CHAPTER 1

INTRODUCTION

One of the most important problems in agricultural industry is to protect the food products from pest and herb. For this reason the use of pesticides become inevitable. Among pesticides, insecticides with high toxicity level such as azinphos-methyl, dichlorvos (DDVP), endosülfan, methamidophos, methyl-bromide and parathion-methyl are highly used (Delen et al. 2005). However the toxicity of pesticides comes out some problems. Their residues harmful for human health and ecological balance (Rekha et al. 2000). In Turkey the use of pesticides has been increasing at 270% from 1979 to 2007 (Durmusoglu et al. 2010). Hazardous foods have been launched due to the lack of pesticide residue controls (Çopur et al. 2010). Besides Turkey becomes second country among the 125 countries that are exported inconvenient food to Europion Union (Durmusoglu et al. 2010). In this manner the extensive and insensible uses of pesticides in agriculture increase the requirement for fast methods in residue analysis.

Chromatographic techniques such as GC, HPLC, or coupled techniques (GC–MS) currently used as reference methods in order to detect and measure pesticide residues at and below maximum residue limit (MRL) levels (Bicchi et al. 1996; Babicka et al. 1986). Besides the some advantages such as very high sensitivity, reliability and precision, these techniques require expensive instrumentation and highly trained personal, are time consuming, and are not easily adapted for in field analysis (Solna et al. 2005). Biosensors could be a reliable and promising alternative to the standard methods applied for detection of pesticide residue. They allow both real time and in situ monitoring (Lee et al. 2002; Ramirez et al. 2008).

Biosensors are usually designed by considering appropriate conditions for enzyme loading to ensure that sufficient biocatalyst activity is available. The biosensor should be easy to use and most important one is cost of the analysis mostly depends on the sensitivity of the enzyme electrode. (Watson 2004). Most of the studies have recently focused on the development of ideal low cost enzyme electrode which influenced by choice of sensor, the number of units to be manufactured, immobilization techniques and the nature of the enzyme (Nade et al. 2003).

Most of enzymes like tyrosinase and acetylcholinesterase are affected by several factors such as pH, which controls the distribution of charges in the active site, is especially critical for enzyme-catalyzed reactions, temperature that can cause inactivation of the enzyme and also organic solvents. Most enzymes are water soluble and a certain amount of water is necessary for the solubilization of them. In the case of low solubility of substrates or other reactant, organic solvents which influence activity of the enzymes have to be used. Adverse conditions of these factors can reduce or destroy the biological activity of the enzyme. (Illanes 2008; Yoruk and Marshall 2003; Drauz and Waldmann 2002). Therefore a great deal of research has been conducted towards the development of enzyme immobilization to extend shelf life and improved length of useful measurement times (Buerk 1993). Enzymes could be immobilized to the supports with entrapment, micro encapsulation, adsorption, covalent cross-linking and covalent binding methods. Wide range of supports such as organic (polyamides, nylon, polyacrylamide, polyethylene, silk, starch, agarose) and inorganic materials (zeolite, clays and metals) present for the immobilization of enzymes by using these methods (Drauz and Waldmann 2002). Among these supports chitosan has been widely used for enzyme immobilization which contains large groups of $-NH_2$ and –OH. This material is also preferable due to its excellent biocompatibility, nontoxicity, cheapness, easy-handling and high mechanical strength (Krajewska 2004; Dung et al. 2009; Bhatt et al. 2010). Gelatin is also another attractive supports for enzyme immobilization due to its high hydrophilicity, nontoxicity, requires only simple equipment and being relatively inexpensive (Seo et al. 2003; Peña et al. 2010). Both supports are transparent and available to study with most optical detection methods for pesticide determination. In this study chitosan and gelatin were used as supports materials due to their distinctive properties.

The detection of pesticide is possible by inhibiting the enzyme such as organophosphorous hydrolase, alkaline phsosphatase, ascorbate oxidase, tyrosinase, acid phosphatase, butyrylcholinesterase and acetylcholinesterase (Du et al. 2007). The decline in the amount of enzymatically produced product is defined as inhibition of enzyme activity (Watson 2004). The conversion of substrate to product causes a change in optical, electrical or mechanical properties of the reaction system. Most of the studies were conducted by using electrochemical detection as technique and acetylcholinesterase as enzyme for pesticide detection. In the study of Du et al. (2007) this enzyme was immobilized by using glutaraldehyde as cross-linker to multiwall

carbon nanotubes–chitosan composite for determination of pesticide (triazophos). Vastarella et al. (2007) also prepared electrochemical biosensors by covalently immobilizing the acetylcholinesterase enzyme on different nylon pre-activated membranes by means of glutaraldehyde for the determination of pesticide (paraoxon). Different immobilization methods for this enzyme such as prussian blue modified screen-printed electrodes by cross-linking with glutaraldehyde (Suprun et al. 2005), immobilization in photocrosslinkable poly(vinyl alcohol) photopolymer, encapsulation in a sol–gel matrix or immobilization by met al–chelate affinity (Andreescu et al. 2002) were used to detect the pesticide in the literature. For these studies the inhibition of pesticides on enzyme was monitored by measuring the decrease in oxidation current of product (thiocholine). However these enzyme sensors show irreversible inhibition on enzyme activity leads to a rapid decrease in the sensitivity and performance (Nade et al. 2003). If the reactivation of the enzyme is impossible in this case disposable biosensors are designed. Tyrosinase (TYR) can be an alternative enzyme for the detection of pesticides. Vidal et al. (2008) reversibly immobilized the tyrosinase to 1,2-naphthoquinone by electropolimerization method to determine the dichlorvos and atrazine.

However electrochemical enzyme sensor is not a hydrodynamic technique. In the other words the solution is generally stagnant and hence process is limited by the diffusion of substrate. Cyclic voltammetry is popular methods among the electrochemical biosensor and has the disadvantage such that processes occurs at a given potential can depend on preceding potentials (Koryta et al. 1993). These techniques are also relatively expensive. Photometric enzyme assay has the advantage to be fast, easily manageable, convenient, capable of rapidly providing accurate and reproducible results, provide controling the mass transfer by stirring the solution and moreover cheap in performance with respect to the electrochemical methods. Enzyme assays based on changes in the absorbed light are more frequently used among the photometric methods (Walz and Schwack 2007; Eisenthal and Danson 2002). Optical biosensors prepared by tyrosinase immobilized on a nylon membrane (Russell and Burton 1999) jellose/agarose membrane (Paranjpe et al. 2001) and pyrrole matrix (Narlı et al. 2006) have been reported. In these studies phenol was detected based on the reaction between the enzymatically produced *o*-quinone and 3-methyl-2-benzothiazolinone hydrazone (MBTH) result in maroon color product. The use of MBTH or other nucleophilic compounds provide stable stoichiometric adduct with the

o-quinones. Because o-quinones are very unstable and undergo to a sequence of non-enzymatic reactions within the short time (Fiorentinoa et al. 2010). However, some MBTH-quinone adducts shows solubility problems. Methods rely on spectrophotometric measurement of o-quinone formation (do not include any nucleophilic compounds) are rapid, practical and perhaps more accurate (Yoruk and Marshall 2003). In this respect it is significant issue to measure the initial reaction rate which is linear for only a short period in the absence of nucleophilic compounds. In this study the change in the absorbance of enzymatically produced product was monitored without using any nucleophilic compounds. The reaction was initiated by stirring the reaction media while the spectrophotometric measurement was started (in situ analysis).

Based on all of these considerations the aims of this study are;

- To prepare tyrosinase immobilized chitosan and gelatin films as optical enzyme sensors for detection of Parathion-methyl (by utilizing the enzyme inhibition ability of pesticide), a model pesticide.
- To investigate the performance of the sensors by measuring their activities with respect to pH, temperature, organic solvent tolerance, storage and operational stabilities without using any nucleophilic compounds.
- To investigate the inhibition mechanism exerted by organic solvents and parathion-methyl by characterizing the apparent kinetic parameters of the enzyme catalyzed reaction.

CHAPTER 2

ENZYMES

Enzymes are proteins composed by a number of amino acid residues. Peptide bond formation occurs between carbon atom of the carboxyl group of one amino acid and the nitrogen atom of the α-amino group of the following as depicted in Figure 2.1.

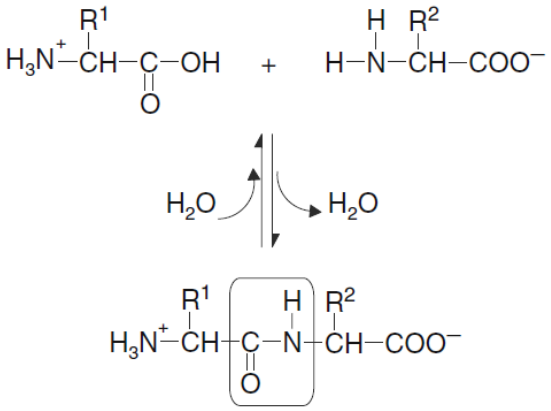
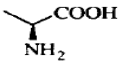
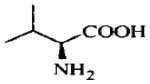
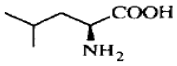
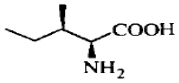
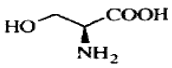
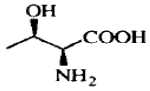
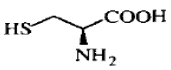
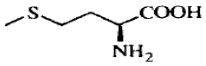
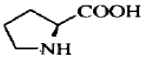
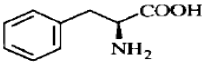
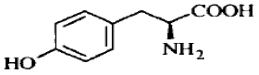
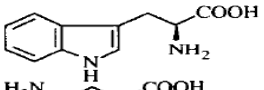
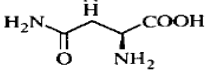
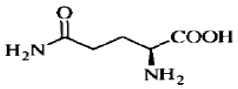
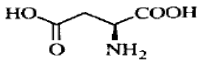
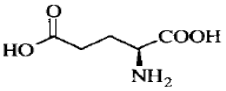
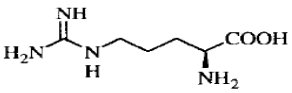
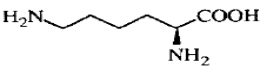
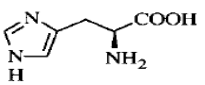


Figure 2.1. Formation of peptide between two adjacent α-amino acids

The distribution of amino acids (can be non-polar (hydrophobic) or polar (charged or uncharged) depending on the nature of the R group) along the protein molecule determines its behavior. Table 2.1 shows the general structure of amino acids. Primary structure is related with the amino acid sequence of protein while secondary three-dimensional structure is the result of interactions of amino acid residues proximate in the primary structure (for enzymes, these interactions dictate a predominantly ribbon-like coiled configuration termed α-helix). The interactions of amino acid residues located apart in the primary structure formed the tertiary three-dimensional structure which is essential for the biological functionality of the protein. Figure 2.2 shows Hierarcy of protein structute.

Table 2.1. Structure and properties of amino acid side chains
(Source: Drauz and Waldmann 2002).

Name	Symbol	Structure	pK_a of ionizing side chain[a]
Glycine	Gly (G)		
Alanine	Ala (A)		
Valine	Val (V)		
Leucine	Leu (L)		
Isoleucine	Ile (I)		
Serine	Ser (S)		
Threonine	Thr (T)		
Cysteine	Cys (C)		9.1–9.5
Methionine	Met (M)		
Proline	Pro (P)		
Phenylalanine	Phe (F)		
Tyrosine	Tyr (Y)		9.7
Tryptophane	Trp (W)		
Asparagine	Asn (N)		
Glutamine	Gln (Q)		
Aspartate	Asp (D)		4.5
Glutamate	Glu (E)		4.6
Arginine	Arg (R)		~ 12.0
Lysine	Lys (K)		10.4
Histidine	His (H)		6.2
α-amino group			6.8–7.9
α-carboxyl group			3.5–4.3

[a]: The pK_a values depend on temperature, ionic strength and, especially on the microenvirronment of the ionizable group

The main types of interactions responsible for the three-dimensional structure are

- Hydrogen bonds which is main determinant of the helical secondary structure and plays a significant role in tertiary structure as well
- Apolar interactions which is rather weak however, contribute to the stabilization of the three dimensional structure.
- Disulphide bridges relevant in the stabilization of the three-dimensional structure
- Ionic bonds between charged amino acid residues which contribute to the stabilization of the three-dimensional structure
- Other weak type interactions, like van der Waals forces, whose contribution to three-dimensional structure is not considered significant.

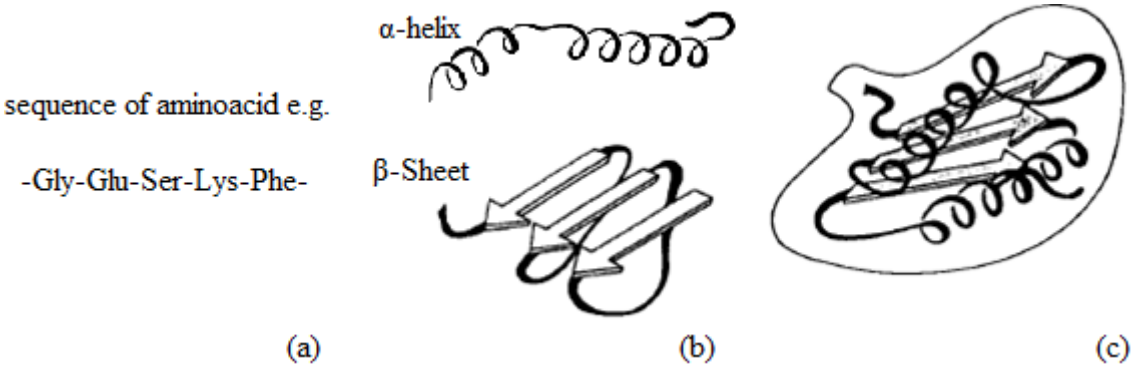


Figure 2.2. Hierarcy of protein structure; primary structure (a), secondary structure (b), teriary structure (c).

The only difference between enzymes and proteins is that enzymes possess catalytic activity. Enzymes are biological catalysts that facilitate the conversion of substrates into products by providing favorable conditions that lower the activation energy of the reaction. The regions of the enzyme that are directly involved in the catalytic process are called the active sites. This small part is usually hydrophilic cleft or cavity formed by very few amino acid residues. Substrate is bound to the active site of enzyme and changes in the distribution of electrons in its chemical bonds cause the reactions that lead to the formation of products. Then products are released from the enzyme and therefore enzyme is ready for the next catalytic cycle.

$$\text{Substrate(s)} \xrightarrow{enzyme} \text{Product(s)}$$

Enzyme activity defined as the capacity of an enzyme to catalyze a chemical reaction is strictly dependent on its molecular structure. Some compounds can decerase (inhibitors) or increase (activators) the enzyme activity. The change in enzyme activiy can be irreversible, reversible or partially reversible. For instance irreversible inhibitors (poisons) completely inactivate the enzyme (process called inactivation). On the other hand reversible inhibitors reduce enzyme activity and when the inhibitor is removed enzyme activity is restored (no chemical changes occurres in the protein molecule). Partial reversibility occurs if some of enzyme activity is restored when the inhibitor is removed. The term "inhibition" is used for fully reversible or partially reversible processes. Factors that affect enzyme activity are also

- The nature of the enzyme and substrate
- The concentrations of enzyme and substrate
- pH
- Temperature
- Organic solvents
- Heavy met als and chelating agents
- External factors such as irradiation and shear stress.

Adverse conditions of these factors can reduce or destroy its biological activity by distorting the proper active site configuration. (Illanes 2008; Missen et al. 1999)

Enzymes contain many polar amino acids which may be protonated or unprotonated depending on medium pH. Change in pH can change the distribution of charges in the active site which may prevent proper conformation of the active site, binding of substrates, and/or catalysis of the reaction. Temperature also affects both enzyme activity and stability. At low temperatures slower reaction observed due to decrease in kinetic energy of the reactant molecules. An increase in temperature increases the rate of the chemical reaction. At higher temperatures the concentration of active enzyme decreases. Thermal inactivation can occur as a consequence of the weakening of the intermolecular forces responsible of the preservation of its three-dimensional structure. Inactivation may involve covalent or non-covalent bond disruption with subsequent molecular aggregation or improper folding. At extremes pH or temperature values the enzyme activity tends to decrease (Illanes 2008; Yoruk and Marshall 2003). Mechanical forces such as shear and surface tension can disturb the the

shape of the enzyme molecule and this may drastically affect enzyme activity (Missen et al. 1999).

Water levels also have important effects on enzyme behavior. In the case of too little water the catalytic activity significantly decreases while enzyme stability increases. At high water levels catalytic activity also falls. This is because water result in mass transfer limitation by promoting agglomeration of catalyst particles, or may act as a competitive inhibitor for any of the substrates. It is difficult to prepare desired water content. Sometimes organic solvents have to be used. Because substrates may not be sufficiently soluble in water or desired reaction may be favorable in the media containing organic solvent which influence activity and stability of enzymes. In recent years, researcher shows that the majority of bulk water in a reaction system may be replaced by organic solvents which influence dielectric properties of the reaction medium responsible for the ordered water structures. (Drauz and Waldmann 2002).

Consequently the medium pH, temperature, solvent concentration (if necessary), water content, flow rates and stirrer speeds must be controlled and optimize to obtain high enzyme productivity.

Enzymes display distinct properties when compared with chemical catalysts due to their complex molecular structure. The advantages and drawbacks of enzymes as catalysts are summarized in Table 2.2.

Table 2.2. Advantage and drawbacks of enzymes as catalysts
(Source: Illanes 2008).

Advantages	**Drawbacks**
High specificity	High molecular complexity
High activity under moderate conditions	High production costs
High turnover number	Intrinsic fragility
Highly biodegradable	
Generally considered as natural products	

Enzymes can be classified into six main classes according to the type of reaction catalyzed as depicted Table 2.3.

Table 2.3. Classification of enzyme according to type of reaction catalyzed (Source: Missen et al. 1999; Drauz and Waldmann 2002)

Class	Type of reaction catalyzed
Oxidoreductases	Various types of oxidation-reduction reactions (dehydrogenases, oxidases, oxygenases and peroxidases)
Transferases	Transfer of functional groups, such as aldehydic or acyl groups
Hydrolases	Hydrolysis reactions (proteases, amylases, acylases, lipases, and esterases
Isomerases	Isomerization and transfer reaction within one molecule (glucose isomerase)
Ligases	ATP (adenosine triphosphate) as a cofactor, lead to the formation of bonds between carbon and other atoms, including carbon, oxygen, nitrogen, and sulfur (glycine ligase)
Lyases	Addition of chemical groups onto double bonds (fumarase, aspartase, decarboxylases, dehydratases, and aldolases)

Among them oxidoreductases have several of technological significance. This class of enzymes catalyze the oxidation/reduction reactions that involve the transfer of electrons, hydrogen or oxygen atoms. Recently electrochemical study of redox enzymes has attracted due to the significance of bioscience and their potential applications in biosensors and biocatalysts. Polyphenol oxidases are immobilized to several supports to construct the enzymatic biosensors. (Mohammadi et al. 2009; Fiorentino et al. 2010)

2.1. Tyrosinase Enzyme

Tyrosinase enzyme is a binuclear copper cluster containing monooxygenase having monophenolase activity (catalyzes o-hydroxylation of monophenols) and diphenolase activity (subsequent oxidation of the formed o-diphenols into o-quinones by consuming the molecular oxygen). Later on, the enzymatic product quinones may undergo nonenzymatic autopolymerization to produce colored compounds melanin (Aytar and Bakir 2008; Xue et al. 2008; Khan et al. 2007; Munoz et al. 2008).

Tyrosinases have been characterized according to its sources, including bacteria, fungi, plants and mammals. The exact structure of tyrosinase is still unknown. However

two related proteins (hemocyanin and catechol oxidase) containing the same type-3 copper according to the spectroscopic similarities. Figure 2.3 shows three-dimensional structures of tyrosinase from S. castaneoglobisporus (type 3 copper proteins). The central domain with the active site consists of the α-helical structure which surrounds the catalytic binuclear copper centre. Binuclear copper site serve as substrate binding centre is located in the bottom of the large concavity according to tertiary structures of tyrosinase (Selinheimo 2008).

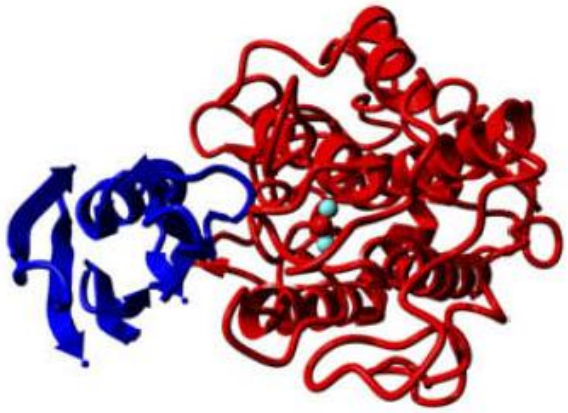


Figure 2.3. Subunit structures of S. castaneoglobisporus tyrosinase (in red: the mature tyrosinase protein and in blue: the carrier protein

Figure 2.4 shows the binuclear copper active site of tyrosinase enzyme. The active site contains a dicopper core. Six nitrogen donor atoms from histidine residues (His-N) bind a pair of copper ions which interacts with both molecular oxygens (O_2). (Garcia-Molina et al. 2007; Koval et al. 2006).

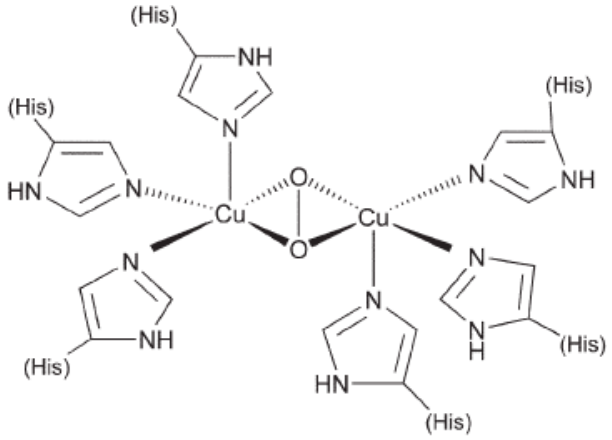


Figure 2.4. Schematic representation of binuclear copper site, C:Cu ion, O:oxygen, and H:His-N

Three forms can exist in the active site. These are met-tyrosinase, oxy-tyrosinase, and deoxy-tyrosinase. In the met-form hydroxyl ion is bound to the active site in which the coppers are in the form of Cu^{2+}-Cu^{2+}. In the oxy-form molecular oxygen is bound to the active site and oxygen is bridged as peroxide between the copper atoms. The enzyme is deoxy-form in instable state which immediately binds molecular oxygen to give the oxy-form In nature the enzyme usually consists of 85-90 % of a met-form, and the rest is an oxy-form (Selinheimo E. 2008). Mushroom tyrosinase has a molecular mass of 120 kD and isoelectric point value of 4.5. This enzyme takes a negative charge at pH > 4.5 in aqueous solution. The pH range from 4-8 enzyme is more active and activity significantly decerase at a higher acid environment (Mohammadi et al. 2009; Xie et al. 2003; Yoruk and Marshall 2003).

Tyrosinase activity can be measured by several methods (Munoz et al. 2008), including

- Using nucleophilic reagents that trap the o-quinones and generate chromophoric adducts
- Using reducing agents such as ascorbic acid, measuring their disappearance as a result of oxidation by the quinones
- Measuring the consumption of oxygen
- Directly measuring the formation of o-quinones or their products

O-quinone can be enzimatically produced from mono-, di- and trihydroxyphenols as substrate by using tyrosinase (Munoz et al. 2008). However this enzyme shows greater affinity for dihydroxyphenols. In addition to this, it was also reported that catechol result in maximum activity as a substrate among the monohydroxyphenols (*p*-cresol and tyrosine), dihydroxyphenols (catechol, L-dopa, D-dopa, catechin, and chlorogenic acid), and trihydroxyphenols (pyrogallol). This suggests that enzyme exhibits highest activity when catechol (o-diphenol) was used as substrate (Seo et al. 2003).

Figure 2.5 shows the basic reaction mechanism of tyrosinase on *o*-diphenols. Firsly *o*-diphenol molecule (D) binds the native form of the enzyme (met-tyrosinase) to form the complex (E_{met}-D). The oxidized form of D is released as product (*o*-quinone) and the enzyme is reduced to the deoxy form (deoxy-tyrosinase). This form binds to an oxygen molecule (oxy-tyrosinase), which reacts again with another D and produces the complex E_{oxy}-D. Then *o*-quinone is to be produced by the oxidation of D and the

enzyme returns to its native form (E_{met}) (Mohammadi et al. 2009, Garcıa-Molina et al. 2007).

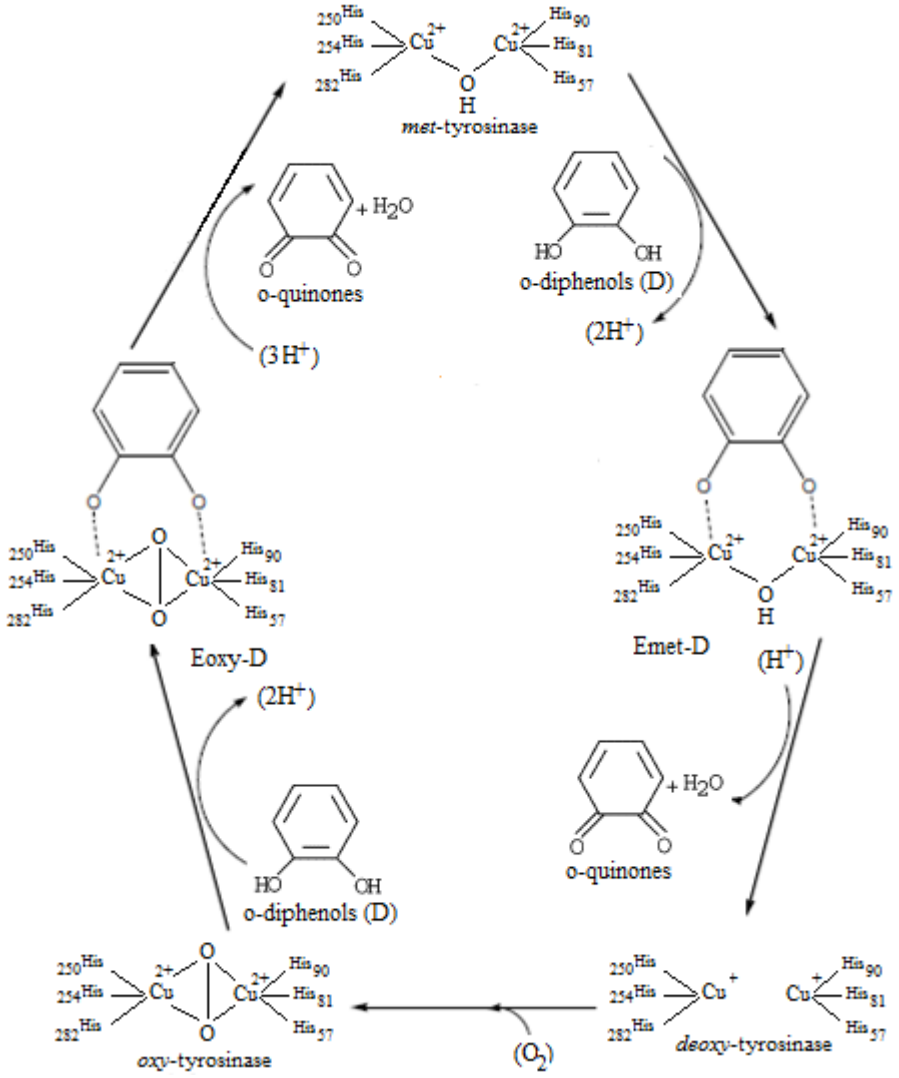


Figure 2.5. Basic reaction mechanism of tyrosinase. E_{met}-D: mettyrosinase o-diphenol binding complex, E_{oxy}-D: oxytyrosinase o-diphenol binding complex

The initial reaction rate is linear for only a short period when o-diphenols is used as substrate. This may be because quick reaction inactivation (suicide inactivation: o-semibenzoquinone free radicals and intermediate products disintegrate the active site's histidine residue(s) consequently tyrosinase enzyme becomes inactive) and irreversible binding of products to the active site of tyrosinase through a Michael reaction (Yoruk and Marshall 2003).

Tyrosinase enzyme have a wide application area including pharmaceutical drug production such as L-DOPA used in the treatment of Parkinson's disease, prodrug

therapy for melanoma, potential tools in treating melanoma patients, Tailoring properties of polymers, using as antibody microarrays, detoxification of phenolic compounds and the detection of phenols by biosensor preparation (Selinheimo 2008; Aytar and Bakir 2008; Xue et al. 2008).

In animals, plants and lower organisms tyrosinases are involved in the melanin pathway starting from l-tyrosine or l-DOPA as substrate leading to the formation of l-dopachrome. In animals, tyrosinase is responsible for melanization (for instance pigmentation in skin, eye, and hair). In some vegetables and fruits, tyrosinase activity is also responsible for browning reactions which cause undesired darkening results in a loss of nutritional and market values (Fiorentino et al. 2010; Selinheimo 2008; Xie et al. 2003).

2.2. Enzymatic Biosensor

Enzymatic biosensors are analytical devices that use a biological element fixed on sensor surface to interact with the analyte leadind to produce a measurable output that can be detected by a transducer. Measurable signal generated by transducer can be monitored by several ways such as hydrogen peroxide formation, changes in NADH concentration, oxygen consumption, fluorescence, absorption, conductivity, pH change, temperature or mass. Biosensor can be classified in several types according to its transduction element outlined in Table 2.4.

Among them, two common forms electrochemical and optical transducers used for pesticide analysis. Electrochemical transducers generate a current or voltage measured proportional to the concentration of analyte in the sample. Optical ones generate a signal measured as a light intensity in proportion to the analyte. Both of them may be an inverse relationship. The change in optical properties (such as UV/vis absorption, reflectance and fluorescence) of analyte is monitored in biosensors based on optical methods

Table 2.4. Commonly used transducers
(Source: Mulchandani and Rogers 1998).

Transducer	**Output**
Electrochemical	
Amperometric	Applied current
Potentiometric	Voltage
Conductimetric	Impedance
Optical	
Colorimetric	Color
Luminescence	Light intensity
Fluorescence	Light intensity
Calorimetric	
Thermistor	Temperature

Enzymes are frequently used as sensing elements in biosensors due to their specificity and catalytic properties. The first enzyme-based sensor was described by Clark and Lyons, who developed amperometric enzyme electrode for glucose. Enzymatic biosensors have been constructed by integrating the variety of enzymes belonging to classes of oxido-reductases, hydrolases, and lyases to different transducers for applications in health care, veterinary medicine, food industry and environmental monitoring (Watson 2004; Mulchandani and Rogers 1998). Recently studies with optical methods to detect polyphenol oxidase (PPO) activity attract attention. Spectrophotometric measurement of o-quinone formation from o-diphenolic compounds by using tyrosinase enzyme is most commonly assayed (Mohammadi et al. 2009; Yoruk and Marshall 2003). In the literature there are some studies about the use of optical biosensor constructed by immobilization of tyrosinase enzyme on different supports as summarized in Table 2.5.

Table 2.5. Some studies about the tyrosinase based optical enzyme sensors

Reference	Enzyme	Support	Application	Measuring techniques
Kıralp et al. 2003	Tyrosinase	Entrapment of enzyme to copolymers of thiophene functionalized menthyl monomer (MM) with pyrrole by electrochemical polymerization coated platinum electrode	Determination of phenolic compounds in wines	UV at 495 nm (Besthorn's Hydrazone method). Optical biosensor immersed in citrate buffer + MBTH + catechol with different concentrations
Rajesh and Kaneto, 2005	Tyrosinase	Covalent attachment of enzyme to the poly-pyrrole film on a stainless steel	Development of novel amine functional poly-pyrrole based biosensor	UV at 280 nm in PBS (pH:6.5)
Abdullah et al. 2006	Tyrosinase	Bi-layer which consist of immobilized MBTH in composite nafion/sol-gel silicate film and immobilized enzme in chitosan film on glass slide	Detection of phenol	UV at 490 nm. (Besthorn's Hydrazone method) Optical biosensor immersed in 3 ml PBS and phenol
Narlı et al. 2006	Tyrosinase	Entrapment of enzyme to poly (terepthalic acid bis-(2-thiophen-3-yl ethyl) ester) (PTATE) with pyrrole as the matrix by electropolymerizatio coated platinum electrode	Determination of phenolic compounds in red wines	UV at 495 nm (Besthorn's Hydrazone method). Optical biosensor immersed in citrate buffer + MBTH + catechol with different concentrations.
Abdullah et al. 2006	Tyrosinase	Physical adsorption of enzyme to chitosan film coated on glass slide vio spin coating	Detection of phenol	UV at 490 nm. (Besthorn's Hydrazone method) Optical biosensor immersed in 3 ml PBS (pH:6.7)and 0.2 mM MBTH (color reagent) and phenol
Ayter and Bakır, 2008	Tyrosinase	Cross-linked enzyme aggregate (CLEA) via precipitation with ammonium sulfate and crosslinking with glutaraldehyde.	Immobilization of enzyme	UV at 420 nm . 0.1 M catechol solution
Fiorentio et al. 2010	Tyrosinase	Cationic polymer poly (dimethyldiallylammonium chloride) (PDDA) on quartz slide by LBL method which provide electrostatic interactions with enzyme	Detection of phenol	UV at 475 nm over first 2 min. Optical biosensor immersed in 2.5 ml PBS (pH:6.7) including substrate without exogenous reagent

Tyrosinase-based optical biosensors have been developed and their performances were investigated by the researchers as outlined in Table 2.2.2. The aim is to obtain the fast, sensitive and inexpensive enzyme biosensor. Therefore the optimum design of enzyme sensor is very important to detect and measure the analyte at and below maximum residue limit (MRL) levels. Optimization of enzyme sensor can be defined as finding the best design or elite designs by taking into consideration the several basic physical properties of the measuring system summarized as; (Cuyper and Bulte 2002; Newman and Setford 2006).

- *Sensitivity*; defined as the magnitude of the enzyme sensor output signal at final steady state change with respect to change in concentration of analyte (slope of the linear range: $\Delta S / \Delta C$) as illustrated in Figure 2.6.

- *Linearity*; an enzyme sensor has to be wide linear range to be practically useful. Hence, linear biosensor will have a constant sensitivity over the substrate concentration range from zero to maximum.

- *Detection limit*; the analyte concentrations must be detectable for a desired application. Therefore, Ideal enzyme sensor can detect the lowest concentration of analyte

- *Stability*; the length of the time that biosensor remain sensitive under normal operational conditions must be long term (storage stability). Also biosensor has to be used for several times without a significant loss of its function (operational stability). The life time may be dependent on the total measurements number.

- *Selectivity and quick response*; the ideal enzyme sensor should have high selectivity and quick response which depend on diffusion of the chemical species to the active surface of the transducer. The changes in analyte concentrations have to be sensed in a short time, and biosensor also should not be influenced by the other chemical species.

- *Hysteresis*; Enzyme sensor would have zero hysteresis or in the other words it should not be affected by its past history of measurements

- *Easy to use and cost effectiveness*; an ideal biosensor is easily designed and being cheaper. It should not require expensive instrumentation and highly

trained personel, are not time consuming, and are easily adapted for in field analysis.

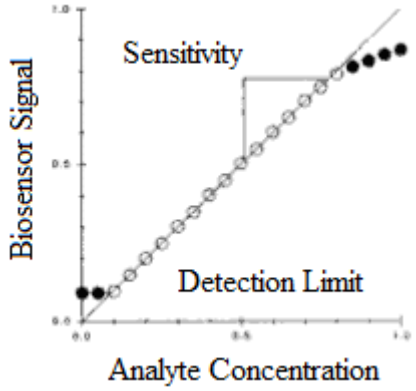


Figure 2.6. Calibration curve for an enzymatic biosensor with a linear range showing lower detection limit.

The success and performances of enzyme sensors are largely depend on the enzyme immobilization techniques. Many different strategies have been used to stabilize the enzyme molecules depending on the nature of the support and the biological molecule (Watson 2004).

2.3. Enzyme Immobilization Strategies

Immobilization is the most commonly used strategy to provide desirable features for enzymes. Enzyme immobilization can be defined as the conversion of an enzyme to a form restricted mobility (allows for containment and recovery of the enzyme) and retention of catalytic function.

The advantages of immobilizations are:

- It is possible to use enzyme repeatedly or continuously.
- Enzymes can be easily separated from reaction media which prevent contamination of the final product.
- pH, temperature, storage stability and catalytic properties can be enhanced.
- Control of microbial contamination becomes simpler.

However there are some practical limitations for immobilized enzymes such as;

- The yield of protein binding is rarely quantitative.
- The cost of the support may exceed the cost of the enzyme.

- Decrease in enzyme activity observed in immobilized form due to chemical modification of the protein, steric hindrance and mass transfer limitations.
- The proportion of active enzyme rarely exceeds 5-10 % w/w in immobilized form, which reduces catalytic activity per weight of solid.

One of the most important issue in immobilization is to enable the subsrate accommodation to the active site of the tyrosinase which depends on structural rigidity (allowing the retention of a specific 3D conformation) and flexibility (allowing the protein to perform its catalytic function) of the enzyme. Therefore the choice of immobilization technique becomes significant and important considerations (reducing the limitations, proper balance between structural rigidity and flexibility) have to be taken into account when enzyme is immobilized to the support (Drauz and Waldmann 2002; Cuyper and Bulte 2002).

The immobilization methods can be classified as;

- Chemical methods
  - ✓ Covalent binding
  - ✓ Covalent cross-linking
- Physical methods
  - ✓ Entrapment
  - ✓ Encapsulation
  - ✓ Adsorption

Figure 2.7. illustrates the types of immobilization. In Chemical methods, a covalent bond is established between the functional groups in the support (also called the matrix or the carrier) and functional groups in the enzyme. Physical immobilization methods do not involve covalent bond formation with the enzyme. Figure 2.7 shows the classification of immobilization methods.

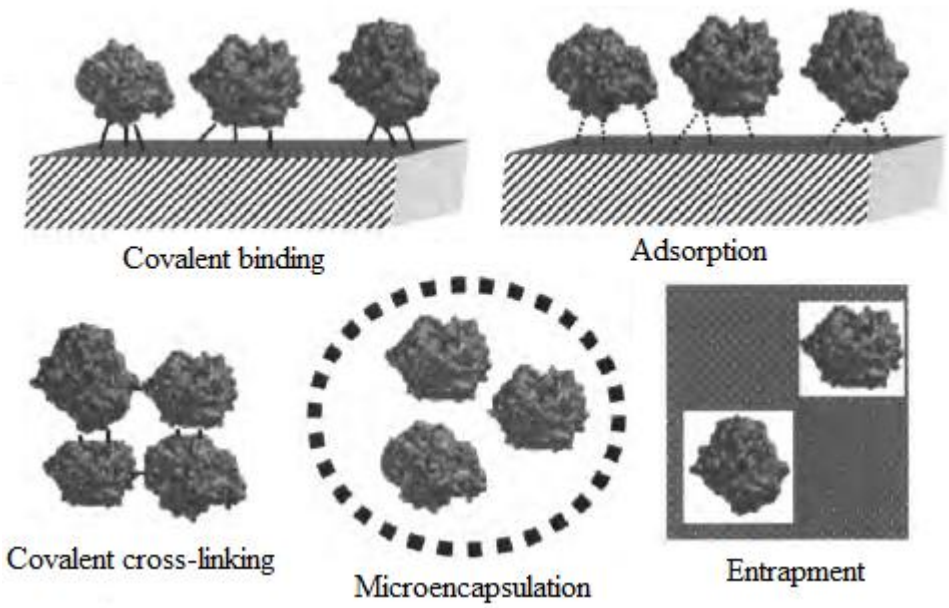


Figure 2.7. Classification of immobilization method
(Source: Drauz and Waldmann 2002).

2.3.1. Chemical Methods

This method includes covalent binding and cross-linking using multifunctional reagents, such as glutaraldehyde and cyanuric chloride and etc. The enzyme can be covalently attached or crosslinked to supports.

Covalent binding: The immobilization of enzymes by covalent attachment to support involves formation of a covalent bond between the functional groups of supports with amino acid residues of the enzyme like OH, SH, NH_2, and COOH. Activation of the support, enzyme coupling, and removal of loosely bound enzymes are three main steps. Silanes, carbodiimide or glutaraldehyde are the chemicals used for covalent attachment of enzyme to the support. In this technique rational control of immobilized enzyme is possible, the enzyme value and operational stability is high and enzyme release from support is minimal. The establishment of the stronger carrier-protein linkage also provides a more stable immobilized enzyme. However significant decrease in (or even loss of) activity occurs. Glyoxyl agarose and amino-epoxy are interesting supports for enzyme immobilization by covalent attachment.

Covalent cross-linking: Enzymes can be insolubilized by cross-linking with bifunctional reagents (like glutaraldehyde or toluene diisocyanate) without the addition of an inert carrier. The obvious advantage is that the specific activity of the enzyme is

very high. This is because the enzyme is auto-immobilized in its own protein mass (no support is involved). This technique can be performed over the soluble enzyme protein (cross-linked enzyme, CLE), over a crystallized enzyme protein (crosslinked enzyme crystal: CLEC) or over a protein enzyme aggregate (cross-linked enzyme aggregate: CLEA). Direct cross-linking of the enzyme followed by precipitation results in low activity and poor mechanically properties. CLECs are produced by cross-linking of purified enzyme crystals which exhibit excellent properties such as high stability under adverse conditions (high temperatures, extreme pHs, organic solvents), excellent mechanical properties and simple biocatalyst recovery. However, a main drawback is their high cost arises from the requirement of a high degree of enzyme purity to crystallize. CLEAs are obtained by cross-linking of protein aggregates produced by conventional protein precipitation techniques which provide insoluble and stable cross-linked enzyme aggregates. In this technique purified enzyme is not required and therefore the cost of production is low. On the other hand mechanical properties and control of particle size are the main drawbacks.

2.3.2. Physical Methods

This immobilization method mainly entrapment, micro encapsulation and adsorption do not involve covalent bond formation and therefore the native composition of the enzyme does not change.

Entrapment: In this method enzyme is held by the support and physically restricted within a confined space or network to to keep them from diffusing away. There is no direct interaction between enzyme and support, therefore, its conformational structure and hence activity can be preserved. The enzymes can be entrapted by the supports such as polymeric matrices, gelation of polyanionic or polycationic polymers, hollow-fiber ultrafiltration, membranes (cellophane, cellulose acetate or nitrate, poly (vinyl alcohol) and polyurethane), entrapment gels (agarose, alginate, κ-carrageenan, gelatin, polyacrylate and polyacrilamide) liposomes, cross-linked arrays and cross-linked whole cells. The pore structure of the support used for entrapment should enable to diffuse small molecules (substrates and products) while keep the macromolecular enzyme within the network. Mass transfer limitations are an issue due to imposible control of pore size and leakage of enzyme is often a problem in these systems. The

retention of enzyme and mechanical strength can be improved by using glutaraldehyde or other covalent cross-linking reagents.

Encapsulation: In this technique there is no covalent attachment of the enzyme to the medium. Enzymes can be encapsulated within the microscopic semi-permeable membranes (microencapsulation) or within the macroscopic hollow-fiber membranes. Reverse micelles and liposomes (composed by a double layer of surfactant) are common microscopic encapsulation technique. Microenvironment is adequate for the enzyme and mass transfer limitations are negligible for enzyme reverse micelles. On the other hand mechanical weakness is the main drawback.

Adsorption: The interactions between the enzyme and the support including non covalent bonds like van der Waals forces (short-range interactions), and also hydrophobic interactions, electrostatic and ionic bonds (stronger ones) are termed as adsorption. Hydrophobic bonds are formed between hydrophobic surfaces and hydrophobic domains of enzymes whereas electrostatic bonds can be formed between charges on the support surface and charged groups on the enzyme. It is the simplest method where the carrier can be easily recovered. Adsorption is frequently used in industrial processes because of the low cost, and high immobilization yields. This type of immobilization does not require any chemical modification and protein structure remains usually unaltered. However, the main problem is that the enzyme can be easily desorbed from the support which can be prevented by cross-linking. Randomly orientated biological molecule is the other drawback for this technique (Illanes 2008; Drauz and Waldmann 2002; Mulchandani and Rogers 1998; Buerk 1993; Lojou and Bianco 2006; Mikkelsen and Corton 2004; Watson 2004).

Table 2.6 summarizes the adventage and disadvantage of immobilization methods.

Table 2.6. Comparison of immobilization methods
(Source: Drauz and Waldmann 2002).

Immobilization Methods	Advantages	Disadvantages
Covalent binding	• Tight binding • Wide variety of supports and linkers available • Rational control of enzyme loading, distribution and microenviroment	• Chemical modification of enzyme • Often expensive • Activity diluted by support • May limit mass transfer
Covalent cross-linking	• High volumetric activity • Compatible with elevated temperature and organic solvents • No carrier required • Tight binding • Efficient for whole cells	• Chemical modification of enzyme • Little control of particle properties (especially for precipitate and whole cell) • Requires crystallization of enzyme (for CLEC) • May limit mass-transfer
Entrapment and Encapsulation	• No chemical modification of enzyme • Can be simple • Efficient for whole cells	• Little or no stabilization • Environmental changes can disrupt network and cause leakage • Often limits mass transfer
Adsorption	• Simple • No chemical modification of enzyme • Reversible • Often inexpensive	• Weak binding, leaching of enzyme • Little or no stabilization • Non-specific binding • May limit mass transfer

2.4. Properties of Immobilized Enzyme

The catalytic behavior of immobilized enzymes may different from that of soluble ones. Reduction of the overall enzyme activity can be observed due to enzyme inactivation, steric hindrance or mass transfer limitations. Reaction kinetics of enzyme can also be affected by conformational (native three-dimensional structure of the

enzyme protein) and micro-environmental (refers to partition and mass transfer limitations) effects caused by immobilization. Mass transfer limitations are usually expressed as diffusional restrictions. Because substrate transportation from the bulk to the enzyme and transportation of products away from the enzyme is governed by molecular diffusion. Diffusional restrictions are usually divided into two categories external and internal. Reaction rate (strongly depend on the stirring in the batch reactor or on the flow rate of a substrate in the column reactor) should not depend on pH or enzyme concentration if a reaction is completely limited by external diffusion. The partitioning of substrates, products, inhibitors, met al and hydrogen ions between the bulk solution and support is another important phenomenon for immobilized enzymes. Mostly, partition occurs with respect to protons due to the redistribution of hydrogen ions between a bulk solution and support. Negatively charged supports result in a shift of pH profile to a basic pH while positively charged supports result in a shift of pH profile to a acidic pH. These electrostatic effects exist in solutions with low ionic strength and the shift in pH displacement is reduced by increasing the ionic strength of the medium (Illanes 2008; Drauz and Waldmann 2002).

2.5. Selection of Support Material

Some desirable characteristics should be common to any supports even there is no universal ones for all enzymes and their applications. These include: high affinity to proteins, availability of reactive functional groups, hydrophilicity, susceptibility, mechanical and chemical stability and rigidity, regenerability and excellent film forming ability. High protein binding capacity, compatibility, insolubility in the reaction medium, recoverability after use and conformational flexibility are the other desired characteristics for the supports. The materials should also be nontoxic, biocompatible biodegradable, and cost-effectiveness (Krajewska 2004; Dung et al. 2009; Bhatt et al. 2010; Peña et al. 2010)

There are lots of supports available for the immobilization of enzymes by using the wide range of methods. Table 2.7 shows the type of supports used for enzyme immobilization (Drauz and Waldmann 2002).

Table 2.7. Type of support materials.

Organic - synthetic polymer	**Organic - biopolymer**	**Inorganic**
Polyamides	**Polysaccharide**	**Minerals**
Nylon	Cellulose	Sand
Poly alkylene	Starch	Pumice
Polystyrene	Agarose	Met al oxides
Polyacrylates	Dextran	Diatomaceous earth
Polyacrylamide	Chitin	Clays
Polyethylene	Polyalginate	
Polypropylene	Carrageenan	
Polyvinyl alcohol	**Proteinaceous**	**Synthetic**
Polyvinyl.acetate	Gelatin	Glass, controlled pore
Polyvinykhloride	Collagen	glass
Polyethylene glycol	Silk	Zeolites
Polyester	Albumin	Silica
Polycarbonate	Bone	Sol-gel
Polyurethane		Alumina
Polysiloxane		Met al Oxides
Phenol-fonnaldehyde		Met als

Special care should be taken in choosing a representative support for enzyme immobilization. Among the many carriers that have been considered and studied for immobilizing enzymes, chitosan and gelatin offer most of desired characteristics.

2.5.1. Chitosan as Support

Chitin and chitosan are natural polyaminosaccharides (Dung et al. 2009; Li et al. 2008; Bhatt et al. 2010). Chemically, chitin which is a long chain linear polymer composed by β(1 → 4) linked 2-acetamido-2-deoxy-β-d-glucose units (or *N*-acetyl-d-glucosamine), as shown in Figure 2.8. It is insoluble in most solvents. Chitosan is a copolymer of *N*-acetyl-d-glucosamine and d-glucosamine (the principal derivative of chitin), and characterized by the degree of deacetylation. It is insoluble in water, but below the pH value about 6.5 (in acidic solutions) the presence of amino groups provides solublility (Krajewska 2004). Chitosan is a promising matrix for enzyme immobilization due to having reactive amino and hydroxyl functional groups. The surfaces of this material are also transparent in the UV and visible regions, therefore should have little effect on most optical detection methods (Abdullah et al. 2006).

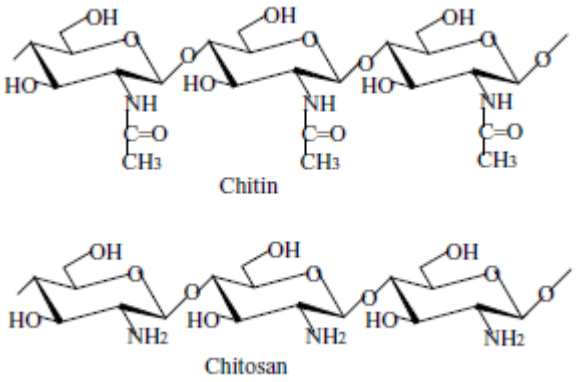
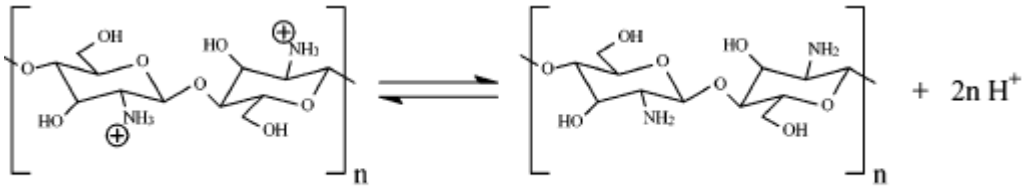


Figure 2.8. Structure of chitin and chitosan

Chitosan properties are largley depends on the pH which substantially alters the charged state of this material. The amines in chitosan are protonated and positively charged at low pH, and chitosan becomes a water-soluble cationic polyelectrolyte. In the case of high pH condition, the polymer loses its charge due to deprotonation of chitosan's amines and the material becomes insoluble as depicted in Figure 2.9 (Yi et al. 2005). The p*K*a value of chitosan is near neutral and the soluble-insoluble transition occurs at pH between 6 and 6.5 which is a particularly convenient range for biological applications.



Soluble Insoluble

Figure 2.9. Soluble and insoluble from of chitosan

2.5.2. Gelatin as Support

Gelatin is a heterogeneous mixture of water-soluble proteins (on dry weight basis, consists of 98 to 99% protein) with high molecular weight. Peptide bonds keep together the coils of amino acids. The unique protein structure provides wide range of functional properties which are still being discussed today despite it is one of the polymers recognized for millennia. These proteins derived from the chemical

degradation of collagen, with average molecular weights from 65,000 to 300,000 g/mol, depending on the grade of hydrolysis and also form a compound (triple) helix in aqueous solution. Gelatin contains mainly glycine, proline and 4-hydroxyproline (for pigskin gelatin 33%, 13% and 9%, respectively). Chemical structure of gelatin is shown in Figure 2.10.

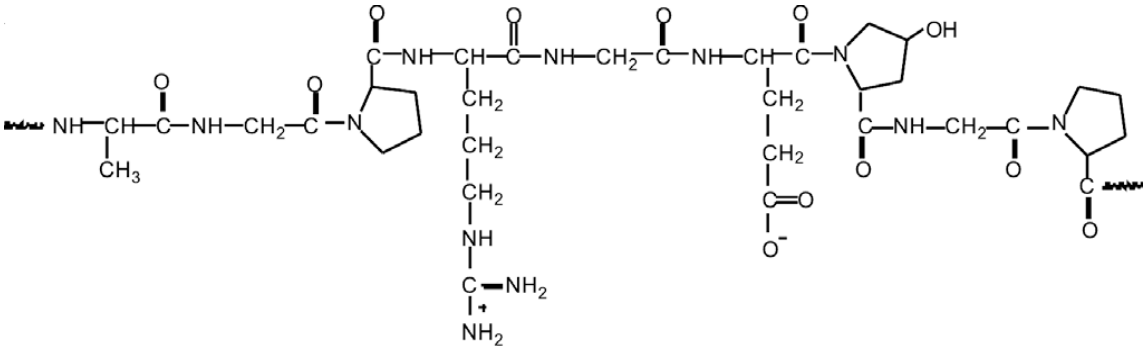


Figure 2.10. Chemical structure of the gelatin

This material is brittle in dry state with high moisture absorption arise from the tightly bounds (hydrogen bonds and hydrophobic interactions) present in its structure and the polar groups of amino acids. These gels are insoluble in organic solvents and soluble in hot water, glycerol, and acetic acid. Their viscosity increases under stress (thixotrophic) and are thermally reversible. Gelatin is neither acidic nor alkali (amphoteric) depending on the nature of the solution. The isoelectric point of gelatin (the pH at which gelatin's charge in solution is neutral) ranges between 4.8 and 9.4. The protein gelatin is also colorless, brittle when dry, odorless slightly yellow and tasteless. Gelatin has wide application area such as photography, pharmaceutical, tissue engineering, food industries and also glue since ancient times. (Peña et al. 2010; Omri 2002). These materials can be successively used for most optical detection methods due to having transparent surfaces.

CHAPTER 3

PESTICIDES

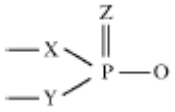
The science dealing with poisons or toxicants is defined as toxicology (Klassen et al. 1996). Although nutritional toxicology and food toxicology are related with eachother and they can be considered as a parallel branch, they are not synonymous. Overall, the effect of natural and synthetic compounds present in food on human health have been investigated taking into account dangerous effects (carcinogenicity, allergic reactions, immunotoxicity, neurotoxicity, and the potential for the prevention of disease) by the researchers working in the area of food toxicology. Pests are living organisms where they are not wanted or that cause damage to crops, humans, or other animals. Pesticides are type of toxicant used in agriculture and play an important role in the protection food plants. They assist in ensuring substantial yield by controling insects, weeds, organisms responsible for plant diseases, and other pests and provide a wide variety of foods at inexpensive prices to the consumers. They are inherently designed to kill but must be selective with regard to target species.

However the use of pesticides results in some degree of risk to human healt, animals and ecological balance. It should be considered the potential health risks of pesticides in the human diet. Because pesticides are toxic chemicals and many of them may potentially leave residues on foods available for human consumption. The possible risks are available caused by pesticide residue such as acute poisoning (exposure to large amounts of pesticides consumed in a short duration) and chronic risks (exposure to low levels of pesticide residues over extended periods of time). Agricultural workers involved in the mixing, loading, or application of pesticides and to those working in fields treated with pesticides are also at risk in addition to consumer concerns. Some epidemiological evidence caused by specific pesticides and occupational illnesses and injuries resulting from pesticide use have been reported frequently. These materials are also present environmental concerns such as air pollution, destruction of natural vegetation, reductions in natural pest populations, water and soil contamination. They can also effect the non-target organisms including fish, wildlife, and livestock, creation of secondary pest problems, and the evolution of pesticide resistance.

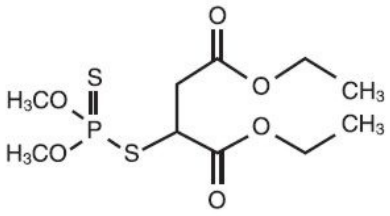
Some pesticides including the organophosphate, carbamate, and pyrethroid pesticides disrupt the movement of nerve impulses. The time for harvesting is very important because some of ones are residual in action and continue to be effective for months after their application. For example the triazine herbicides persist in the soil and kill emerging weeds over the lifetime of a crop (Watson et al. 2004). Many common pesticides and herbicides are categorized as; organochlorine pesticides, organophosphorus pesticides, carbamate pesticides, triazine herbicides, chlorophenoxy acid herbicides and urea-type herbicides. In many countries, the application of organochlorine pesticides in agriculture has been banned while other classes of pesticides such as organophosphates and carbamates have become popular.

Organophosphorus (OP) pesticides are small molecules. They are derived from phosphoric acid with the oxygen atoms being either replaced by other atoms, such as sulphur, and/or linked to aliphatic, aromatic, anhydrides or heterocyclic groups. Table 3.1 lists the more important categories of OP compounds with their particular side chains, and Figure 3.1 shows the structure of three examples of common OPs.

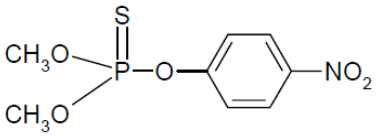
Table 3.1. Main side groups on different classes of organophosphate compounds (Source: Watson et al. 2004)



Class of organophosphorus	X	Y	Z
Phosphate	-O	-O	O
Phosphorothionate	-O	-O	S
Phosphorothiolate	-S	-O	O
Phosphorodithionothiolate	-S	-O	S
Phosphorodithiolate	-S	-S	O
Phosphoramidate	=N	-O	O
Phosphordiamidate	=N	=N	O
Phosphoramidothionate	=N	-O	S
Phosphoramidothiolate	=N	-S	O
Phosphonate	≡C	-O	O
Phosphonothionate	≡C	-O	S
Phosphonothionothiolate	≡C	-S	S



(a)



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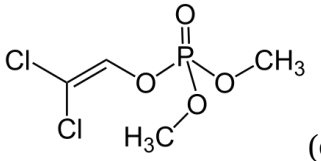

(c)

Figure 3.1. Three examples of organophosphates (a) Malathion (b) Parathion-methyl (c) Dichlorvos

Some common pesticides of OP and their alternative names are also listed in Table 3.2 (Patnaik et al. 2004).

OPs have been extensively used as insecticides, and are also used as nematocides, helminthicides and have fungicidal and herbicidal properties. These compounds exert their toxic effects by tendency to inhibit a number of important enzymes such as acetylcholine esterase. The importance of this enzyme is related with the inactivation of the fast-acting neurotransmitter acetylcholine found in the nerve synapses of the neuromuscular junction and brain nicotinic junctions. The use of OP is strictly regulated to prevent harmful effects to the population and the maximum permissible level of OP residue, known as the maximum residue limit (MRL) in ppm is measured.

Table 3.2. Common Organophosphorus Pesticides, their formulas and synonyms

Pesticide	Molecular formula	Alternative Names
Abate	$C_{16}H_{20}O_6P_2S_3$	Difenphos, Temefos, Bithion
Acephate	$C_4H_{10}NO_3PS$	Acetamidophos, Orthene
Akton*	$C_{12}H_{14}Cl_3O_3PS$	Axiom
Aspon	$C_{12}H_{28}O_5P_2S_2$	ASP 51, Propyl thiopyrophosphate
Azinphos methyl	$C_{10}H_{12}N_3O_3PS_2$	Gusathion, Guthion, Carfene
Azinphos ethyl	$C_{12}H_{16}N_3O_3PS_2$	Gusathion ethyl, Ethyl guthion, Bionex
Bolstar sulfone	$C_{12}H_{19}O_4PS_3$	—
Bomyl	C H O P	GC 3707
Carbofenthion	$C_{11}H_{16}ClO_2PS_3$	Trithion, Hexathion, Acarithion
Chlorfenvinphos	$C_{12}H_{14}Cl_3O_4P$	Birlane, Enolofos, Sapecron
Chlorofos	$C_4H_8Cl_3O_4P$	Metrifonate, Anthon, Trichlorfon
Coumaphos	$C_{14}H_{16}ClO_5PS$	Muscatox, Asuntol, Baymix
Crotoxyphos	$C_{14}H_{19}O_6P$	Ciodrin, Decrotox
Demeton-O	$C_8H_{19}O_3PS_2$	Mercaptophos, Thiodemeton
Demeton-S	$C_8H_{19}O_3PS_2$	Isosystox, Thioldemeton
Diazinon	$C_{12}H_{21}N_2O_3PS$	Basudin, Dimpylate, Neocidol
Dicapthon	$C_8H_9ClNO_5PS$	Isochlorthion, Chlorthion, Dicaptan
Dichlofenthion	$C_{10}H_{13}Cl_2O_3PS$	Nemacide, Mobilawn, Bromex
Dichlorvos	$C_4H_7Cl_2O_4P$	Chlorvinphos, Cyanophos, Atgard
Dicrotophos	$C_8H_{16}NO_5P$	Carbicron, Ektafos, Bidrin
Dimethoate	$C_5H_{12}NO_3PS_2$	Fosfotox, Cygon, Phosphamid
Dioxathion	$C_{12}H_{26}O_6P_2S_4$	Navadel, Ruphos, Delnatex
Disulfoton	$C_8H_{19}O_2PS_3$	Dithiodemeton, Glebofos, Dithiosystox
Dursban	$C_9H_{11}Cl_3NO_3PS$	Chlorpyrifos, Lorsban, Eradex
EPN	$C_{14}H_{14}NO_4PS$	Santox, EPN 300
Ethion	$C_9H_{22}O_4P_2S_4$	Rodocide, Phosphotox E, Ethanox
Ethephon	$C_2H_6ClO_3P$	Camposan, Ethrel
Famophos	$C_{10}H_{16}NO_5PS_2$	Famphur, Warbex, Cyflee
Fenamiphos	$C_{13}H_{22}NO_3PS$	Nemacur, Phenamiphos
Fensulfothion	$C_{11}H_{17}O_4PS_2$	Terracur P, Desanit, Hexazir
Fenthion	$C_{10}H_{15}O_3PS_2$	Baycid, Mercaptophos, Baytex
Folithion	$C_9H_{12}NO_5PS$	Fenitrothion, Metathion, Nitrophos
Fonofos	$C_{10}H_{15}OPS_2$	Dyphonate, Difonate
Isazophos	$C_9H_{17}ClN_3O_3PS$	Miral
Isofenphos	$C_{15}H_{24}NO_4PS$	Oftanol, Amaze
Leptophos	$C_{13}H_{10}BrCl_2O_2PS$	Phosvel, Abar, MBCP
Malathion	$C_{10}H_{19}O_6PS_2$	Fosfothion, Carbofos, Malafos, Cythion
Merphos	$C_{12}H_{27}PS_3$	Folex, Tributyl trithiophosphite
Methamidophos	$C_2H_8NO_2PS$	ENT, Tamaron, Monitor
Methidathion	$C_6H_{11}N_2O_4PS_3$	Supracide, Medathion, Ultracide
Mevinphos	$C_7H_{13}O_6P$	Phosdrin, Duraphos, Mevinex
Monocrotophos	$C_7H_{14}NO_5P$	Azodrin, Bilobran
Montrel	$C_{12}H_{19}ClNO_3P$	Crufomate, Amidophos, Ruelene
Naled	$C_4H_7Br_2Cl_2O_4P$	Dibrom, Bromex, Dibromfos
Oxydemeton-methyl	$C_6H_{15}O_4PS_2$	Metasystox R, Metaisosystox sulfoxide
Oxydemetonmethyl sulfone	$C_6H_{15}O_5PS_2$	Metasystox R sulfone
Parathion-ethyl	$C_{10}H_{14}NO_5PS$	Parathion, Thiophos, Foliodol
Parathion-methyl	$C_8H_{10}NO_5PS$	Metaphos, Azofos, Metron, Nitrox
Paraoxon	$C_{10}H_{14}NO_6P$	Phosphacol, Diethyl paraoxon
Phenamiphos	$C_{13}H_{22}NO_3PS$	Nemacur
Phorate	$C_7H_{17}O_2PS_3$	Thimate, Aastar, Granutox
Phosalone	$C_{12}H_{15}ClNO_4PS$	Rubitox, Zolone, Benzophosphate
Phosfolan	$C_7H_{14}NO_3PS_2$	Cyolane, Cylan

(Continued in next page)

Table 3.2. (cont.)

Pesticide	Molecular formula	Alternate Names
Phosphamidon	$C_{10}H_{19}ClNO_5P$	Famfos, Dimecron, Apamidon
Phosmet	$C_{11}H_{12}NO_4PS$	Imidan, Decemthion, Safidon
Profenofos	$C_{11}H_{15}BrClO_3PS$	Polycron, Selecron
Prophos	$C_8H_{19}O_2PS_2$	Ethoprophos, Mocap, Ethoprop
Sulfotepp	$C_8H_{20}O_5P_2S_2$	Dithiophos, Thiotepp, Pirofos
TEPP	$C_8H_{20}O_7P_2$	Fosvex, Nifos, Hexamite
Terbufos	$C_9H_{21}O_2PS_3$	Counter, ST-100
Tetrachlorvinphos	$C_{10}H_9Cl_4O_4P$	Stirofos, Rabon, Gardona
Tokuthion	$C_{11}H_{15}Cl_2O_2PS_2$	Protothiophos
Trichloronate	$C_{10}H_{12}Cl_3O_2PS$	Agritox, Phytosol, Phenophosphon
Zinophos	$C_8H_{13}N_2O_3PS$	Cyanophos, Menafos, Thionazine

MRL levels are set for different pesticides used for varied crops and additional variation can also be seen between the different regulating authorities. In addition, only certain OPs are licensed, with many OPs being banned. Table 3.3 gives examples of MRLs for three different OP compounds for three different foods.

Table 3.3. Maximum residue limits for three different OP compounds and for three different crops (Source: Budak 2001)

Organophosphate compound	Crop	Maximum Residue Limit (MRL in ppm)
Malathion	Peach	2
	Tomato	8
	Corn	0.5
Parathion-methyl	Peach	0.05
	Tomato	0.1
	Corn	0.2
Dichlorvos	Peach	0.1
	Tomato	0.2
	Corn	1

3.1. Detection of Pesticide Residue by Enzymatic Biosensor

Traditionally, separation techniques such as chromatography or electrophoresis have been used in order to determine the OPs by exploiting their chemical and

physicochemical properties. Quantification is achieved using gas chromatography (GC) or high performance liquid chromatography (HPLC). One of the sensitive and relatively quick techniques GC has the disdaventage of being expensive equipment that must have a gas supply, so is limited to laboratory use. The other preferred method for OP analysis is HPLC which does not have the disadvantage of thermal degradation of the sample as occurs in GC. However there are a number of limitations for this technique on its application such as expensive equipment, uses solvents and requires trained operators and as such is limited to laboratory use. The identification of OP can be achieved using spectral methods such as NMR and mass spectroscopy. NMR can identify a single pesticide residue whereas mass spectroscopy, when interfaced to either GC or HPLC, allows identification of more than one pesticide residues. Being highly specialised, expensive and limited to laboratories where trained personel perform the analysis has some disadvantages for these techniques.

In recent years, immunoassay and enzyme inhibition techniques developed by using the biochemical and immunological properties of pesticide residues are now widely used. These methods depend on the interaction between the pesticide residue and biological molecule differs from the techniques described in the previous methods. This interaction may be specific or non-specific for a particular pesticide as in the interaction with an antibody or as in the way a number of different pesticides interfere with an enzyme reaction respectively, (Watson 2004). The popularity of these methods comes from their advantages such as they are relatively simple, require little or no sample pretreatment, and do not require expensive instrumentation and provide rapid detection of pesticide residues (Mikkelsen and Corton 2004).

First known enzymatic assay was repotted by Osann in 1845: hydrogen peroxide (H_2O_2) was quantified using the enzyme peroxidase. In enzymatic methods OP compounds inhibit the biological activity of particular enzymes and prevent to form their products from given substrates. Measuring the activity of the enzyme in the absence and presence of the sample gives an indication of total OP concentration. There will be a decrease in enzyme activity (measured by monitoring the disappearance of substrate or the accumulation of product) in the presence of organophosphate residues. The most commonly used enzymes in these methods are acetylcholine esterase (AChE), butyrylcholine-esterase, organophosphorus hydrolase and ascorbate oxidase, alkaline phsosphatase, tyrosinase and acid phosphatase (Du et al. 2007; Solna et al. 2005). The detection of pesticide by tyrosinase enzyme sensor rely on the fact that the pesticide

inhibit the activity of tyrosinase enzyme by preventing the formation of enzymatically produced o-quinones (cause decrease in product amount) while catechol is consumed.

The conversion of analyte to another chemical species by the enzyme through a biochemical reaction causes a change in optical, electrical or mechanical properties of the analyte. Recently optical detection becomes mostly preferred ones among these detection techniques. Because they are convenient methods and rapidly providing accurate and reproducible results can be obtained. Sometimes incorporation of appropriate additional reagents such as 3-methyl-2-benzothiazolinone hydrazone (MBTH) allows the reaction to be monitored photometrically. Among the photometric methods enzyme assays based on changes in the light absorbed as the reaction proceeds are more frequently used one (Eisenthal and Danson 2002).

Detection of pesticide residue is generally performed in aqueous solutions. Besides this, water solubility of most pesticides is very low with respect to organic solvents which are used for the extraction of their residues from food before analysis. However enzymes show limited tolerance for the solvents. Therefore it is necessary to be considered the influence of different solvents on enzyme activity and applied solvent has to be carefully chosen, as well. The effects of organic solvents differ according to the hydrophobicity, solvating ability, molecular geometry, concentration of the solvents and the nature of the enzyme (Turdean and Turdean 2008). Their effects on enzymatic reaction rates are two types. One of them is distortion of enzymes arise from displacement of water molecules both in the hydration layer surrounds the protein molecules in aqueous solution and in the interior of the protein. The flexibility of the enzyme is restricted in the case of too low water content (Krishna 2002). Besides this polar organic solvents can interact with the essential water layer of enzyme which is required for maintaining the proper polypeptide conformation (Micaelo and Soares 2007). Therefore they disturb the non-covalent and hydrophobic interactions, which hold the enzyme in the proper folded state. The other type of effect is that, organic solvents behave as probable inhibitors or activators through specific interactions with enzymes, which cause to changes in the reaction kinetics and substrate specificity (Pazhang et al.2006). In the case of high solvent concentrations a partition effect of the solvent between the bulk solution and the enzyme microenvironment is produced (Illanes 2008).

CHAPTER 4

THEORETICAL ESTIMATION OF SOME KINETIC PROPERTIES FOR ENZYME IMMOBILIZED FILMS

The immobilized enzyme activity in operational modes could be reduced. Because the stagnant film surrounded around the non-soluble enzyme molecules prevent the transportation of substrate and products into or from the catalytic micro-environment. In general the mass transfer resistance tries to be minimized by adjusting the operating parameters. In this chapter, some theoretical approach for the estimation of some important kinetic parameters is discussed for enzyme immobilized films.

4.1. Characterization of Kinetic Parameters

The interaction between the enzyme (E) and substrate (S) is usually weak forces (van der Waals force and hydrogen bonding). The substrates can bind to the enzyme solution which has a fixed number of active site. At high substrate concentration the enzyme is saturated. In the other words all these sites may be occupied by substrates. Saturation kinetics can be explaibed by a simple reaction scheme which involves a reversible step for enzyme-substrate complex formation and dissociation step for the ES complex.

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} ES \overset{k_2}{\rightarrow} E + P \tag{4.1}$$

It is assumed that the rate of the reverse reaction of the second step is negligible and the ES complex is established rather rapidly . The rate of product (P) formation is;

$$V = \frac{d[P]}{dt} = k_2[ES] \tag{4.2}$$

In the development of quasi-steady state approach, the rate expression suggests that initial substrate concentration greatly exceeds the initial enzyme concentration [E_0]. Since [E_0] was small and d[ES]/dt ≈ 0. This can be written as;

$$k_1[E][S] = k_{-1}[ES] + k_2[ES] \tag{4.3}$$

By arranging the equation $[ES] = \frac{k_1[E][S]}{k_{-1} + k_2} = \frac{[E][S]}{K_m}$ (4.4)

Where $K_m = \frac{k_{-1} + k_2}{k_1}$ corresponds to the substrate concentration at the half-maximal reaction velocity. The meaning of K_m is complicated. For a two step reaction with $k_2 << k_{-1}$, K_m reduces to (k_{-1}/k_1). This is the dissociation constant for the ES complex, and K_m also measures the affinity of the enzyme for substrate. Low Km value indicates greater affinity of the enzyme for the substrate that the enzyme requires little amount of substrate to achieve saturated conditions.

In the system the total enzyme is $[E]_0 = [E] + [ES]$ (4.5)

Multiplying the total enzyme to rate of product formation;

$$\frac{V}{[E]_0} = \frac{k_2[ES]}{[E] + [ES]} \tag{4.6}$$

Where, V_m is the maximal velocity. When all of the [E_0] enzyme is in the ES complex represented as;

$$V_m = k_2[E_0] \text{ or } [E_0] = \frac{V_m}{k_2} \tag{4.7}$$

By applying these assumptions and substitution of equation 4.7 ($[E_0]$) and equation 4.4 ($[ES]$) to the equation 4.6, the rate equation becomes;

$$v = \frac{V_m[S]}{K_m + [S]} \tag{4.8}$$

Therefore the reaction kinetics is expressed with Michaelis-Menten equation (Equation 4.8) (Marangoni 2003; Shuler and Kargi 2002).

The enzyme activity is influenced in the presence of any inhibitor (such as organic solvents and pesticides) which decreases the rate of an enzyme-substrate reaction. The enzyme inhibition can be irreversible or reversible. In the case of reversible inhibition competitive, uncompetitive and noncompetitive ones are most commonly types. The values of V_m and K_m can be changed for each types of reversible enzyme inhibition as shown in Table 4.1.

$$\begin{array}{ccccc} E + S & \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} & ES & \xrightarrow{k_2} & E + P \\ \downarrow [\mathrm{I}] & & \downarrow [\mathrm{I}] & & \\ \mathrm{EI} & & \mathrm{EIS} & & \end{array} \tag{4.9}$$

Competitive inhibitors are usually substrate analogs and compete with substrate for the active site of the enzyme. The net effect of competitive inhibition is an increased value of K_m and therefore reduction reaction rate. Uncompetitive inhibitors bind to ES complex and have no affinity for the enzyme itself. The net effect of uncompetitive inhibition is a reduction in both V_m and K_m values and net result is a reduction in reaction rate. Noncompetitive inhibitors are not substrate analogs, bind at the enzyme (E) or at the ES complex. This type of inhibitor has no effect on the K_m value but lowers the V_m value. This is because the inhibitor binds all enzyme species with the same affinity.

Table 4.1. Summary of the effect of reversible inhibitors on enzyme catalytic parameters V_m and K_m (Source: Marangoni 2003).

	Competitive	**Uncompetitive**	**Noncompetitive**
V_m^*	No effect (-) V_m	Decrease (↓) V_m/α	Decrease (↓) V_m/α
K_m^*	Increase (↑) αK_m	Decrease (↓) K_m/α	No effect (-) K_m

Where, V_m^* and K_m^* correspond to the enzyme maximum velocity and enzyme–substrate dissociation constant in the presence of an inhibitor respectively.

$$\alpha = 1 + \frac{[I]}{K_i} \tag{4.10}$$

Where $[I]$ is the inhibitor concentration and K_i is the enzyme-inhibitor dissociation constant.

4.2. Characterization of Mass Transfer Resistance

During enzymatic processes the external and internal mass transfer resistances should be minimized to apply the Michaelis-Menten kinetics which assumes that;

- The substrate-binding step and formation of ES complex are fast with respect to the breakdown rate which leads to the approximation that the substrate binding reaction is at equilibrium.
- The substrate concentration remains essentially constant during the reaction ($[S_0] \approx [S_t]$) due partly to the fact that initial velocities are used.
- The conversion of product to the substrate is negligible. Because very little product has had time to accumulate during the reaction time.
- The system does not involve any mass transfer limitations during the enzymatic process.

These assumptions are based on the following conditions:

- The enzyme is stable during the measurements time used for determination of the reaction velocities.
- Reaction velocities are calculated from the initial rates of the reaction
- The reaction velocity is directly proportional to the total concentration of the enzyme (Marangoni 2003).

In the case of immobilized enzyme to the surface of the chitosan and gelatin film, external mass transfer resistance might be expected. Because homogenous catalytic reaction becomes heterogeneous which leads to hindrance of substrate transportation by the stagnant liquid surrounding solid enzyme. Figure 4.1 shows concentration profiles of substrate and products at the interface of optical biosensor under static condition.

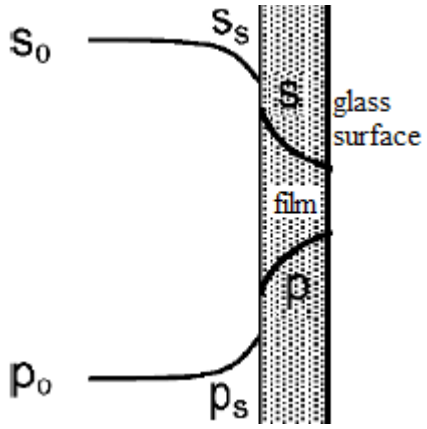

Figure 4.1. Substrate and product profiles in the immobilized enzyme system as a consequence of partition and mass transfer limitations under static condition

As can be seen from the figure substrate conversion occurs in three steps

i) Substrate transport from the bulk medium to the biocatalyst surface

ii) Enzymatic conversion of substrate to product

iii) Product transport from the biocatalyst surface to the bulk medium.

At steady-state, the average rate of substrate transport from the bulk fluid to the surface of the support is balanced by the enzymatic reaction rate.

$$r = k_s A(S_0 - S_s) = \frac{V_{max} S_0}{K_m + S_0} \tag{4.11}$$

Where S_s and S_0 are the substrate concentrations at the interface and in the bulk fluid respectively. If we arrange the equation (Engasser 1978);

$$S_s = S_0 - \frac{V}{k_s A} \tag{4.12}$$

The substrate or product diffusion can limit the enzyme catalytic efficiency. Any of these steps is the rate-limiting. The influence of external mass transfer resistance on the overall enzymatic reaction can be represented by effectiveness factor, η, which is defined physically by

$$\eta = \frac{(observed\ reaction\ rate)}{(rate\ in\ the\ case\ of\ no\ mass\ tranfer\ resistance\ S_s = S_0)} \tag{4.13}$$

If η is equal to 1, in this case there is no external mass transfer resistance. Mathematically, η is described by the following equation

$$\eta = \frac{\frac{V_{max}S_s}{K_m+S_s}}{\frac{V_{max}S_0}{K_m+S_0}} = \frac{\beta_s(1+\beta_0)}{\beta_0(1+\beta_s)} \tag{4.14}$$

Where

$$\beta_0 = S_0/K_m \tag{4.15}$$

$$\beta_s = \frac{-(1+\lambda-\beta_0) \pm \sqrt{[(1+\lambda-\beta_0)^2+4\beta_0]}}{2} \tag{4.16}$$

$$\lambda = V_{max}/k_s K_m \tag{4.17}$$

Only the positive root will give the positive value of β_s. λ can be calculated by maximum velocity (V_{max}) and Michaelis constant (K_m) . These values are determined from the Michaelis-Menten equation. The mass transfer coefficient k_s during the enzymatic reaction can be calculated by empirical correlation developed by Cussler (1984).

$$\frac{k_s.r_{film}}{D_{i,\infty}} = \alpha \,\mathrm{Re}^{0.5}.Sc^{0.33} \tag{4.18}$$

$$Re = \frac{D_p^2 N \rho_w}{\mu_w} \tag{4.19}$$

$$Sc = \frac{\mu_w}{\rho_w D_{i,\infty}} \tag{4.20}$$

Where r_{film} is the radious of the film, α is the device geometry (dimensionless: 0.323), D_p is the propeller diameter (m), N is the rotational speed (rate/s), $D_{i,\infty}$ is the

diffusion (1.13×10^{-9} m^2/s) coefficient of the solute (m^2/s), μ_w is the viscosity of water and ρ_w is the density of water (Miyabe and Okada 2002).