. Immobilized enzyme in PPy matrix also revealed a maximum enzyme activity at 40°C and it lost 40% of its activity at 50°C. On the other hand, PTATE/PPy/PPO electrode showed maximum enzyme activity at 70°C and after this temperature up to 80°C, it lost only %40 of its activity. Inactivation by heat denaturation has a profound effect on the enzymes productivity. These results imply that PTATE/PPy matrix provides better protection to tyrosinase against high temperatures [50].

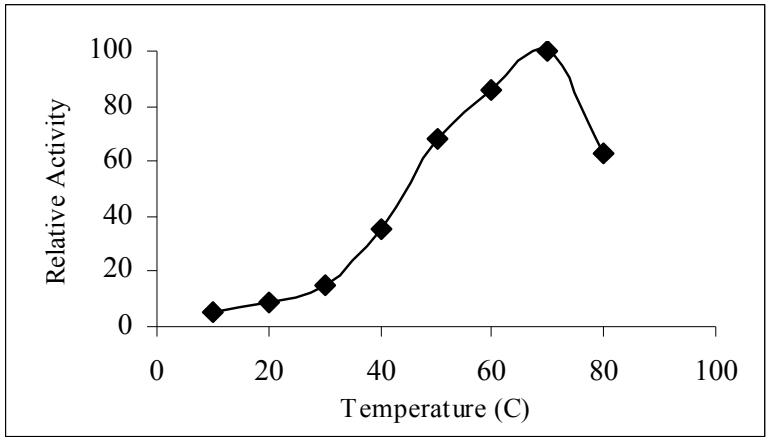


(a)



(b)

Figure 3.8 Effect of temperature on (a) free tyrosinase (b) tyrosinase immobilized in PPy (c) tyrosinase immobilized in PTATE/PPy



(c)

Figure 3.8 (Cont.)

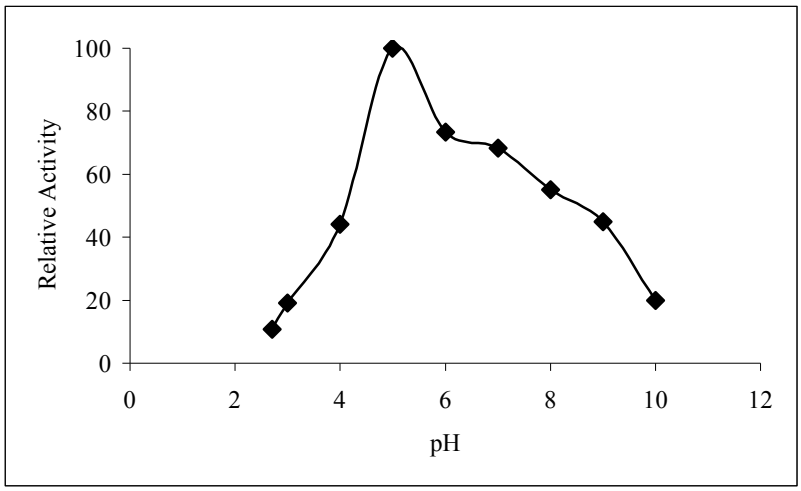
3.4.5.2 Effect of pH on Enzyme Activity

The effect of pH is another environmental condition to be investigated (Figure 3.9). Results showed that free tyrosinase had an optimum pH of 5. Immobilized tyrosinase in PPy matrix revealed an optimum pH as 7 which imply that pH stability is increased due to immobilization. For both PPy/PPO and PTATE/PPy/PPO electrodes, the activity of immobilized tyrosinase was less sensitive to the pH changes at levels of pH lower than 6. This indicates a better resistance of immobilized protein molecules to the ionization since the ionic state of the functional groups in or close to the active center has a great effect on enzyme activity.

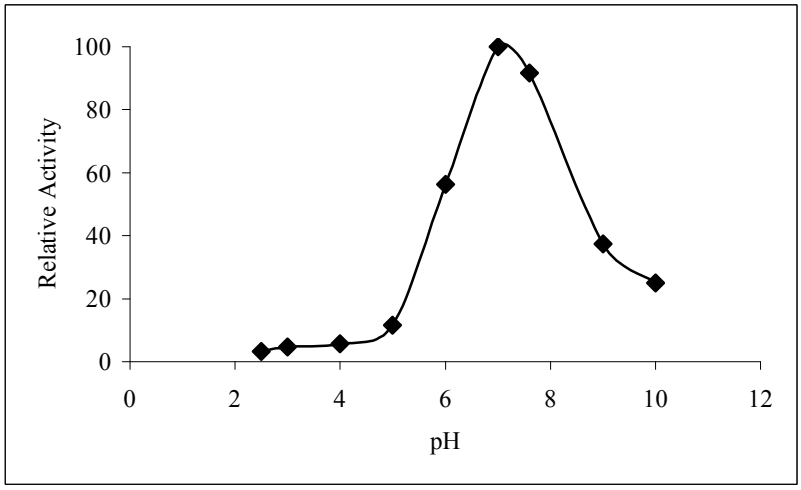
It is observed that the optimum pH values of the enzyme electrodes were shifted towards alkaline side when compared to that of free enzyme. The reason may be partitioning of protons. Charges are always present on the surface of the immobilized enzyme molecules due to amphoteric nature of enzymes. A partitioning of the charged particles occurs between the bulk solution and the microenvironment. The pH of the microenvironment may differ considerably from the pH of the bulk solution if protons are partitioned into or out of the immobilized enzyme matrix. The binding of substrate and the activity of immobilized enzyme both depend on the microenvironmental pH. This causes apparent shifts in the behavior of the kinetic constants with respect to the solution pH. In this case, protons are concentrated around the enzymes and protect them against high concentration of OH^-. As a result of this, the pH around enzyme lowers pH than that of the bulk. As the tendency of matrix to concentrate protons in it increases, the pH stability of the enzyme in this matrix increases.

The isoelectric point of the tyrosinase is 6.1. Isoelectric point of a protein is the pH at which the protein has an equal number of positive and negative charges. At a pH below isoelectric point, proteins carry a net positive charge while above it; they carry a net negative charge. The activity of immobilized tyrosinase in PPy matrix was much affected by the pH changes than that of the free enzyme at pH values greater than the maximum value. The repulsive forces generated by changing pH might have caused a split in the electrodes resulting in a less favorable conformation of the enzyme molecules.

When PTATE/PPy/PPO electrode is considered, it is determined that the optimum pH value was shifted towards alkaline side. However, there is almost no change in the enzyme activity between pH 7 to pH 11. This shows that this electrode can protect enzyme molecules against high OH^- concentration [50].

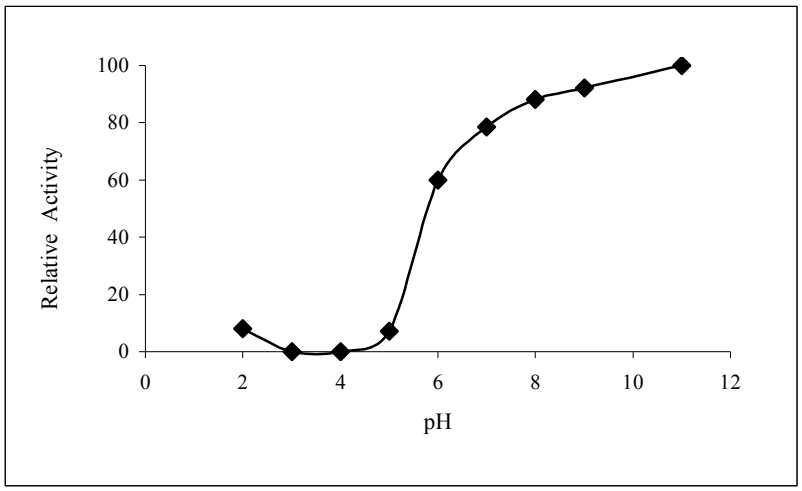


(a)



(b)

Figure 3.9 Effect of pH on (a) free tyrosinase (b) tyrosinase immobilized in PPy (c) tyrosinase immobilized in PTATE/PPy



(c)

Figure 3.9 (Cont.)

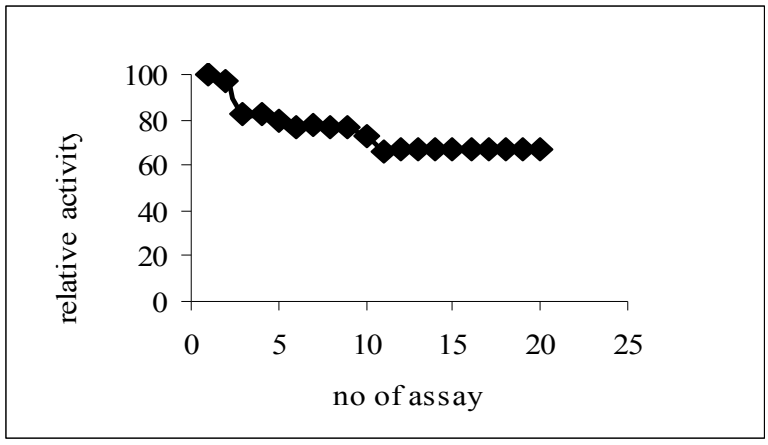
3.4.5.3 Operational Stability and Shelf-Life of Enzyme Electrode

Operational stability and shelf-life are crucial important parameters on constructed enzyme electrodes. The stability is mainly dependent on the lifetime, as well the rate of denaturation or inactivation of the enzyme employed.

Operational stability shows the repeatability of the usage of tyrosinase modified electrodes. The measurement depends on the 20 successive measurements. The activity of tyrosinase immobilized in PPy matrix decreased gradually up to 10^{th} use and then stayed constant at 60% activity (Figure 3.10a). However, immobilized tyrosinase in PTATE/PPy matrix lost 80% of its original activity till the end of the 10^{th} use. After that, it showed 20% constant activity (Figure 3.10b).

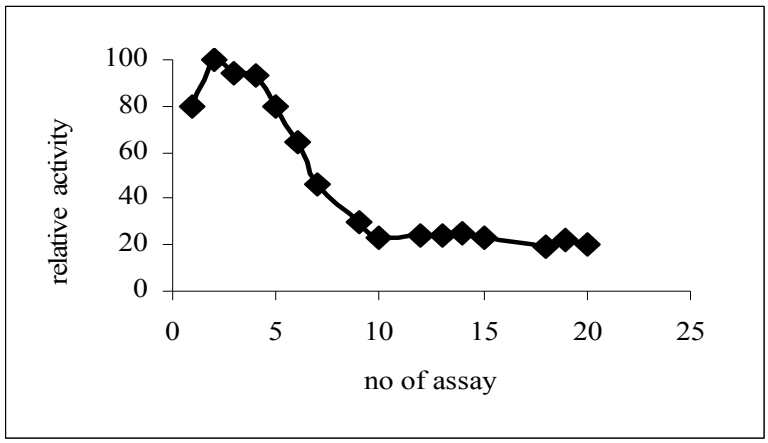
Shelf-life refers to the stability of enzyme electrodes when not in use. It was reflected by the changes in activity of tyrosinase over a period of 30 days. There is a

gradual decrease in the activity of tyrosinase immobilized in PPy matrix. At the end of the experiment, it exhibited 40% activity (Figure 3.11a) In the case of PTATE/PPy/PPO electrode, an increase in tyrosinase activity was observed after 5th day. It can be explained by the reorganization of enzyme molecules in the matrices. Up to 20th day, the activity was almost constant but it is seen that 60% of its original activity was lost till the end of the experiment (Figure 3.11b).



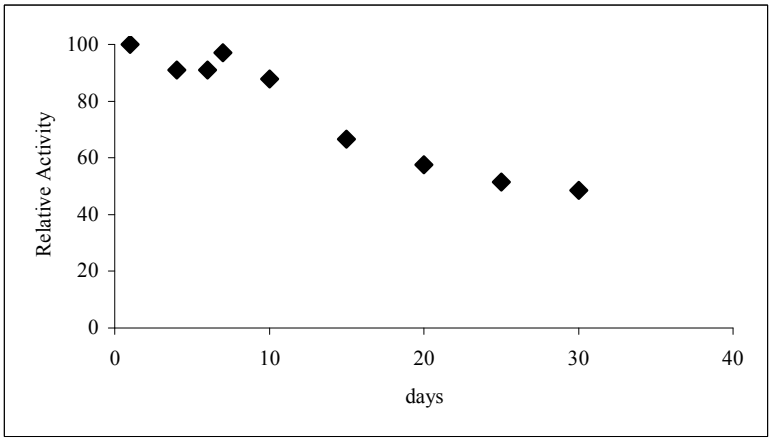
(a)

Figure 3.10 Operational stability for (a) PPy/PPO (b) PTATE/PPy/PPO



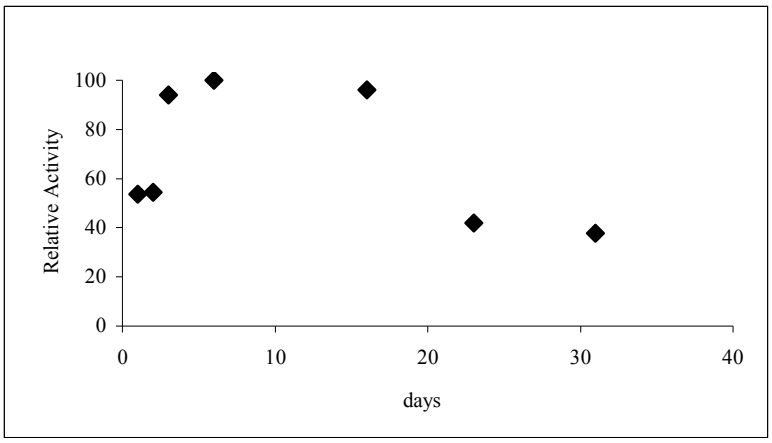
(b)

Figure 3.10 (Cont.)



(a)

Figure 3.11 Shelf –life for (a) PPy/PPO (b) PTATE/PPy/PPO



(b)

Figure 3.11 (Cont.)

3.4.6 Determination of Phenolic Compounds in Red Wines

Two Turkish red wines (Brand K and Brand D) were analyzed for their amount of phenolic compounds. Experiments were carried by using free tyrosinase and immobilized tyrosinase in PPy and PTATE/PPy matrices as described in section 2.3.4.8.

Tyrosinase enzyme acts on the –OH groups on phenolic compounds. Total phenolics are expressed as gallic acid equivalents [53].

As shown in Table 3.3, in Brand K, 220 mg/L phenolics were found by using free tyrosinase, 4000 mg/L phenolics were found by using PPy/PPO electrode and 830 mg/L phenolics were found by using PTATE/PPy/PPO electrode. According to results found in Brand D, there are 270 mg/L phenolics when free tyrosinase was used, 2200 mg/L and 920 mg/L phenolics when PPy/PPO and PTATE/PPy/PPO

electrodes were used, respectively. As known from literature, total phenolics in Turkish red wines were reported as 2000-3000 mg/L [54-56].

In order to double check, Folin-Ciocalteau method was also used for the determination of amount of phenolics. According to this method, Brand D contains 2360 mg/L phenolics and Brand K contains 4040 mg/L phenolics. These results imply that PPy/PPO electrode can be used as an effective biosensor for the determination of phenolic compounds in red wines. By using free tyrosinase, the amount of phenolics was detected with values lower than the expected ones. The reason is the inhibitors that are naturally found in the wines. They inhibit tyrosinase before it completes its enzymatic reaction. It is seen from scanning electron micrograph and Km value that PPy matrix having a compact environment provides a better protection for tyrosinase against inhibitors as also given in the previous studies [57-59]. However, PTATE/PPy matrix could not prevent the entry of inhibitors into the matrix hence, enzyme was affected. Km and micrograph of matrix also support the idea of easy diffusion of inhibitors besides substrates; hence results obtained for phenolic amount by using PTATE/PPy/PPO electrode do not reflect the actual values.

After detection of such results by using enzyme electrodes, the inhibitory effect of benzoic acid, cinnamic acid and sorbic acid which are naturally found in wines were studied.

Table 3.3 Total phenolic compounds in two Turkish red wines determined by free tyrosinase, two enzyme electrodes and Folin Ciocalteau method.

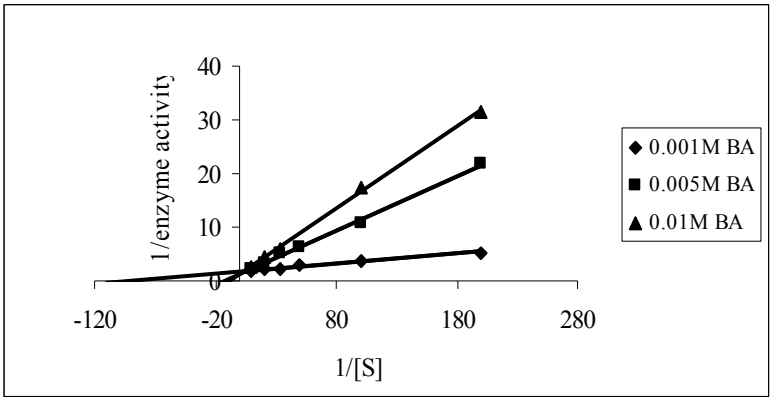
	Free Tyrosinase	**PPy/PPO**	**PTATE/PPy/PPO**	**Folin-Ciocalteau Method**
Brand K	220 mg/L	4000 mg/L	830 mg/L	4040 mg/L
Brand D	270 mg/L	2200 mg/L	920 mg/L	2360 mg/L

3.5 Inhibitory Studies

3.5.1 Inhibitory Effect of Benzoic Acid

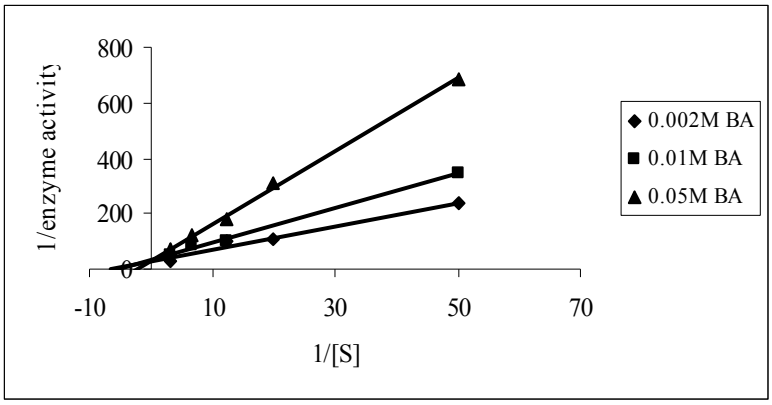
When benzoic acid was used as the inhibitor, Ki was found as 0.6 mM for free tyrosinase, 16 mM for tyrosinase immobilized in PPy matrix and 4 mM for tyrosinase immobilized in PTATE/PPy matrix. Benzoic acid showed competitive inhibition for all cases. Increasing Ki value decreases the inhibitory power of the molecule. When the results are compared, PTATE/PPy/PPO electrode provides better protection against benzoic acid than free enzyme, but less protection than

PPy/PPO electrode. Ki values show entirely parallelism with Km values (Table 3.4). Also, SEM micrographs of electrodes are considered, the less protection of PTATE/PPy/PPO electrode can be explained in terms of large pores. Figure 3.12 shows the Lineweaver-Burk plot showing the effect of different benzoic acid concentrations on immobilized PPO.

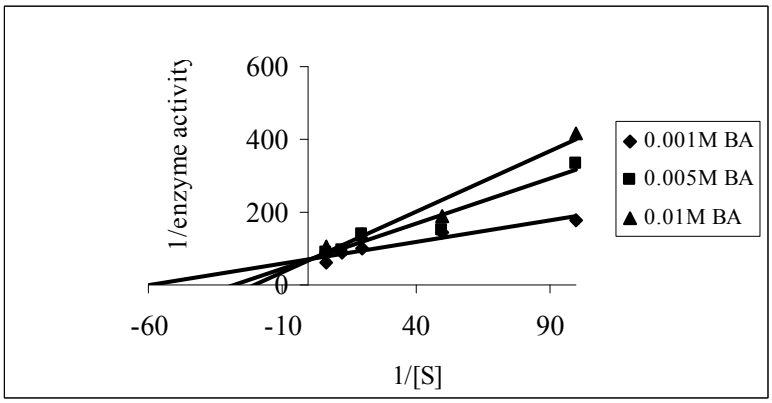


(a)

Figure 3.12 Lineweaver-Burk plots showing the effect of different concentrations of benzoic acid (BA) on (a) free PPO (b) PPO entrapped in PPy matrix (c) PPO entrapped in PTATE/PPy matrix.



(b)



(c)

Figure 3.12 (Cont.)

Table 3.4 Michaelis-Menten constants and inhibition constants in the presence of benzoic acid (BA)

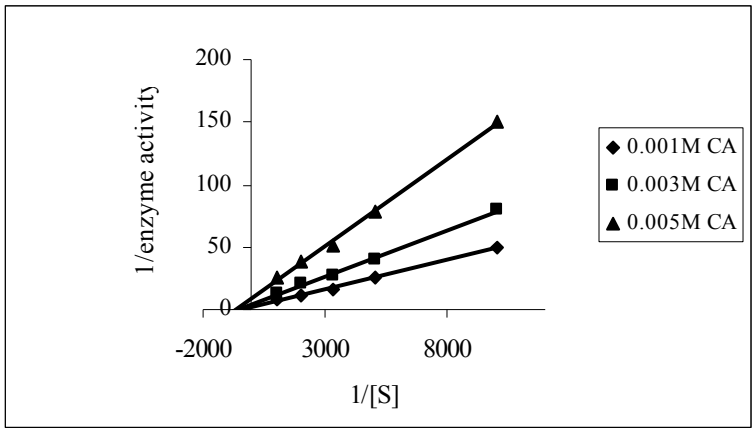
	Km (mM)	**Ki (mM)**
Free PPO	0.6	0.6
PPy/PPO	100	16
PTATE/PPy/PPO	22	4

3.5.2 Inhibitory Effect of Cinnamic Acid

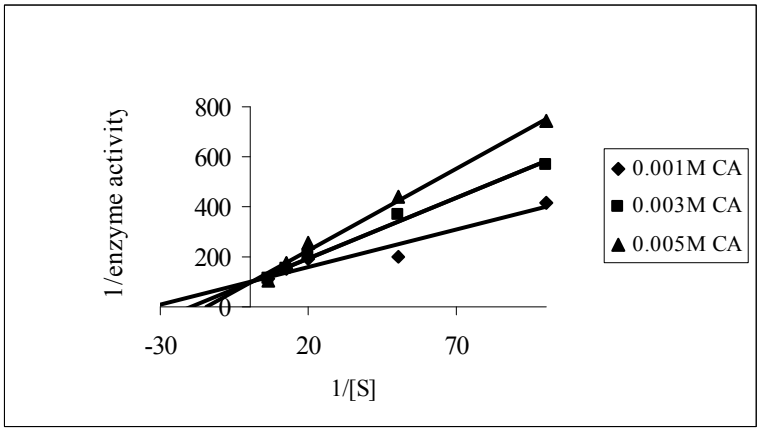
As shown in Figure 3.13a, cinnamic acid affected free PPO with a non-competitive manner. But immobilized PPO exhibited a competitive inhibition. The reason may be the change in the conformation of enzyme when it is entrapped in a medium. While inhibitor can bind both the enzyme and enzyme-substrate complex, after conformational change, it can only bind to enzyme itself.

When Ki values are compared, it is seen that PPy matrix provides the best protection against the cinnamic acid like in the case of benzoic acid. The Km and

Ki values for immobilized enzyme are proportional to each other which is a logical result of morphologies of the electrodes (Table 3.5).

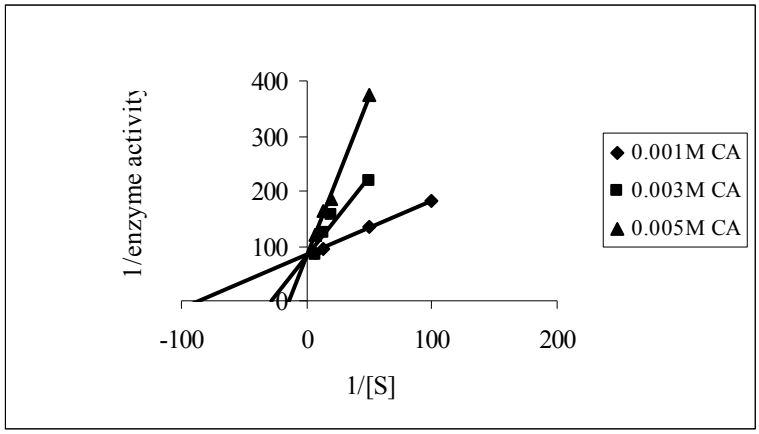


(a)



(b)

Figure 3.13 Lineweaver-Burk plots showing the effect of different concentrations of cinnamic acid (CA) on (a) free PPO (b) PPO entrapped in PPy matrix (c) PPO entrapped in PTATE/PPy matrix.



(c)

Figure 3.13 (Cont.)

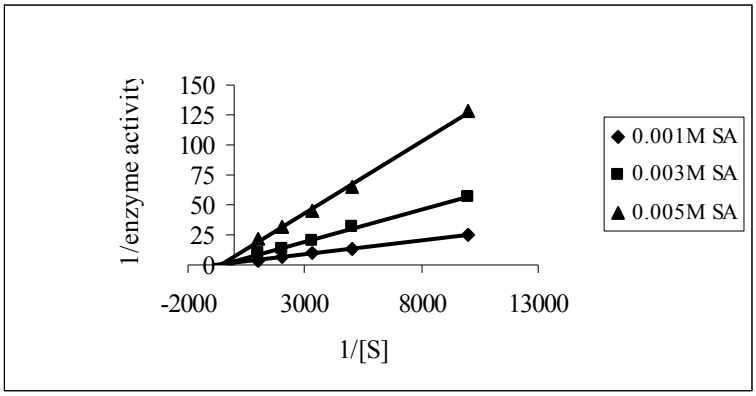
Table 3.5 Michaelis-Menten constants and inhibition constants in the presence of cinnamic acid (CA)

	Km (mM)	**Ki (mM)**
Free PPO	0.6	0.1
PPy/PPO	100	2
PTATE/PPy/PPO	22	0.4

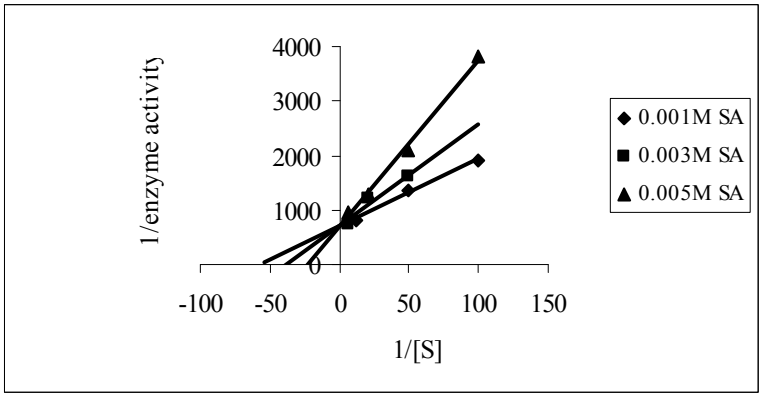
3.5.3 Inhibitory Effect of Sorbic Acid

Figure 3.14 shows the behavior of free and immobilized enzymes in the presence of sorbic acid. While a non-competitive type of inhibition was obtained for free enzyme, immobilized enzymes show a competitive inhibition because of the conformational changes.

Sorbic acid is an aliphatic carboxylic acid. Due to this structural difference (from benzoic acid and cinnamic acid), the Ki values of sorbic acid are not comparable with the Km values. If the results are taken into account, it is seen that PTATE/PPy/PPO electrode shows better protection than PPy/PPO electrode. In this case, the diffusion of sorbic acid through the matrices is easier for PPy matrix (Table 3.6).

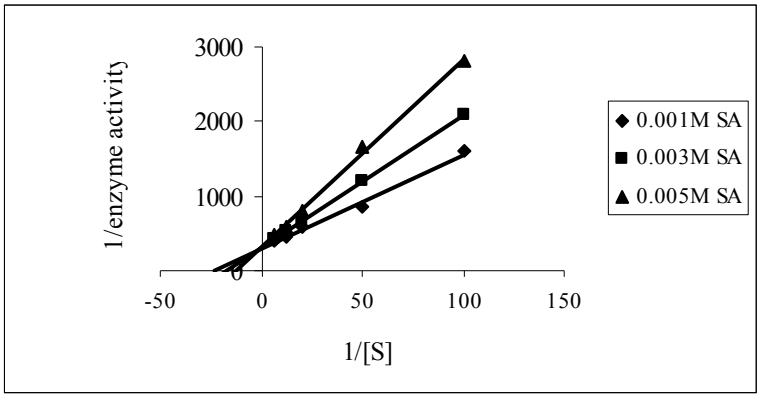


(a)



(b)

Figure 3.14 Lineweaver-Burk plots showing the effect of different concentrations of sorbic acid (SA) on (a) free PPO (b) PPO entrapped the PPy matrix (c) PPO entrapped in the PTATE/PPy matrix.



(c)

Figure 3.14 (Cont.)

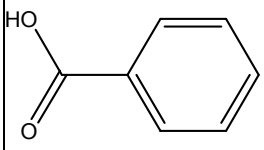
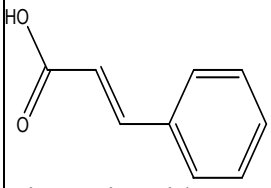
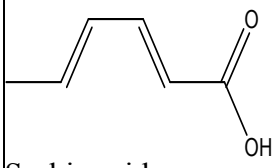
Table 3.6 Michaelis-Menten constants and inhibition constants in the presence of sorbic acid (SA).

	Km (mM)	**Ki (mM)**
Free PPO	0.6	0.2
PPy/PPO	100	1.5
PTATE/PPy/PPO	22	3.2

3.5.4 Comparison of the Inhibitory Powers of Inhibitors

Table 3.7 shows the effect of inhibition of three different acids on both free and immobilized enzymes. If the free tyrosinase inhibition is considered, cinnamic acid has the greatest inhibitory effect, and sorbic acid is a stronger inhibitor than benzoic acid. The same inhibitory pattern was also observed in literature [60]. When tyrosinase is entrapped in PPy matrix, the inhibitory power of cinnamic acid and sorbic acid is almost equal. However, benzoic acid and sorbic acid shows almost same inhibitory effect on immobilized tyrosinase in PTATE/PPy matrix. This difference may be due to conformational changes of the tyrosinase when entrapped in a medium. If benzoic acid and cinnamic acid are compared according to structural differences, it is seen that double bond between the benzene ring and carboxylic acid group improves inhibitory power. Pifferi et al. reported that the difference in the inhibitory behavior between benzoic acid and cinnamic acid may be due to the size of degree of unsaturation of their molecules [61]. Yet, benzene ring has no effect on the inhibition as far as sorbic acid's inhibitory power is considered. A conjugated system involving the carboxylic acid group enhances the inhibition power of the molecule.

Table 3.7 Comparison of the inhibitory power of the inhibitors

	Ki (mM) free PPO	Ki (mM) PPy/PPO	Ki (mM) PTATE/PPy/PPO
 Benzoic acid	0.6	16	4
 Cinnamic acid	0.1	2	0.4
 Sorbic acid	0.2	1.5	3

CHAPTER 4

CONCLUSION

PTATE/PPy copolymer can be used as entrapment matrix for immobilizing tyrosinase. As regards to the kinetic parameters, temperature and pH analysis, the results imply that the fabricated enzyme electrode by using PTATE/PPy is superior to PPy matrix. Especially strange pH behavior which protects enzymes against high pH is a treatment which is a required property in enzyme immobilization studies. Stability measurements, either operational stability or shelf life, may exhibit a little inefficient results compared to PPy matrix, but an overall evaluation of PTATE/PPy matrix reveals that it is suitable for the purpose.

Immobilization of tyrosinase in a conducting polymer matrix was studied as an alternative method for determination of phenolic compounds in wines. Results show that free tyrosinase and immobilized tyrosinase in PTATE/PPy matrix were affected from inhibitors that are naturally found in red wines. So, the amount of phenolics was detected lower than the expected values by using them. Benzoic acid, cinnamic acid and sorbic acid were studied in order to understand the effect of inhibitors. As a result, cinnamic acid is the most powerful inhibitor and the inhibitory powers of benzoic acid and sorbic acid are the same for immobilized tyrosinase in PTATE/PPy matrix.

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