DEVELOPMENT OF A BIOSENSOR BASED ON AMINE OXIDASE FROM *Cicer arietinum* FOR THE DETECTION OF BIOGENIC AMINES

(PEMBANGUNAN BIOSENSOR BERDASARKAN *AMINE OXIDASE* DARIPADA *Cicer arietinum* BAGI MENENTUKAN *BIOGENIC AMINES*)

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2007

ACKNOWLEDGEMENT

I would like to express my appreciation to all those who have assisted me, whether directly or indirectly, in conducting this research. There are too many names to list down. However, special thanks goes to the research assistant, Mohd. Hafiz Kassim for his dedications and tireless efforts in making this research work a success.

ABSTRACT

Biogenic amines such as histamine, cadaverine and putrescine have been confirmed as useful chemical indicators to estimate bacterial spoilage of foods, particularly fish and fish products, cheese, meat and fermented foods. Histamine is toxic at high intakes, while cadaverine and putrescine potentiate the effects of histamine. The regulated level of histamine is 200 mg/kg (200 ppm). For biogenic amines biosensor, the basic principle is the action of diamine oxidase (DAO) that catalyzes the oxidative deamination of primary amines to the corresponding aldehydes, hydrogen peroxide and ammonia. Biogenic amines concentration can be measured by monitoring either the decrease in oxygen or the increase of hydrogen peroxide concentration. Recently, it has been found that DAO from pea seedlings shows higher activity compared to commercial porcine kidney diamine oxidase (PKAO). For that reason, in this research, DAO from *Cicer arietinum* (chick pea) seedlings will be used to develop the biogenic amines biosensor. Amine oxidases from chick pea (CPAO) was successfully purified following three chromatographic steps, giving a specific activity of 12.7, 11.7 and 0.45 U/mg with putrescine, cadaverine and histamine as substrate, respectively. The molecular mass of the CPAO was 73 kDa, determined by SDS-PAGE. Immobilization of PKAO in cross-linked poly (vinyl alcohol) (PVA) has been performed, and PVA concentration of 10% and cross-linking ratio (CR) of 0.06 were found to be the optimum parameters for CPAO immobilization. Immobilization of partially purified CPAO has also been done. Result showed that at equal enzyme loading, CPAO-PVA membranes gave higher apparent activities compared to commercial PKAO-PVA membranes.

ABSTRAK

Biogenic amines seperti histamin, kadaverin dan putresin telah disahkan sebagai penunjuk kimia yang sangat berguna untuk menganggarkan kerosakan makanan hasil tindakan bakteria, khususnya ikan dan produk ikan, keju, daging dan makanan yang diperam. Histamin adalah toksik dalam kadar pengambilan yang tinggi, manakala kadaverin dan putresin merangsang kesan-kesan histamin. Had yang dibenarkan bagi histamin ialah 200 mg/kg (200 ppm). Bagi biosensor *biogenic amines*, prinsip asas ialah tindakan *diamine oxidase* (DAO) yang memangkinkan tindakbalas deaminasi oksidatif terhadap amina utama kepada aldehid yang bersepadanan, hidrogen peroksida dan ammonia. Kepekatan *biogenic amines* boleh ditentukan dengan memantau samada tahap pengurangan kepekatan oksigen atau pertambahan kepekatan hidrogen peroksida. Terkini, DAO dari sumber bebenih kekacang menunjukkan aktiviti yang lebih tinggi berbanding DAO komersil dari sumber buah pinggang khinzir (PKAO). Atas sebab itu, dalam penyelidikan ini, DAO dari bebenih *Cicer arietinum* (kacang kuda) akan digunakan untuk membangunkan biosensor *biogenic amines*. *Amine oxidase* dari bebenih kacang kuda (CPAO) telah ditulenkan melalui tiga peringkat kromatografi, memberikan nilai aktiviti spesifik 12.7, 11.7 dan 0.45 U/mg dengan masing-masing putresin, kadaverin dan histamin sebagai substrat. Berat molekul bagi CPAO ialah 73 kDa, ditentukan melalui SDS-PAGE. Immobilisasi PKAO di dalam poly (vinyl alkohol) (PVA) yang disambung-silang telah dilakukan, dan kepekatan PVA 10% dan nisbah penyambungan-silang (CR) 0.06 di dapati merupakan parameter optima bagi immobilisasi CPAO. Immobilisasi terhadap CPAO separa tulen juga telah dilakukan. Keputusan yang diperolehi menunjukkan, pada muatan enzim yang sama, membran CPAO-PVA memberikan aktiviti bandingan yang lebih tinggi berbanding membran PKAO-PVA.

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SYMBOL	MEANING
α	Alpha
A_{280}	Absorbance at 280 nm
A_{450}	Absorbance at 450 nm
A_{562}	Absorbance at 562 nm
β	Beta
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CE	Counter electrode
CPAO	Chick pea seedling amine oxidase
CR	Cross-linking ratio
DAO	Diamine oxidase
DEAE	Diethyl amino ethyl
EPR	Electron paramagnetic resonance
GOD	Glucose oxidase
HDC	Histidine decarboxylase
HMT	Histamine-N-methyl transferase
HRP	Horseradish peroxidase
HPLC	High Performance Liquid Chromatography
kat	Katal; SI unit of enzyme activity
kDa	Kilo Dalton

LC	Liquid Chromatography
MW	Molecular weight
PEG	Poly(ethylene glycol)
PKAO	Porcine kidney amine oxidase
PPB	Potassium phosphate buffer
ppm	Part per million
PSAO	Pea seedling amine oxidase
PVA	Poly (vinyl alcohol)
RE	Reference electrode
SDS-PAGE	Sodium dodecylsulphate-polyacrylamide gel electrophoresis
TEMED	(*N,N,N',N'*-tetramethylene-ethylenediamine
U	Enzyme activity unit; 1 unit (U) is the amount of enzyme that catalyses the conversion of 1 micromole of substrate per minute under defined conditions.
WE	Working electrode

CHAPTER 1

INTRODUCTION

1.1   Introduction

Histamine poisoning, also known as 'scombroid fish poisoning' is an illness that results from eating spoiled fish because of inadequate refrigeration or preservation after it is being caught. It is most commonly reported with fish from *Scombridae* and *Scomberesocidae* families. One of the toxic agents implicated in scombroid poisoning is a chemical called histamine. For years, studies had been conducted to find the best method to detect and determine the level of histamine in food. This can avoid people from consuming spoiled food, instead of giving them treatment after being poisoned.

Traditionally, histamine has been measured by derivatization with fluorescent reagents followed by chromatographic separation (e.g. HPLC in most cases). As the HPLC analysis of biogenic amines is tedious with regard to sample clean-up prior to the analysis and it requires trained personnel in combination with quite expensive equipment, further analysis methods have been described such as capillary electrophoresis, immunochemical methods such as ELISA, and even some gas chromatographic methods, thin-layer liquid chromatography (LC), reversed phase

LC and LC with pre-column, post column and on-column derivatization techniques (Tombelli and Mascini, 1998; Lange and Wittmann, 2001). To reduce the time needed for analysis and to offer a rapid screening method for industrial food quality testing, some enzymatic methods and several enzyme sensors have been described so far by previous researchers (Chemnitius *et al*., 1992; Esti *et al*., 1998; Loughran *et al*., 1995; Wimmerova *et al*., 1999). Biosensor applications, in general, exhibit various advantages such as allowing a more rapid analysis with less sample treatment being required (Lange, J. and Wittmann, C., 2001).

In this work, an enzyme-based histamine amperometric biosensor will be developed. In developing a biosensor for detecting and determining the content of histamine in fish and its product, the basic principles used are the same as a glucose biosensor, except it needs to have a layer of diamine oxidase membrane instead of glucose oxidase. Extensive studies in constructing histamine biosensor have been conducted by lots of researcher before, which majority of them used diamine oxidase from porcine kidney as the immobilized enzyme. However, recently some researchers such as Tombelli *et al*., (1998), Niculescu *et al*., (2001), Wimmerova *et al*., (1999) and Frebort *et al*., (2000) have found that diamine oxidase extracted and purified from pea seedling shows higher activity compared to the commercial diamine oxidase from porcine. Consequently, the sensitivity of sensor was improved. An additional reason for using the enzyme is because since Malaysia is an Islamic country, it is better to avoid the usage of components or substances from pigs.

For that reason, in this research, diamine oxidase from pea seedlings will be used. Since pea seedling diamine oxidase is not available in the market, it has to be purified. The type of pea that will be used for enzyme purification is *Cicer arietinum* (chick pea) (Tombelli *et al*., 1998).

The purified chick pea diamine oxidase (CPAO) will be entrapped in cross-linked poly (vinyl alcohol) (PVA) membrane. PVA is a non-toxic water-soluble synthetic material that has good film forming properties, resulting in tough membranes. PVA can also stabilize the activity of various enzymes such as horseradish peroxidase. The stabilization effect is achieved through the inhibition of the formation of non-functional conformations due to the extensive hydrogen bonding between the H-atoms of the alcohol groups in PVA and the O-atoms of the carbohydrate groups in diamine oxidase. These properties make PVA an appropriate matrix for diamine oxidase immobilization. Glutaraldehyde, a bifunctional agent that can react with organic hydroxyl groups and lysine amino acid residues in the enzyme, will be used as the cross-linking agent. The cross-linking process overcomes the loss of enzyme activity due to diffusional loss, which is a prevalent problem for enzyme immobilized in physical entrapment.

1.2   Research Objectives

The objectives of this research are:

1. To purify diamine oxidase from *Cicer arietinum* seedlings (chick pea) as an alternative source of enzyme to porcine kidney diamine oxidase.

2. To develop highly active and stable chick pea seedling diamine oxidase (CPAO) membranes for histamine biosensor.

1.3   Research Scopes

The scopes of this research are:

1. Purification of chick pea seedling diamine oxidase (CPAO).

2. Comparison of the enzyme activity and stability between chick pea seedling diamine oxidase (CPAO) and the commercial porcine kidney diamine oxidase (PKAO).

3. Development of chick pea seedling diamine oxidase membranes which are highly active, stable and have long operational life using appropriate immobilization methods.

CHAPTER 2

LITERATURE REVIEW

2.1   Biogenic Amines

Biogenic amines are low molecular-weight organics compounds which are derived from the corresponding amino acids when the carboxylic acid is removed by enzymatic reactions. They are termed biogenic amines because they are formed in raw food by bacterial action. Biogenic amines may present in various foods, particularly fish and fish products, cheese, meat and fermented foods (Eerola *et al*., 1993). During storage and processing, if foods are mishandled, certain protein within the foods might break down to free amino acids, which may also be naturally present within the food. If the food is contaminated with bacteria containing decarboxylase enzymes, these free amino acids undergo decarboxylation to produce biogenic amines. For example, lysine is decarboxylated to produce cadaverine, histidine is decarboxylated to produce histamine, while glutamine, agmatine and arginine is decarboxylated to produce putrescine (Halasz *et al*., 1994) (Figure 2.1).

Decarboxylase enzymes

Histidine	$\rightarrow$	Histamine
Lysine	$\rightarrow$	Cadaverine
Glutamine, Arginine or Agmatine	$\rightarrow$	Putrescine

Figure 2.1 : Decarboxylation of certain amino acids to produce biogenic amines.

Biogenic amines often appear in conjunction with food intoxication. It were generated by microbial spoilage of food which high in protein content or through processing, ripening and storage of fermented foodstuff. Therefore, certain biogenic amines could be used as an indicator for food quality and hygiene during processing (Leuschner *et al.*, 1998)

The biogenic amines content of food depends on the biotechnological processes involved in the production procedures. It is influenced by certain factors such as microbial growth, availability of free amino acids, the presence of decarboxylase enzymes and elevated temperature conditions (Halasz *et al.*, 1994). The enzymes that are involved in the production of histamine, histidine decarboxylase, require temperature greater than 15°C and 30°C is the optimum temperature. In tropical areas of the world, fish are often caught in temperature exceeding $20°$C. If these fish are not refrigerated immediately, conditions are favorable for biogenic amines production providing bacteria containing decarboxylase enzymes are present. Bacterial growth will cease at temperature lower than 5°C, however enzymatic activity will still continue, resulting in further amine production (Ahmed, 1991).

2.2   Diamines

Many biogenic amines have been studied in scientific literature, however diamines such as histamine, putrescine and cadaverine are often documented in clinical studies with histamine being linked to food poisoning and putrescine and cadaverine potentiating the toxicity of histamine (Public Health Division, 2002). Histamine, cadaverine and putrescine have been confirmed as useful chemical indicators to estimate bacterial spoilage. Consumption of high level of histamine can lead to scombrotoxicosis while the presence of other biogenic amines is described to potentiate the effects. The significance of histamine is well known. Person being highly sensitive to histamine often develops pseudoallergic symptoms shortly after ingestion. For healthy individuals, the putrescine or cadaverine is not considered to be toxic. In general, dietary polyamines at levels normally present in food are nontoxic, while biogenic amines, particularly histamine is toxic at high intakes. The Food Standards Code stated that the regulated level for histamine is 200 mg/kg (200 ppm). Histamine itself is not destroyed by cooking, freezing, smoking, curing and canning (Lange and Wittmann, 2001). This is the same histamine that causes problems for some people when high levels are produced in cheese and red wine.

Histamine has an important role in human metabolism, such as the release of stomach acid. In small doses it has little effect, but in larger doses it has toxic effects. The intestinal tract of humans contains the enzymes diamine oxidase (DAO) and histamine-N-methyl transferase (HMT) which convert histamine to harmless degradation products. Putrescine and cadaverine can inhibit these enzymic reactions and are therefore potentiators of histamine toxicity. The presence of low levels of histamine, in the diet normally has no toxic effect as humans do not absorb histamine efficiently from the gastrointestinal tract. If a high level of histamine is present in the diet, then the capacity of DAO and HMT to detoxify histamine will be limited and histamine will enter into the bloodstream resulting in histamine poisoning (Taylor, 1986).

2.3   Histamine Poisoning

Histamine poisoning, also known as 'scombroid fish poisoning', histamine overdose, pseudo allergic fish poisoning or mahi-mahi flush is among the most common toxicities related to fish ingestion. The poisoning directly relates to improper preservation and inadequate refrigeration. Histidine decarboxylase (HDC) found in *E. coli* and in *Proteus* and *Klebsiella* species, converts histidine which present in fish tissue to histamine (Figure 2.2). The bacteria also live on fish tissue. Without adequate cooling, these bacteria multiply, increasing the conversion rate of histidine to histamine, thus increase the histamine levels.
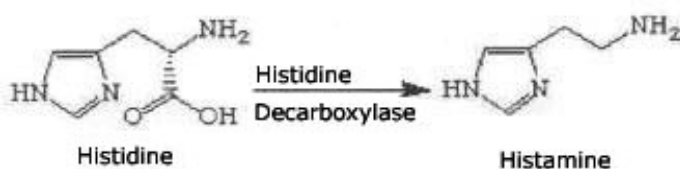
Figure 2.2 : Conversion of histidine to histamine by histidine decarboxylase (HDC).

Histamine poisoning described as a food-borne chemical intoxication. It is most commonly reported with fish from Scombridae and Scomberesocidae families. This includes tuna, mackerel, skipjack and bonito. Nonscombroid fish such as mahimahi, bluefish, amberjack, herring, sardines and anchovies, as well as Swiss cheese have also been implicated as causes of scombrotoxism (Hughes and Potter, 1991). Histamine poisoning can be resulted from inappropriate handling of fish during storage or processing. The poisoning actually is caused by ingestion of the toxins within the fish's tissues. Other chemicals have been found in decaying fish flesh, but their association to scombroid poisoning has not been clearly established. Generally, the symptoms of histamine or scombroid fish poisoning are a `sharp', `metallic' or `peppery' taste while eating the fish, flushing (reddening of the face and sometimes the neck, arms and upper part of the trunk), severe headache, palpitations (rapid heartbeat), stomach cramps and/or diarrhea, itching on the face or around the mouth,

a burning sensation in the throat or dryness of the mouth, difficulty in swallowing and/or breathing, muscle weakness and nausea (Wu *et al*., 1997; Bardocz, 1995; Hughes and Potter, 1991).

The symptoms listed above usually appear within one hour after eating decayed fish but the onset can range from a few minutes to several hours. It is often last for 8 to 12 hours, after which most persons recover rapidly. The treatments usually given to patients are drugs containing antihistamines, which have been shown to help in many cases. However, in more serious cases, prompt medical attention may be required.

The toxic levels for histamine are estimated at 200-500 mg/kg (200-500 ppm) (Noltkamper, 2002) while the recent regulated level is 200 mg/kg (200 ppm). Regulatory limits for histamine content in fish vary with countries. In the United States of America, 200 ppm denotes mishandling of fish while 500 ppm levels indicate `hazard action level'. In Germany and Sweden, 200 ppm histamine in fish results in the rejection of a consignment (Mohd Nasir Azudin and Nazamid Saari, 1988).

2.4 Amines in Plants

Plant amines can be considered simply as the products of decarboxylation of amino acids, formed by the reaction:

$$RCH(NH_2)CO_2H \rightarrow RCH_2NH_2 + CO_2$$
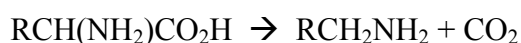
The most widespread plant amines are conveniently divided into three groups; aliphatic monoamines, aliphatic polyamines and aromatic amines. Aliphatic amines are volatile compounds (e.g. methylamine (CH_3NH_2) to n-hexylamine ($CH_3(CH_2)_5NH_2$). They are widely distributed in higher plants and fungi and when

present in any concentration, have unpleasant fish-like smell. They function in flowers (e.g. in the cow parsley, *Heracleum sphondvlium*) as insect attractants by simulating the smell of carrion.

By contrast to the monoamines, diamines and other polyamines are less volatile, although they still possess offensive odors. Widespread polyamines include putrescine $NH_2(CH_2)_4NH_2$, agmatine $NH_2(CH_2)_4NHC(=NH)NH_2$, spermidine $NH_2(CH_2)_3NH(CH_2)_4NH_2$ and spermine $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$. There are several others of more limited occurrence such as cadaverine $NH2(CH2)_5NH_2$. Polyamines are of topical research interest because of their growth stimulating activity in relation to their effect on ribosomal RNA.

The best known aromatic amine from plants is probably mescaline, the active principle of the flowering heads (peyote) of the cactus, *Lophophora williamsii*, a powerful natural hallucinogenic compound. Indeed, many of the known aromatic amines are physiologically active and for this reason they are sometimes classified with the alkaloids. Three substances which are very important in animal physiology are noradrenaline, histamine and serotonin; all three occur in plants, noradrenaline for example being present in the banana and the potato.

All the amines so far mentioned are primary amines, have the general formula RNH_2. Secondary amines, general formula R_2NH and tertiary amines, R_3N, are known in plants but they are not very common. A typical secondary amine found in plants is dimethylamine, a typical tertiary amine is hordenine N-dimethyltyramine, which is the principal `alkaloid' of barley (Harborne, 1973).

2.5   **Diamine Oxidase**

Diamine oxidase (DAO, EC 1.4.3.6) [amine: O_2 oxidoreductase (deaminating)] is a member of the class of copper-containing amine oxidases and catalyzes the oxidative deamination of histamine and other biogenic amines. Other names are diamino oxhydrase, histaminase, histamine deaminase, histamine oxidase, amine oxidase, amine oxygen oxidoreductase, Cu-amine oxidase, monoamine oxidase and others. Diamine oxidase was originally characterized as the enzyme degrading histamine and was therefore earlier called histaminase. It is characterized by possessing the active-site cofactor topa quinine, formed post-translationally by modification of a conserved tyrosine residue. Although diamine oxidase appears to play an important role in histamine catabolism, the enzymes efficiently converts many diamines besides histamine and is expressed in many tissues, suggests that it might have additional function. These ubiquitous soluble enzymes catalyze the oxidative deamination of primary amines to form the corresponding aldehydes, ammonia and hydrogen peroxide (Wilflingseder and Schwelberger, 2000).

$$RCH_2NH_2 + H_2O + O_2 \xrightarrow{DAO} RCHO + NH_3 + H_2O_2$$

Measurements of the oxygen consumption or the hydrogen peroxide production are commonly used for assays of the enzyme activity (Wimmerova and Macholan, 1999).

These Cu-amine oxidase enzymes have been found in several microorganisms such as bacteria and fungi, various plants and animals (Wimmerova and Macholan, 1999). Analyses of genes and cDNAs encoding copper amine oxidases revealed that all members of this enzyme family have homologous sequences with several absolutely conserved amino acid residues. The conserved residues appear to be important for the overall protein structure and for the catalytic function and include the tyrosine that is converted to topa quinone, three histidine residues that bind to the copper ion and an aspartic acid residue important for

substrate conversion (Wilflingseder and Schwelberger, 2000). Plant diamine oxidases are of widespread occurrence in Leguminaceae such as *Cicer arietinum* (chick pea), *Lathyrus sativus* (grass pea) and *Vigna radiata* (mungbean) (Choundhary *et al.*, 1999).

Disclosure of topa quinine as the organic cofactor (Janes *et al.*, 1990, 1992) and determination of amino acid sequences of some amine oxidases (Rossi *et al.*, 1992; Mu *et al.*, 1994; Tipping and McPherson, 1995) brought new aspects into the study of the molecular and structural properties of these enzymes. For the formation of topa quinone derived from the specific tyrosyl residue in the premature protein, a self-oxidation mechanism catalyzed by cupric ions has been proposed (Cai and Klinman, 1994; Matsuzaki *et al.*, 1994). Copper ion and quinone cofactor mediate the catalytic reaction following a ping-pong mechanism (Hartman and Klinman, 1991). Electron paramagnetic resonance (EPR) studies have shown the occurrence of a Cu(I)/topa-semiquinone state as an intermediate in the catalytic cycle at the substrate reduced enzyme (Dooley *et al.*, 1991; Turowski *et al.*, 1993)

Diamine oxidase was first purified by Mann (1955) from pea seedlings. There after, the enzyme has been purified to homogeneity and characterized from different plant sources. Pea seedling amine oxidase (PSAO) has wide substrate specificity, oxidizing preferably natural diamines and polyamines (Kenten and Mann, 1952). However, the commercially available diamine oxidase is from porcine kidney. This porcine diamine oxidase has a low specific activity, even when purified, in contrast to the plant homogenous enzyme. PSAO has already been used in amperometric biosensors for the assay of biogenic amines and for the assay of some pseudosubstrates and drugs (Wimmerova and Macholan, 1999).

The crystal structure of amine oxidase from *E. coli* has been determined in both an active and inactive form (Parson *et al.*, 1995). Each subunit of the mushroom-shaped dimer comprises of four domains: a large C-terminal β-sandwich domain, which contains the active site and provides the dimer interface, and three

smaller peripheral α/β domains. The active sites are buried in the protein and lay some 35 Å apart, connected by a pair of β-hairpin arms. Copper binds directly to topa quinone in the inactive form, but not in the active form.

Recently, the structure of amine oxidase from pea seedlings (*Pisum sativum*) has been reported. The protein structure is quite similar to that of the enzyme from *E. coli*, and the study provided additional information on the arrangement and catalytic mechanism of the active site (Kumar *et al.*, 1996). Plant copper-containing amine oxidases have been mostly isolated from seedlings of members of the Fabaceae, particularly of pea and lentil (Medda *et al.*, 1995). Two copper-containing amine oxidases from plants of the genus *Lathyrus* have also been characterized. The enzyme from *Lathyrus sativus* was first reported by Suresh *et al.*, (1976). Substrate specificity and sensitivity of this enzyme to several inhibitors were rather similar to the oxidases from lentil and pea (Medda *et al.*, 1995).

Some of the molecular properties of this enzyme have been determined using improved purification method which gave a homogeneous enzyme (Padiglia *et al.*, 1991). This enzyme is a homodimer of 70 to 90 kDa subunits, each containing a single copper ion and a covalently bound cofactor formed by the post-translational modification of the catalytic tyrosyl residue to 2,4,5-trihydroxyphenylalanine quinone (TPQ). The molecular mass of 150 kDa determined by gel permeation chromatography and of 72 kDa found by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) confirmed the enzyme dimeric structure. Similar properties were found for the amine oxidase from seedlings of *Lathyrus cicera*, which was purified using the same procedure (Cogoni *et al.*, 1989).

In mammalian tissues, Cu-amine oxidases enzymes prefer either monoamines or diamines as substrates. Mammalian DAO is a homodimeric glycoprotein with subunits of a relative molecular weight of approximately 100,000 linked by disulfide bonds. DAO primary structures are highly conserved in mammals. It posses a classical signal peptide and is *N*-glycosylated indicating that the protein enters the

secretory pathway. It is soluble enzyme mainly found inside cells. In porcine kidney and intestine, it is localized in vesicular structures in proximity to the plasma membrane. Recent crystallographic studies of Cu-amine oxidases from microorganisms and plants have extremely large contributed to the understanding of the enzymology and structural organization of these proteins (Wilflingseder and Schwelberger, 2000).

2.6   Chickpeas (*Cicer arietinum*)

Chickpeas, as shown in Figure 2.3, are the pea-like seeds of a bushy plant, an irregularly round with a nutty flavour and frequently used in Central Asian and Middle Eastern cuisine. The chick pea, also known as garbanzo bean, bengal gram, channa dhall or in Malay called 'kacang kuda', is an edible pulse of the *Leguminosae* or *Fabaceae* family, subfamily of *Faboideae* or *Papilionoideae*. The botanical name for chickpeas is *Cicer arietinum*. The scientific classification of chickpea is shown in Table 2.1. The plant is 20-50 cm high and has small feathery leaves on both sides of the stem. One seed-pod contains 2-3 peas. The flowers are white or reddish blue. Chickpeas need a warm climate and more than 400 mm annual rain.

Figure 2.3 : The fruit and seeds of the chickpeas (*Cicer arietinum*).

Table 2.1 : Scientific classification of chickpeas.

Kingdom :	Plantae
Division :	Magnoliophyta
Class :	Magnoliopsida
Order :	Fabales
Family :	Fabaceae
Subfamily :	Faboideae
Genus :	*Cicer*
Species :	*arietinum*

2.6.1   Chickpea Varieties

Several dozen distinct chickpea varieties are cultivated. There are two groups of chickpeas, European and Asian, distinguished by seed size, shape and colour. They also have different growth requirements and end uses. The European varieties, also known as `kabuli' types, are large (typically around 15 mm diameter) and more rounded or brain-shaped types which are most commonly pale yellow in color. There were several other varieties, with black and reddish seeds that are rarely grown today. The black variety was mainly used as fodder. Asian varieties, also known as `desi' types, are smaller (typically 5-8 mm) and angular seeds, which are dark brown in color.

The chickpea is not known in a wild state. The kabuli types are commonly found throughout southern Europe, Western Asia, the Nile Valley, North Africa and South America, whereas the desi types are mostly found in the Indian subcontinent, Iran, Ethiopia and parts of central America. They are available mainly dried whole or split. In parts of the world where chickpeas are grown they are frequently sold as the whole green plant from which the seeds are consumed fresh as a snack or the whole

plant can be placed in a fire and the parched seeds eaten as a snack. They are also available tinned whole or as a puree. Chickpea flour is also available in some countries.

2.6.2   Chickpea Nutrition and Uses

Chickpeas are an excellent source of carbohydrates and proteins, which constitute about 80% of the total dry seed weight. Dried chickpeas contain about 20% protein. The bulk of the seed is made up of carbohydrates (61%) and 5% fat. Crude fiber is mostly located within the seed coat. They are relatively rich in lecithin, potassium, phosphorus, calcium, folate and vitamin C, and also have small quantities of vitamins A and B. 100 g of chickpeas can supply about 350 calories.

Chickpeas can be eaten in salads, cooked in stews and ground into a flour called `gram flour' (also known as besan, garbanzo bean flour or in Malay `tepung kacang kuda', and usually used in Indian cuisine). They can also be ground and shaped in balls and fried as `falafel', cooked and ground into a paste called hummus, or roasted and spiced and eaten as a snack. The plant can also be used as a green vegetable. Dried chickpeas can be kept almost indefinitely. Tinned chickpeas last well for up to 5 years. Once cooked, the chickpeas can be stored covered for several days in the fridge.

2.6.3   History of Cultivation

The earliest chickpeas found on the Hacilar site near Burdur in Turkey, have been estimated to be 7500 years old. It is not known if these were cultivated or collected from the wild but it is near this area of the fertile crescent that chickpeas are believed to have been first domesticated and where the wild progenitor *Cicer reticulatum* was recently discovered. They have been found in the late Neolithic in Thessaly, at Kastanas, Lerna and Dimini at ca. 3500 BC. In the southern French cave of L'Abeurador Dept. Aude, chickpeas have been found in Mesolithic layers, dated to 6790+90 BC with the radiocarbon method.

They have also been found at about the same time in Iraq and are known to have been grown at a later date in the hanging gardens of Babylon. By the Bronze age, they were known in Italy and Greece. In classical Greece, they were called `erebinthos', and eaten both as a staple and as dessert, and eaten raw when young. The Romans knew several varieties known, for example venus-, ram- and punic chickpeas. They were eaten as a broth and roasted as a snack. The Roman gourmet Apicius gives several receipes for chickpeas. Carbonized chickpeas have been found at the Roman legionary fort at Neuss (Novaesium), Germany in layers of the 1st century AD, as well as rice.

Chickpea are mentioned in Charlemagne's Capitulare de villis (ca. 800 AD) as Cicer italicum, to be grown in each imperial demesne. Albertus Magnus knows three varieties, red, white and black. In India, the acid secretion of chickpea leaves is sometimes collected by spreading a cloth over the plants at night. The acid mixed with dew is wrung out and used medicinally and as vinegar. Eastern Sicily has a dish made by putting chickpeas and hot pebbles in the same container and stirring them vigorously until the heat from the pebbles has cooked the chickpeas. It would appear that chickpeas have been eaten by man since earliest civilization.

2.7   Introduction of Biosensor

A biosensor may be broadly defined as any measuring device that contains a biological element (Buerk, 1993) or a device incorporating a biological sensing element connected to a transducer (Eggins, 1996). It is also frequently described as a 'reagentless' system, or it is more correct to say that the reagents are already part of the reaction chamber and do not therefore have to be added by user, most likely be concerned with immobilized reagent (Hall, 1991). The biological element involved might be tissue, microorganisms, organelles, cell receptors, enzymes, antibodies or nucleic acids (Rogers and Gerlach, 1996). The sensing element which responds to the substance being measured is biological in nature. It has to be connected to a transducer of some sort so that a visually observable response occurs. A transducer converts an observed change, physically or chemically into a measurable signal, usually an electronic signal whose magnitude is proportional to the concentration of a specific chemical or set of chemicals. It is an apparently alien marriage of two contrasting disciplines which combines the specificity and sensitivity of biological systems with the computing power of the microprocessor. Biosensors are generally concerned with sensing and measuring particular chemicals which need not be biological components themselves, although sometimes they are. They are referred as the *substrate,* although the more general term *analyte* is often used. (Eggins, 1996). A schematic diagram drawing for a generalized biosensor is shown in Figure 2.4.
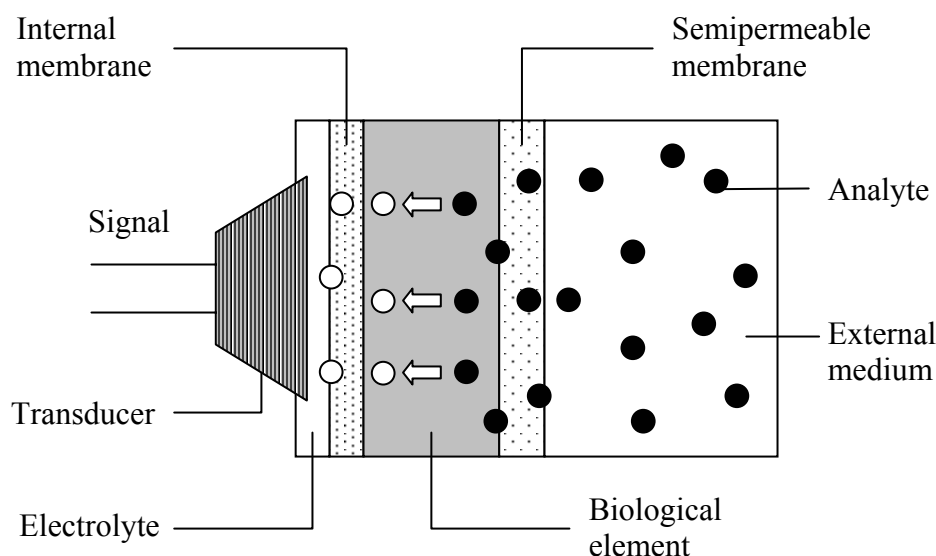
Figure 2.4 : A schematic drawing for a typical biosensor. The specific chemical target (analyte) is recognized by the biological element, creating a stimulus to the detecting transducer from which a reproducible signal is measured.

A target analyte (illustrated by solid circles) in the external medium must be able to enter the biosensor. The external membrane of the biosensor must be permeable to the analyte, and if possible, exclude other chemical species that the biosensor might also be sensitive to. The biological element inside the biosensor then interacts with the analyte, and responds in the same manner that can be detected by a transducer. The biological element may convert the analyte to another chemical species (represented by open circles) through a biochemical reaction; produce or release optical, electrical or mechanical properties: or make some other response that can be reliably quantified. There may be another internal membrane near the transducer which might have different permeability properties than the external membrane. The output signal from a biosensor depends on the type of transducer used. The transducer may be a conventional electrochemical sensor or may be based on another technology. (Buerk, 1993).

2.7.1 Chemical Sensor and Biosensor

A chemical sensor is a device that transforms chemical information, ranging from the concentration of a specific sample component to total composition analysis, into analytically useful signal. Chemical sensor usually contains two basic components connected in series that is a chemical recognition system (receptor) and a physicochemical transducer. Biosensors are chemicals sensors in which the recognition system utilizes a biochemical mechanism.

The main purpose of the recognition system is to provide the sensor with a high degree of selectivity for the analyte to be measured. The biological recognition system translates information from the biochemical domain, usually an analyte concentration, into a chemical or physical output signal with a defined sensitivity. While all biosensors are more or less selective (non-specific) for a particular analyte, some are, by design and construction, only class-specific, since they use class enzymes such as phenolic compound biosensors or whole cells used to measure biological oxygen demand. Because in sensing system presents in living organisms or systems, such as olfaction and taste, the actual recognition is performed by a cell receptor, the word 'bioreceptor' is often be used for recognition system of a biosensor.

The transducer is a part of the biosensor serves to transfer the signal from the output domain of the recognition system to, mostly the electrical domain. A transducer provides bidirectional signal transfer (non-electrical to electrical). The transducer part of sensor is also called a detector or electrode, but the term transducer is often used to avoid confusion. Table 2.2 shows the types of receptors used in electrochemical and biosensors measurement techniques. Indicated in *italic* types are the biological receptors, which part of the electrochemical biosensors.

Table 2.2 : Types of receptors used in biosensors and electrochemical measurement techniques.

Analytes	Receptor/chemical recognition system	Measurement technique/ Transduction mode
1. Ions	Mixed valence metal oxides, perselective, ion conductive inorganic crystal, trapped mobile synthetic or *biological ionophores*, ion exchanges glasses and *enzymes*.	*Potentiometric, Voltametric.*
2. Dissolved gases, vapors, odors.	Bilayer lipid or hydrophobic membrane, inert metal electrode, *enzymes, antibody, receptor.*	In series 1; *Amperometric, potentiometric, impedance,* piezoelectric, optical.
3. Substrates	*Enzymes, whole cells, membrane receptors, plant or animal tissue.*	*Amperometric or potentiometric;* In series with 1 or 2, *metal or carbon electrode, conductometric,* piezoelectric, optical, colorimetric.
4. Antibody/ antigen	*Antigen/antibody* *Oligonucleotide duplex* *Enzyme labeled,* Chemiluminenescent or fluorescent labeled.	*Amperometric, potentiometric or impedimetric,* piezoelectric, optical, surface plasmone resonance. In series with 3, Optical.
5. Various proteins and low molecular weight substrates, ions.	*Specific ligands, protein receptors and channels; Enzyme labeled,* Fluorescent labeled.	*Amperometric, potentiometric or impedimetric,* piezoelectric, optical.

Besides quantification of the above mentioned analytes, biosensors are also used for detection and quantification of micro-organisms, receptors are bacteria, yeast or oligonucleotide probes coupled to electrochemical, piezoelectric, optical or calorimetric transducers.

2.7.2   Electrochemical Biosensor

A biosensor with an electrochemical transducer is called an electrochemical biosensor. It is an integrated receptor-transducer device, which is a capable of providing selective quantitative or semi-quantitative analytical information using a biological recognition element. A biosensor can be used to monitor either biological or non-biological matrices. Non-electrochemical transducers are used within biosensors, these includes piezoelectric, calorimetric (thermistor) and optical (planar wave guide, fiber optic).The different types of electrochemical transducer for classified types of measurement, with corresponding analytes to be measured are listed in Table 2.3.

Table 2.3 : The different types of electrochemical transducer.

Measurement	Transducer	Transducer Analyte
1. Potentiometric	Ion Selective Electrode (ISE), Glass Electrode, Gas Electrode, Metal Electrode	K^+, Cl^-, Ca^{2+}, F^- H^+, Na^+ CO_2, NH_3 Redox Species
2. Amperometric	Metal or Carbon Electrode, Chemically modified electrodes (CME)	O_2, sugars, alcohols Sugars, alcohols, phenols, etc.
3. Conductometric	Interdigitated electrode,	Urea,

	Metal electrode.	oligonucleotides, charged species.
4. Ion charge or field effect	Ion-sensitive field-effect transistor, Enzyme FET	H^+, K^+

2.7.3   Electrochemical Detection

2.7.3.1 Amperometry

Amperometry is based on the measurement of the current resulting from the electrochemical oxidation or reduction of an electroactive species. The resulting current is directly correlated to the bulk concentration of the electroactive species or its production or consumption rate within the biocatalytic layer.

2.7.3.2 Potentiometry

Potentiometric measurements involves the determination of the potential difference between either indicator and a reference electrode or two reference electrodes separated by a permiselective membrane, when there is no significant current flowing between them. The most common potentiometric devices are pH electrodes, several other ion (F^-, I^-, CN^-, Na^+, K^+) or gas (CO_2, NH_3) selective electrodes are also available.

2.8   The First Biosensor

These were first described by Clark and Lyons (1962) for the determination of glucose and often called *enzyme electrodes*. This is by far the most studied and developed biosensor application. Glucose is of special importance because of its involvement in human metabolic processes. In particular sufferers from diabetes mellitus do not produce sufficient insulin in their pancreas to control adequately the level of glucose in their blood. Doses of insulin have to be administered and it is vital that the diabetic regularly monitors the level of glucose in the blood. Previously, substantial blood sample had to be taken and analyzed in a medical laboratory with consequent time delays and consequent uncertainty about the diabetic's condition. With the availability of glucose biosensors, the patient himself or herself can extract one small drop of blood and obtain a direct digital readout of the glucose concentration inside 1 min. Glucose biosensors are based on the fact that the enzyme glucose oxidase catalyses the oxidation of glucose to gluconic acid. In the early biosensors oxygen was used as the oxidizing agent. The consumption of oxygen was followed by electrochemical reduction at a platinum electrode, as in the Clark oxygen electrode (invented in 1953), shown in Figure 2.5 (Eggins, 1996).
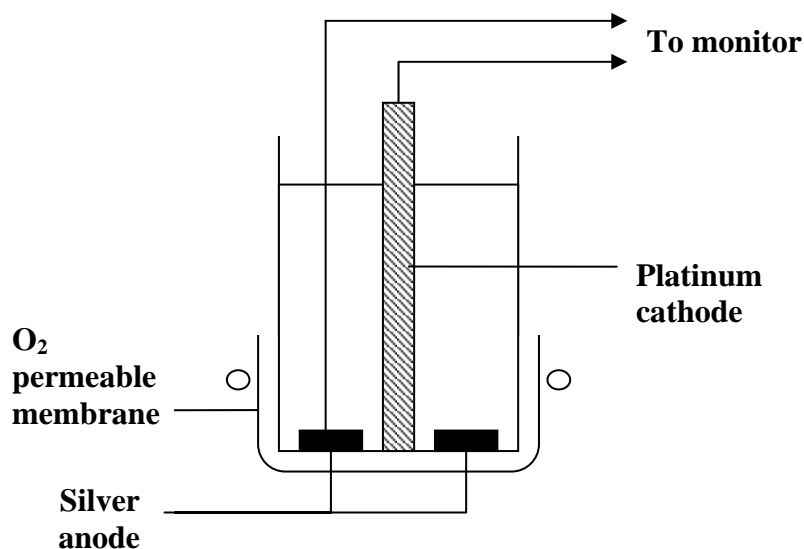
Figure 2.5: A Clark oxygen electrode.

The glucose oxidation reaction, catalyzed by glucose oxidase (GOD) is

$$\text{glucose} + O_2 + H_2O \xrightarrow{\quad GOD \quad} \text{gluconic acid} + H_2O_2$$

At the electrode:

$$O_2 + 2e^- + 2H^+ \longrightarrow H_2O_2$$
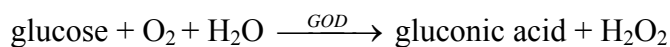
A voltage of -0.7 V is applied between the platinum cathode and the silver anode, sufficient to reduce the oxygen, and the cell current, which is proportional to the oxygen concentration, is measured. The concentration of glucose is then proportional to the decrease in current (oxygen concentration). The oxygen electrode has an oxygen permeable membrane (such as PTFE, polythene or cellophane) covering the electrodes. A layer of enzyme (glucose oxidase) is placed over this and held in place with a second membrane such as cellulose acetate, as shown in Figure 2.6 (Eggins, 1996).
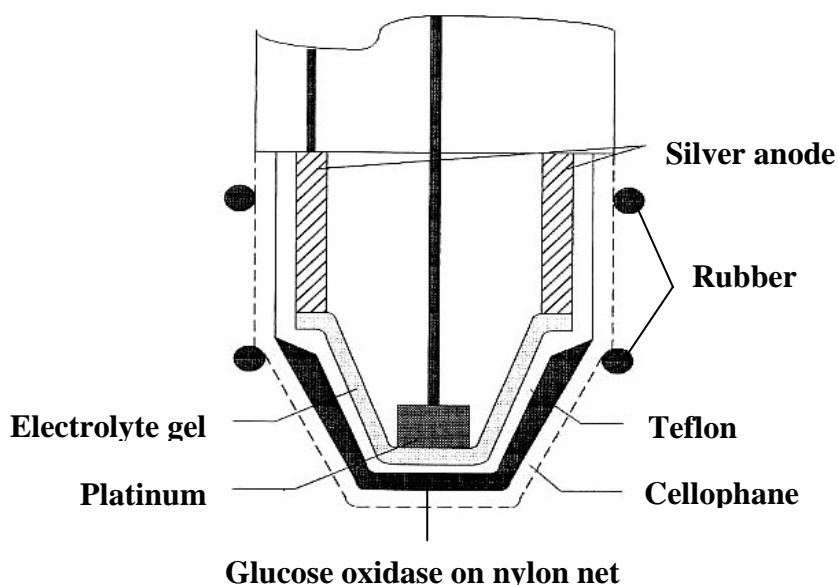
Figure 2.6 : A glucose biosensor based on Clark oxygen electrode.

2.9 Fundamental of Amperometric Biosensor

Amperometric measurements can be measured when a defined potential is applied at a working electrode with respect to the reference electrode. This result current that can be related to the concentration of an electroactive substance in the solution. At low current densities, it is sufficient for most of elementary electrochemical setup to have two electrodes, which are working electrode (WE) and reference electrode (RE). However, at higher current densities, the potential of reference electrode may change with current, so it is not possible to obtain reproducible determination of analyte. To avoid this problem, a third electrode is required (three-electrode setup) which is known as counter electrode (CE). For the three-electrode setup, current is measured between working and counter electrodes, while potential is measured based on reference electrode. The measured current is directly related to the rate of the overall process in the electrochemical cell. (Wagner, and Guilbault, 1994).

2.10 Histamine Biosensor

In histamine biosensor, the basic principles are the same as the principles of the glucose biosensor. The basic underlying chemistry is the action of diamine oxidase (DAO) that catalyzed the oxidative deamination of histamine to imidazoleacetaldehyde, hydrogen peroxide (H_2O_2) and ammonia (NH_3). The reaction involved is shown in Figure 2.7 (Niculescu *et al.*, 2001).
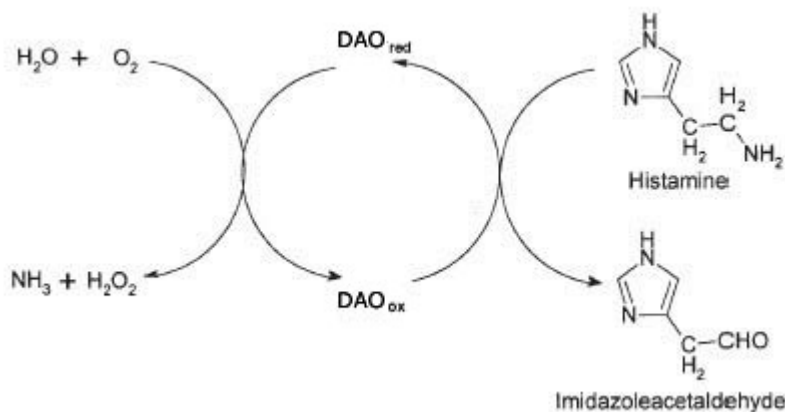
Figure 2.7 : Oxidative deamination of histamine to imidazoleacetaldehyde, hydrogen peroxide and ammonia by diamine oxidase.

The reaction will become rate limited if either histamine or oxygen concentration is too low. Histamine concentration can be measured by monitoring either the decrease in oxygen concentration or the increase of hydrogen peroxide, as the reaction proceeds (Lange and Wittmann, 2001).

For oxygen-based histamine sensor, since oxygen is consumed during the reaction, oxygen concentration in the diamine oxidase membrane will be a linear function of histamine concentration. The oxygen concentration can be measured by coupling the immobilized diamine oxidase membrane to an electrochemical oxygen sensor. Since oxygen is also available in the sample, a similar reference oxygen sensor without the enzyme needs to be incorporated in the system. The signal current is then subtracted from the reference electrode and gives the result of histamine-dependent difference current. The advantage of this type of sensor is that it has low electrochemical interference due to the use of a nonporous hydrophobic membrane. This membrane only allows gaseous molecules to reach the electrode and also can provide information on oxygen variations in the system. The immobilization of diamine oxidase will help prolong the working lifetime of diamine oxidase as catalase promotes the degradation of hydrogen peroxide to oxygen and water.

The hydrogen peroxide-based histamine sensor has found wide application in the development of such a sensor, especially an implantable version, due to its simple sensor configuration that facilitates ease of miniaturization. Unlike oxygen, hydrogen peroxide is not present in the sample to be analyzed. This make no differential setup needed. However, it suffers from an intrinsic problem, the interference from small endogenous analytes, which may be electro-active at the detection potential of hydrogen peroxide which is quite high (Azila Abdul Aziz, 2001).

Those two types of sensors mentioned above are known as the first generation amperometric biosensors. The second generation of histamine biosensors makes use of mediators to shuttle electrons from the enzyme to the electrode, instead of oxygen, which are reversible, had appropriate oxidation potentials and whose concentration could be controlled. If the system is oxygen, the biosensor will become insensitive to histamine, thus will only respond to changes in oxygen concentration. As oxygen remains in the system, the mediator must be able to compete effectively for the electrons (Azila Abdul Aziz, 2001).

The use of mediator in determining the content of histamine and other biogenic amines has been studied by Tombelli and Mascini (1998), compared to a single enzyme sensor and a flow system based on hydrogen peroxide generated by enzymatic reaction. A bi-enzyme FIA system with amperometric detection was used based on the following enzyme reactions, with ferrocene carboxylic acid (Fc-COOH) as the mediator facilitating the electron transfer between the electrode and horseradish peroxidase (HRP). Pea seedling amine oxidase (PSAO) catalyses the oxidation of the amine and subsequently the co-substrate, molecular oxygen, is reduced to hydrogen peroxide. Hydrogen peroxide is then expends in the following peroxidase catalyzed reaction using Fc-COOH as the mediator. The amperometric signal is monitored reductively at the electrode. Figure 2.7 illustrated the basic principle of the electron transfer during measurements (Wimmerova and Macholan, 1999).

$$RCH_2NH_2 + O_2 + H_2O \xrightarrow{\;PSAO\;} RCHO + NH_3 + H_2O_2$$

$$H_2O_2 + Fe\,(II) \xrightarrow{\;HRP\;} H_2O + Fe\,(III)$$
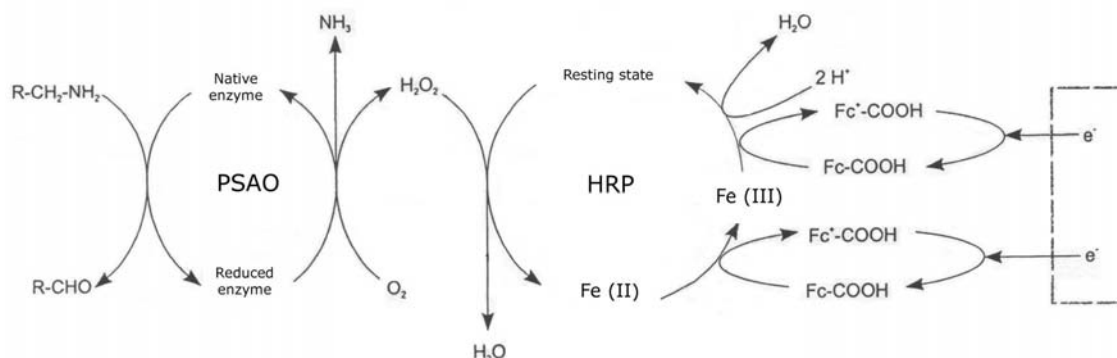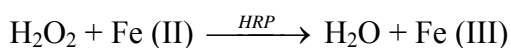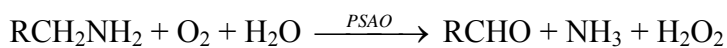
Figure 2.8 : The basic principle of the electron transfer during measurements in amperometric bi-enzyme system.

According to Tombelli (1998) by using second enzymatic reaction and a mediator, two distinct advantages are obtained. First, it allows low potential detection (0.00 mV) of substrates, hence avoiding interferences from electroactive species which enhances the specificity of the assay. Second, the assay sensitivity is enhanced by increasing the efficiency of the electrochemical detection.

The third generation amperometric biosensors are based on the use of conducting organic salts or polymers. These conducting salts can be built into electrodes in three ways; as single crystals, as pressed pellets or as a paste with graphite powder (Eggins, 1996). The films are grown electrochemically and enzyme is entrapped in the membranes. The advantage of this system is that manipulation of the electropolymerization can give a film that extends the linear range for substrate detection and reduces oxygen dependence.

2.11   Enzyme Immobilization

Enzymes are biocatalytically active entities upon which the metabolisms of all living organisms are based. Enzymes are usually proteins of high molecular weight (15,000<MW<several million Daltons) that act as catalyst. Enzymes are specific, versatile and very effective biological catalyst resulting in much higher reaction rates compared to chemically catalyzed reaction under ambient condition. They speedup biochemical reactions by lowering the energy of activation, without themselves appearing in the reaction products. The catalytic actions of enzymes involve their ability to alter the distribution of charges on the compound to be converted, thus bringing about a lowering of the energy of activation. Furthermore, they are highly specific, thus side reactions can be avoided by employing enzymatic breakdown.

Enzymes are proteins that are constructed from chains of amino acids. Different types of amino acid links have a different shapes and properties. As an unfolded chain, the enzyme has no catalytic activity. Only the folded structure forms the catalytic or active site, which is brought about by their tertiary and quaternary (oligometric enzyme) structures. However, this folded structure will generally be held together by non-covalent interactions, unlike the covalent bonds that hold the amino acid links together. These consist of interactions such as ionic bridges, hydrogen bonds, hydrophobic and hydrophilic interaction (Trevor Palmer, 1985). The molecular structure of enzymes that is essential for their catalytic activity is liable to be destroyed under conditions such as high temperature, high or low pH, with presence of organic solvents, or even conditions suitable for catalysis. The recovery of active enzymes from spent reaction mixtures is another problem when free or non-immobilized enzymes are utilized.

A biocatalyst is termed 'immobilized' if its mobility has been restricted by chemical or physical means. This limitation of mobility may be achieved by widely different methods. For example, by trapping in the network of a polymer matrix or

by membrane confinement. An essential criterion for defining a system as immobilized is that human interference has to be involved. A variety of immobilization methods have been used in the development of a successful biosensors. Immobilized enzyme preparations may be more effective since they are recoverable and possibly more stable than free enzymes. Moreover, being in their native environment, operational denaturation of the enzymes can be minimized.

Enzymes are largely used as biocatalysts in chemical, pharmaceutical and food industries, and as specific ligands in clinical and chemical analysis. Since the recovery and the reusability of the free enzyme are limited, immobilization of enzyme has attracted the attention. Immobilized enzymes have the advantages of using in batch and continuous systems, removing easily from the reaction medium and providing the facility of controlled production. However, immobilized enzyme systems do also have limitations, such as loss activity due to the immobilization technique and decrease in the apparent activity due to mass transfer resistance for the substrate and product. An optimal support material should provide large surface area per unit volume (or mass) of the carrier for effective immobilization of the desired amount of enzyme and should allow substrate and product transport with the least diffusional resistance and also should be easily available and non toxic. The immobilization technique should also lead high immobilization and activity yields (Bulmus *et al.*, 1998).

In order to construct a viable biosensor, the enzyme used has to be properly attached to the transducer. There are five regular methods in enzyme immobilization, as follows (Eggins, 1996).

i. *Adsorption*
    This is the simplest and involves minimal preparation. However, the bonding is weak and this method is only suitable for exploratory work over a short time-span.

ii.   *Microencapsulation.*

This was the method used in the early biosensors. The biomaterial is held in place behind a membrane, giving close contact between the biomaterial and the transducer. It is adaptable. It does not interfere with the reliability of the enzyme. It limits contamination and biodegradation. It is stable towards changes in temperature, pH, ionic strength and chemical composition. It can be permeable to some materials, e.g. small molecules, gas molecules and electrons.

iii.   *Entrapment*

The biomaterial is mixed with monomer solution, which is then polymerized to a gel, trapping the biomaterial. Unfortunately, this can cause barriers to the diffusion of substrate, thus slowing the reaction. It can also result in loss of bioactivity through pores in the gel. This can be counteracted by crosslinking. The most commonly used gel is polyacrylamide, although starch gels, nylon and silastic gels have been used. Conducting polymers such as polypyrroles are particularly useful with electrodes.

iv.   *Cross-linking*

In this method, the biomaterial is chemically bonded to solid supports or to another supporting material such as a gel. Bifunctional reagents such as glutaraldehyde are used. Again, there is some diffusion limitation and there can be damage to the biomaterial. Also, the mechanical strength is poor. It is a useful method to stabilize adsorbed biomaterials.

v.   *Covalent bonding*

This involves a carefully designed bond between a functional group in the biomaterial to the support matrix. Some functional groups which are not essential for the catalytic activity of an enzyme can be covalently bonded to the support matrix (transducer or membrane). This method uses nucleophilic groups for coupling such as COOH, OH, C_6H_4OH , SH and amidazole.

Overall, the lifetime of the biosensor is greatly enhanced by proper immobilization. Typical lifetimes for the same biosensor, in which different methods of immobilization are used, are as follows:

Adsorption	1 day
Membrane entrapment	1 week
Physical entrapment	3 - 4 weeks
Covalent entrapment	4 - 14 months

2.11.1 Poly (vinyl alcohol) (PVA)

Poly (vinyl alcohol) is manufactured by the hydrolysis of polyvinyl acetate. Even after a prolonged hydrolysis, PVA generally retains around 1 to 2 mole percent of acetate groups. The amount of residual acetate groups affects the physical and chemical properties of PVA, as they are hydrophobic relative to the hydroxyl groups. The residual acetate groups can interfere with inter-molecular and intra-molecular forces such as hydrogen bonding. Highly hydrolyzed PVA has strong hydrogen bonds within and between molecules.

PVA is used as a basic material for a variety of biomedical applications because of their inherent non-toxicity, non-carcinogenicity, good biocompatibility and desirable physical properties, such as elastic nature, good film forming property, high degree of swelling in aqueous solutions, and their water content matches that of biological tissue (M. Masuda, 1992). PVA gives a hydrophilic tough membrane which can stabilize the activity of various enzymes, including diamine oxidase (DAO) and horseradish peroxidase (HRP), through the inhibition of the formation of non-functional conformations due to the extensive hydrogen bonding between the H atoms of alcohol groups in PVA and the O atoms of the carbohydrate groups in DAO. Moreover, PVA is water-soluble polymer that readily reacts with different

cross-linking to form gel. However, owing to strong internal hydrogen bond, it only goes into solution at higher temperature, around 90°C. Aqueous solutions of PVA exhibit non-Newtonian behavior at room temperature.

PVA can be cross-linked chemically or physically to form a hydrogel. The polymer can be cross-linked chemically by any bifunctional agent that can react with organic hydroxyl groups. Some of the various chemicals that can cross-link PVA are glutaraldehyde, formaldehyde, maleic acid and boric acid. Cross-linking can also be achieved physically using ultraviolet (UV) light in the presence of photo-sensitizers, by electron beam or by y-radiation. Another physical method of cross-linking PVA is through freeze-thaw cycles, where physical bonds are formed.

Cross-linking causes PVA to be insoluble in water. Furthermore, by controlling the cross-link density of this material, variety of transport properties can be obtained. In addition, poly (vinyl alcohol) is also considered biocompatible. Protein adsorption onto cross-linked PVA has been shown to be negligible, thus the potential of minimizing fibrotic capsule development around implantable PVA exists. Hence, due to its promised biocompatibility, ease of manipulation and hydrophilicity, PVA has been used extensively in biomedical applications. In the biomedical area, PVA has found use in hernia treatment, in artificial heart valve replacement and as a drug carrier in controlled drug release system, among others.

In dense hydrogels, diffusion of solutes is determined by the cross-linking density of the network, or mesh size and the degrees of swelling. The `pores' are the open spaces between the cross-link points and are not fixed. Different theories have been proposed to describe solute diffusion in hydrogels. Polymer chains are hypothesized to hinder solute diffusion through one or a combination of these methods: by physically obstructing the passage of solute, by increasing the hydrodynamic drag on the solute molecule or by reducing the available free volume for the solute (Azila Abdul Aziz, 2001).

2.11.2 Freeze-thawed PVA

Physically cross-linked PVA through freeze-thawing method may be a good choice for immobilizing the enzymes. It can minimize the problems associate with chemically cross-linked PVA, which maintaining the good properties of PVA. The exposition of aqueous PVA solution to several freeze-thawing cycles leads to reinforced gels owing to a densification of the macromolecular structure, which is a function of the cycling time and temperature (Christie M. Hassa and Nikolas A. Peppas, 2000). After the freeze-thawing process, fine crystallites are formed due to the slow heat treatment. The chains are physically cross-linked by semi-permanent entanglements, molecular associations or crystalline (Lucio Doretti *et al.*, 1997). Formations of crystallites serve as physical cross-links to render the material insoluble in water.

Some characteristics of the physical cross-linked PVA gels include high degree of swelling in water, a rubbery and elastic nature, and a high mechanical strength because the mechanical load can be distributed along the crystallites of the three-dimensional structure.

The freeze-thawing method as characterized by the absence of chemical cross-linking agents that could compromise is biocompatibility or physical agent, such as gamma radiation that could deactivate the biological substrates, due to the damage caused mostly by the indirect effect of water radiolysis. Generally, physical entrapment of enzyme molecules in polymeric membranes is one of the most advantages method because it is rapid, simple and the retained activity is high (Lucio Doretti *et al.*, 1998).

2.11.3 Immobilization Methods in Constructing Histamine Biosensor

Several immobilization methods have been used and investigated in order to improve the sensitivity of biosensors. Lange *et al.* (2001) were firstly used 20% of transglutaminase solution for immobilization of diamine oxidase, plasma amine oxidase and tyramine oxidase. The immobilization technique improved the sensitivity of sensor, but unfortunately, when lots of transglutaminase was finished, difficulties occurred with the regular quality (sensor reproducibility was low). Therefore, they investigated the conventional immobilization method, which was based on glutaraldehyde-albumin cross-linking. In their research, they compared the results obtained (to determine biogenic amines: specifically histamine and tyramine) between enzyme sensor array and high performance liquid chromatography (HPLC). From the results, it can be concluded that by using enzyme sensor array, less time was required to conduct experiments and was not tedious as when handling HPLC, but still the reproducibility, data validity, detection limit and so on were still poor.

Glutaraldehyde was always chosen as the cross-linking agent to entrapped diamine oxidase. Tombelli and Mascini (1998) used glutaraldehyde solution on cellulose acetate membrane to immobilize diamine oxidase on a platinum electrode. It was also helped Bouvrette *et al.* to develop their membranes. Poly (vinyl alcohol) is a non-toxic water-soluble synthetic material that has good film forming properties, resulting in tough membranes. Glutaraldehyde, a bifunctional agent that can react with organic hydroxyl groups, was used as the cross-linking agent. Glutaraldehyde can also react with the lysine amino acid residues in the enzyme.

The cross-linking process overcomes the loss of enzyme activity due to diffusional loss, which is a prevalent problem for enzyme immobilized in physical entrapment. PVA can stabilize the activity of various enzymes such as horseradish peroxidase. The stabilization effect is achieved through the inhibition of the formation of non-functional conformations due to the extensive hydrogen bonding between the H-atoms of the alcohol groups in PVA and the O-atoms of the

carbohydrate groups in diamine oxidase. These properties make PVA an appropriate matrix for diamine oxidase immobilization.

CHAPTER 3

METHODOLOGY

3.1 Purification of Pea Seedling Amine Oxidase

The purification of pea seedling amine oxidase will be done according to the method given by Sebela *et al.*, (1998) with some modifications. The type of pea that will be used is *Cicer arietinum* (chick pea).

3.1.1 Enzyme Purification

The chick pea seeds were soaked in distilled water for 12 hr, transferred onto wet cotton/tissue paper and germinated in the dark for 7 days at room temperature. The whole seedlings were homogenized in Waring blender with 3 volumes (w/v) of chilled 0.1 M potassium phosphate buffer (PPB) pH 7.0 for about 10 min. The crude homogenate was filtered through a nylon or cotton mesh cloth, and centrifuged at 10,000 x g for 30 min using refrigerated centrifuge. The precipitate was discarded and the supernatant was treated with 30% ammonium sulfate, $(NH_4)_2SO_4$, stirred for

30 minutes at 4°C and centrifuged at 10,000 x g for 30 min using refrigerated centrifuge. The precipitate was discarded and the supernatant obtained was further treated with 70% ammonium sulfate, stirred for 30 minutes at 4°C and centrifuged at 10,000 x g for 30 min using refrigerated centrifuge. The precipitate was then collected, resuspended in 2 volumes (v/v) of chilled 0.1 M PPB (pH 7.0) and dialysed overnight against the same buffer at 4°C. The dialysate was rapidly heated to 55-58°C and kept at 60°C for 5 min with constant stirring. The solution was cooled to 4°C on an ice-water bath, and centrifuged at 10,000 x g for 30 min using refrigerated centrifuge. The insoluble material was discarded. The supernatant was dialysed overnight against 20 mM PPB (pH 7.0) at 4°C.

The dialysate was then loaded onto a DEAE-Cellulose (Sigma) column (2.5 x 20 cm) equilibrated with 20 mM PPB (pH 7.0) until the eluate showed no further absorbance at 280 nm (A_{280}). During loading and washing with buffer, the eluate/fractions that shows the amine oxidase activity were pooled. The enzyme solution was then applied directly to a Hydroxyapatite (Bio-Gel HTP, BioRad) column (2.5 x 20 cm) equilibrated with 20 mM PPB (pH 7.0). The column was washed with the same buffer until the eluate showed no further A_{280}. The amine oxidases were eluted with 0.2 M PPB (pH 7.0). Fractions with the highest amine oxidase activity were pooled and dialysed overnight against 20 mM PPB (pH 7.0) at 4°C. The dialysate was then concentrated using ultrafiltration. Finally, the enzyme was submitted to size-exclusion chromatography on a Sephacryl S-300 HR (Sigma) column (1.5 x 100 cm) equilibrated and eluted with 20 mM PPB (pH 7.0). Fractions with the highest enzymatic activity were pooled and concentrated by ultrafiltration. Absorption spectra of the purified enzymes were recorded on a spectrophotometer.

3.1.2   Ammonium Sulfate Precipitation

When high concentrations of salt are present, proteins tend to aggregate and precipitate out of solution. This technique is referred to as "salting out". Since different proteins precipitate at different salt concentrations, salting out is often used during protein purification. Ammonium sulfate is the salt of choice because it combines many useful features such as salting out effectiveness, pH versatility, high solubility, low heat of solution and low price. Ammonium sulfate concentrations are generally expressed in percent saturation. A simple equation for calculation of m grams of ammonium sulfate needed to make an x% solution starting from x_o% for 1 L solution at 0^oC is $m = 515(x - x_o) / (100 - 0.27x)$. Since most proteins will precipitate at 55% saturation, a good value for obtaining maximum protein precipitation is 85%. For 100 ml solution containing no ammonium sulfate at the start, the following protocol is recommended (Bollag, 1996).

3.1.2.1 Methods

A beaker containing protein solution was placed in a cooling bath on top of a magnetic stir plate. This can be accomplished by placing the beaker within another beaker containing ice-water slurry. While agitating gently on a magnetic stirrer, 56.8 g ammonium sulfate was slowly added. Salt was added very slowly as final saturation is approached. This step should be completed within 5-10 min. After all salt has been added, solution was continually stirred for 10-30 min. Solution was then centrifuged at 10,000 x g for 10 min or at 3,000 x g for 30 min. Supernatant was discarded and the precipitate was resuspended in 1-2 pellet volumes of buffer. Any insoluble material remained was probably denatured protein and should be removed by centrifugation. Ammonium sulfate can be removed by dialysis, ultrafiltration or a desalting column.

3.1.3   Dialysis

Dialysis is typically used for changing the buffering solution of a protein, but it can also be used as a method for concentrating protein solutions if carried out in a vacuum or hygroscopic environment (e.g. poly(ethylene glycol) (PEG), Sephadex). The protein solution is contained within a membrane whose pore size prevents the protein from escaping and which permits solute exchange with either air at reduced pressure or a surrounding solution (Bollag, 1996).

3.1.3.1 Methods

Dialysis tubing was boiled in 10 mM sodium bicarbonate ($NaHCO_3$)/1 mM EDTA for at least 30 min to remove chemical contaminants from the manufacturing process. Following the boiling step, the tubing was then washed extensively in distilled water (for storage, tubing was stored in 1 mM EDTA at $4^{o}C$ to prevent microbial contamination). Two tight knots were made at one end of the tubing. Protein solution was delivered into the dialysis tubing using a pipette or funnel. The other end of the tubing was tied with a double knot and the closed dialysis sack was placed in more than 10 volumes of dialysis buffer. The buffer should be gently stirred with magnetic stirrer bar to improve solute exchange. Equilibrium occurs after several hours of dialysis, and the dialysis buffer was changed several times (If dialyzing against a buffer with a lower salt or organic solvent concentration than the dialysate, be aware that osmotic forces will cause water to influx into the dialysis sack. Allow space for volume increases to avoid the risk of the membrane bursting).

3.1.4 Enzyme Activity Determination

The enzyme activity is most frequently expressed in terms of units (U) such that one unit is the amount of enzyme that catalyses the conversion of 1 micromole of substrate per minute under defined conditions. In some cases, the unit is too large and the activity can be more conveniently expressed in terms of nmol/min or pmol/min. The SI unit of enzyme activity is the *katal (kat)* which represents the transformation of 1 mole of substrate per second. This unit is big and more manageable figures are obtained by expressing activities in microkatals (μkat), nanokatals (nkat) or pikokatals (pkat).1 U = μkat/60 = 16.67 nkat. The purity of an enzyme is expressed in terms of the specific activity, which is the number of enzyme units (U) per milligram of protein.

Free and immobilized diamine oxidase activity was measured using a coupled reaction with horseradish peroxidase (HRP) and chromogen solution, with putrescine, cadaverine and histamine as the substrate, respectively. The activity was determined from standard calibration curve using commercial diamine oxidase (Sigma).

3.1.4.1 Chemicals

An o-dianisidine tablet (10 mg) (Sigma) was dissolved in 1 ml of distilled water to produce 1% (w/v) of o-dianisidine solution. Chromogen solution was prepared by diluting 0.1 ml of 1% o-dianisidine solution in 12 ml of potassium phosphate buffer (pH 7.4); 2 mg of horseradish peroxidase (HRP) (Sigma) was weight and dissolved in 1 ml of distilled water. The solution was further diluted to produce 200 μg/ml of HRP solution; 46.025 mg of histamine (Sigma) was dissolved in 5 ml of distilled water to produce 0.05 M of histamine solution; 4 M hydrochloric acid (HCl).

3.1.4.2 Methods

1.25 ml of chromogen solution, 150 µl of 0.05 M histamine and 50 µl of 200 µg/ml HRP was mixed into a clean test tube. The test tube was duplicated. The solution was preincubated in a waterbath at 37^oC for several minutes for temperature equilibrium. 50 µl of a sample (with the unknown amount of diamine oxidase) was pipetted into the test tube and the solution was stirred using vortexer. Reaction was allowed to proceed at 37^oC for exactly 10 min. The reaction was stopped by adding 150 µl of 4 M HCl and the final solution was stirred using vortexer. The amount of color formed was measured using spectrophotometer by reading the absorbance at 450 nm (A_{450}).

The diamine oxidase activity in the sample was determined from a calibration curve. One unit (1 U) of activity causes the oxidation of 1 micromole of o-dianisidine per minute under defined conditions (at 37^oC and pH 7.4). To construct a calibration curve, 50 µl of sample (with the unknown amount of diamine oxidase) of the previous step was replaced with 50 µl of known amount/unit of standard diamine oxidase. The total unit of standard diamine oxidase was varied between $50 - 500$ mU. For immobilized diamine oxidase, 50 µl of sample was replaced with enzyme membrane. After exactly 10 min of reaction, the membrane was removed from the solution and 150 µl of 4 M HCl was added and stirred using vortexer to stop the reaction.

3.1.5 Protein Concentration Determination – Bicinchoninic Acid (BCA) Assay

Bicinchoninic acid (BCA) assay has become the preferred method for quantifying protein in many laboratories. It is a recently developed variation of Lowry assay. The reaction is simpler to perform and has fewer interfering substances than Lowry assay. Besides, other advantages are it involve single reagent and the end product is stable. Range of sensitivity is $10 - 1200$ µg/ml for standard assay and $0.5 - 10$ µg/ml for microassay (Bollag, 1996).

3.1.5.1 Chemicals

Reagent A (1 L); 10 g BCA (1%) – bicinchoninic acid, 20 g $Na_2CO_3 \cdot H_2O$ (2%) – sodium carbonate, 1.6 g $Na_2C_4H_4O_6 \cdot 2H_2O$ (0.16%) – sodium tartrate, 4 g NaOH (0.4%) – sodium hydroxide and 9.5 g $NaHCO_3$ (0.95%) – sodium bicarbonate was added with distilled water to 1 L. NaOH or solid $NaHCO_3$ was added to adjust pH to 11.25 if needed. Solution was stable for at least 12 months at room temperature, and commercially available (Pierce).

Reagent B (50 ml); 2 g $CuSO_4 \cdot 5H_2O$ (4%) – copper sulfate was added with distilled water to 50 ml. Solution was stable for at least 12 months at room temperature, and commercially available (Pierce).

Standard Working Reagent (SWR); 50 volumes Reagent A was mixed with 1 volume Reagent B. Solution was stable for 1 week.

3.1.5.2 Methods

Bovine serum albumin (BSA) (Pierce) at a concentration of 1 mg/ml was used as protein standard. For the calibration curve, the concentration of BSA was varied from 0.1 – 1 mg/ml. 1 volume of sample was mixed with 20 volumes of SWR (e.g. 100 μl sample, 2 ml SWR). Solution was incubated either at room temperature for 2 hours or at 37°C for 30 min. Solution was cooled to room temperature if it was incubated at 37°C. Absorbance at 562 nm (A_{562}) was read (Bollag, 1996).

3.1.6 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is a low-cost, reproducible and rapid method for quantifying, comparing and characterizing proteins under denaturing conditions. This method separates proteins based primarily on their molecular weight (Laemmli, 1970). SDS binds to hydrophobic portions of a protein, disrupting its folded structure and allowing it to exist stably in solution in an extended conformation. As a result, the length of the SDS-protein complex is proportional to its molecular weight. The ease of execution and wide application of SDS-PAGE have made it an important analytical technique in many fields of research. Sensitivity of staining; Coomassie Blue (0.1 – 1 μg per band) (Smith, 1984) and Silver Staining (2 – 10 ng per band) (Giulian *et al.*, 1983). Optimal resolution ranges (acrylamide percentage – separating resolution); 15% gel – 15 to 45 kDa, 12.5% gel – 15 to 60 kDa, 10% gel – 18 to 75 kDa, 7.5% gel – 30 to 120 kDa, and 5% gel – 60 to 212 kDa (Hames, 1981). The experimental procedures and reagents in this method have been calculated for use with mini-gel system (Bollag, 1996).

3.1.6.1 Equipment

Mini-Gel apparatus, Bio-Rad Mini-Protean II apparatus was used. Minigel systems were highly recommended due to the savings in material and time, and also because they provide high resolution protein separation.

3.1.6.2 Chemicals

Acrylamide (electrophoresis grade), bis-acrylamide (*N,N'*-methylenebisacrylamide), Tris (2-hydroxymethyl-2-methyl-1,3-propanediol), SDS (sodium dodecyl sulfate), TEMED (*N,N,N',N'*-tetramethylene-ethylenediamine), ammonium persulfate, 2-mercaptoethanol, glycerol, bromophenol blue, glycine, hydrochloric acid (HCl), dithiothreitol (DDT), standard protein molecular weight marker (Promega), Coomassie blue R-250, methanol, glacial acetic acid, silver nitrate ($AgNO_3$), sodium hydroxide, 14.8 M (30%) ammonium hydroxide (NH_4OH), citric acid, 38% formaldehyde, Kodak rapid fix and Kodak hypo clearing agent were used as received.

3.1.6.3 Pouring a Gel

Stock Solutions; (1) 2 M tris-HCl (pH 8.8) (100 ml); 24.2 g of Tris base was weighed and distilled water was added to 50 ml. Concentrated HCl was slowly added to pH 8.8 (about 4 ml). pH will increase as solution was allowed to cool to room temperature. Distilled water was added to a total volume of 100 ml. (2) 1 M Tris-HCl (pH 6.8) (100 ml); 12.1 g of Tris base was weighed and distilled water was added to 50 ml. Concentrated HCl was slowly added to pH 6.8 (about 8 ml). pH will increase as solution was allowed to cool to room temperature. Distilled water was added to a total volume of 100 ml. (3) 10% SDS (w/v) (100 ml); 10 g of SDS was weighed and distilled water was added to a total volume of 100 ml. Solution can be stored at room

temperature. (4) 50% glycerol; 50 ml of 100% glycerol was poured and added with 50 ml of distilled water. (5) 1% bromophenol blue (w/v) (10 ml); 100 mg of bromophenol blue was weighed and distilled water was added to 10 ml. Solution was stirred until bromophenol blue became completely dissolved.

Working Solutions; (1) Solution A (Acrylamide Stock Solution, 30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide, 100 ml); 29.2 g of acrylamide and 0.8 g of bis-acrylamide were mixed. Distilled water was added to a total volume of 100 ml and stirred. Solution can be stored for months in the refrigerator. (Caution: Unpolymerized acrylamide is a skin irritant and a neurotoxin. Always handle with gloves and work under hood and acrylamide solution was kept covered with Parafilm until completely dissolved). (2) Solution B (4x Separating Gel Buffer, 100 ml); 75 ml 2 M Tris-HCl (pH 8.8), 4 ml 10% SDS and 21 ml distilled water were mixed and stirred. Solution can be stored for months in the refrigerator. (3) Solution C (4x Stacking Gel Buffer, 100 ml); 50 ml 1 M Tris-HCl (pH6.8), 4 ml 10% SDS and 46 ml distilled water were mixed and stirred. Solution can be stored for months in the refrigerator. (4) 10% Ammonium persulfate (5 ml); 0.5 g ammonium persulfate was added with 5 ml of distilled water. Solution can be stored for months in a capped tube in the refrigerator. (5) Electrophoresis Buffer (1 L); 3 g Tris, 14.4 g glycine and 1 g SDS (0.1%) was added with distilled water to 1 L. pH should be approximately 8.3. Solution can be stored for months in the refrigerator. The solution is stable indefinitely at room temperature. (6) 5x Sample Buffer (10 ml); 0.6 ml 1 M Tris-HCl (pH 6.8), 5 ml 50% glycerol, 2 ml 10% SDS, 0.5 ml 2-mercaptoethanol, 1 ml 1% bromophenol blue and 0.9 ml distilled water were mixed and stirred. The solution is stable for weeks in the refrigerator or for months at -20°C.

Amount of Working Solutions to Use; Volumes necessary for pouring gels of different thickness (for two 6 x 8 cm gels).

Gel thickness	Separating	Stacking
0.5 mm	5.6 ml	1.4 ml
0.75 mm	8.4 ml	2.1 ml
1.0 mm	11.2 ml	2.8 ml
1.5 mm	16.8 ml	4.2 ml

Calculation for x% Separating Gel.

Solution A	x/3 ml
Solution B	2.5 ml
Distilled water	(7.5 – x/3) ml
Ammonium persulfate	50 μl
TEMED	5 μl
Total Volume	10 ml

Pouring the Separating Gel: Gel sandwich was assembled according to the manufacturer's instruction. For Mini-Gel apparatus, make sure that the bottom of both gel plates and spacers are perfectly flushed against a flat surface before tightening clamp assembly. A slight misalignment will result in a leak. Solution A, B and distilled water were combined in a small Erlenmeyer flask or test tube. Plastics gloves should be worn all times because acrylamide (Solution A) is a neurotoxin. Ammonium sulfate and TEMED were added and the solution was mixed by swirling gently (excessive aeration will interfere with polymerization). Work rapidly at this point because polymerization will be under way. The solution was carefully introduced into the gel sandwich using pipette so that it descends along a spacer. This minimizes the possibility of air bubbles becoming trapped within the gel. When the appropriate amount of separating gel solution has been added (in the case of the Mini-Gel, about 1.5 cm from top of front plate or 0.5 cm below level where teeth of comb will reach), about 1 – 5 mm of distilled water was gently layered on top of the separating gel solution. This keeps the gel surface plat. Gel was allowed to polymerize (30 – 60 min). When the gel has polymerized, a distinct interface will appear between the separating gel and the water.

Pouring the Stacking Gel (Example of Standard Stacking Gel Preparation for Two 5% Stacking Gels (for two 60 x 80 x 0.75 mm gels): 2.3 ml distilled water, 0.67 ml Solution A, 1.0 ml Solution C, 30 μl ammonium persulfate and 5 μl TEMED was needed. Distilled water covering the separating gel was poured off. The small droplets remaining will not disturb the stacking gel. Solution A, C and distilled water

were combined in a small Erlenmeyer flask or test tube. Ammonium sulfate and TEMED were added and solution was mixed by swirling gently. Stacking gel solution was pipetted onto separating gel until solution reaches top of front plate. Comb was carefully inserted into gel sandwich until bottom of teeth reach top of front plate. Be sure no bubbles are trapped on ends of teeth. Tilting the comb at a slight angle is helpful for insertion without trapping air bubbles. Stacking gel was allowed to polymerize (about 30 min). After stacking gel has polymerized, comb was carefully removed, making sure not to tear the well ears. Gel was placed into electrophoresis chamber. If using the Mini-Gel system, both gels were attached to electrode assembly before inserting into electrophoresis tank. Electrophoresis buffer was added to inner and outer reservoir, making sure that both the top and the bottom of the gel are immersed in buffer. Wells were checked for trapped air bubbles and damaged well ears. Distorted well ears can be repositioned using Hamilton syringe. Air bubbles clinging to bottom of gel should be removed to insure even current flow. It is useful to rinse wells with electrophoresis buffer prior to loading in order to remove unpolymerized acrylamide and any contaminants.

3.1.6.4 Preparing and Loading Samples

Capacity per Well (Mini-Gel System).

Gel Thickness	5 Wells	10 Wells	15 Wells
0.5 mm	45 μl	16 μl	9 μl
0.75 mm	68 μl	24 μl	14 μl
1.0 mm	90 μl	32 μl	18 μl
1.5 mm	135 μl	48 μl	27 μl

Protein sample and buffer were combined in an eppendorf tube in a 4:1 volume ratio (i.e. 20 μl sample + 5 μl sample buffer). Protein solution was heated at 100^oC for 2 – 10 min. Solution was spin down for 1 second in microfuge (longer if large quantities of debris are present). Sample solution was introduced into well

using Hamilton syringe or disposable gel loading tip. Protein solution was layered on the bottom of the well and the syringe tip was raised as dye level rises. Be careful to avoid introducing air bubbles as this may allow some of sample to be carried to adjacent well. Syringe was rinsed thoroughly with electrode buffer or distilled water before loading different samples. A standard protein molecular weight marker (Promega) was included in one or both outside wells.

3.1.6.5 Running a Gel

Electrode plugs were attached to proper electrodes. Current should flow towards the anode. Power supply was turned on to 200 V (constant voltage; current will be about 100 mA at start and 60 mA at end of electrophoresis for two 0.75 mm gels; 110 mA at start and 80 mA at end of electrophoresis for two 1.5 mm gels). The dye front should migrate to 1 – 5 mm from the bottom of the gel in 30 – 40 min for two 0.75 mm gels or 40 – 50 min for two 1.5 mm gels. Power supply was turned off and electrode plugs were removed from electrodes. Gel plates were removed from electrode assembly. A spacer was carefully removed. The spacer was inserted in one corner between the plates, and the gel plates were gently pried apart. The gel will stick to one of the plates.

3.1.6.6 Staining a Gel with Coomassie Blue

Working Solutions: (1) Coomassie Gel Stain (1 L); 1.0 g Coomassie Blue R-250, 450 ml methanol, 450 ml distilled water and 100 ml glacial acetic acid were mixed and stirred. (2) Coomassie Gel Destain (1 L); 100 ml methanol, 100 ml glacial acetic acid and 800 ml distilled water were mixed and stirred.

Staining Procedure: Gloves must be worn all the times to prevent transfer of fingerprints to the gel. Gel was picked up and transferred into a small container containing a small amount of Coomassie Gel Stain solution (20 ml is sufficient), or glass plate was gently agitated in stain solution until the gel separated from plate. Container was agitated on slow rotary shaker (5 – 10 min for 0.75 mm gels, 10 – 20 min for 1.5 mm gels). Container was covered with lid or plastic wrap during staining and destaining. Stain solution was poured out (can be reused several times), and the gel was rinsed with a few changes of distilled water. The water was discarded. Coomassie Gel Destain solution (about 50 ml) was added into the container. Strong bands were visible immediately on a light box, and the gel was largely destained within an hour. To destain completely, destain solution was changed and agitated overnight. If Coomassie staining was not sensitive enough, the gel can be rinsed and then silver stained.

3.1.6.7 Silver Staining a Gel

Working Solutions: (1) 0.36% NaOH, 1% citric acid (can be stored for several weeks), 50% methanol/10% acetic acid and 1% acetic acid. (2) Solution A: 0.8 g silver nitrate in 4 ml distilled water; Solution B: 21 ml 0.36% NaOH mixed with 1.4 ml 14.8 M (30%) ammonium hydroxide; Solution C: Solution A was added to Solution B dropwise with constant vigorous stirring, allowing brown precipitate to clear. Distilled water was added to 100 ml. Solution must be used within 10 min; Solution D: 0.5 ml 1% citric acid was mixed with 50 μl 38% formaldehyde. Distilled water was added to 100 ml. Solution must be fresh.

Staining Procedure: The gel was picked up and transferred to a small container by wearing gloves. The gel was soaked in 50% methanol/10% acetic acid for at least 1 hour with 2 – 3 changes of solution. The gel was then rinsed with water for 30 min with at least 3 changes. Solution A, B and C was prepared. The gel was removed to a clean container and stained in Solution C for 15 min with gentle agitation. Solution D was prepared. The gel was removed to a clean container and

developed by washing gel in Solution D. Bands should appear in less than 10 min or else Solution D must be changed. Reaction should be stopped if a pale yellow background appears. Development was stopped by rinsing gel in 1% acetic acid. The gel was washed in distilled water for at least 1 hour with at least 3 changes of water. If protein was too dark, the gel was stained with Kodak Rapid Fix or Kodak Unifix. Destain was stopped with Kodak hypo clearing agent such as Orbit. The gel then washed in 50% methanol/10% acetic acid. The gel was stored in distilled water or dried.

3.2   Immobilization of Diamine Oxidase (Cross-linking Method)

Diamine oxidase (DAO) membrane was prepared by entrapment and cross-linking the enzyme in a poly (vinyl alcohol) (PVA) membrane cross-linked with glutaraldehyde. The cross-linking ratio (CR) is defined as moles of glutaraldehyde per moles of PVA repeat unit (CR = moles glutaraldehyde/moles PVA). The PVA concentration and the cross-linking ratio was varied between 5 – 15% for PVA concentration and 0.02 – 0.12 for cross-linking ratio to determine the optimum parameter for enzyme immobilization.

3.2.1   Chemicals

Poly (vinyl alcohol) (PVA), diamine oxidase (Sigma, 0.16 U/mg), 10% (v/v) acetic acid, 50% (v/v) methanol, 10% (v/v) sulfuric acid and 1.2% (w/v) glutaraldehyde were used as received.

3.2.1.1 Working Solutions

DAO Solution: 200 mg of DAO was dissolved in 1 ml of distilled water to produced 200 mg/ml of DAO solution (32 U/ml). PVA Solution: To prepare 10% (w/v) of PVA solution, 5 g of PVA powder was mixed with 50 ml of distilled water. The solution was heated up to approximately $90^{o}C$ and stirred to make the PVA easy to dissolve. Cross-linking Solution: 30 μl 10% PVA was mixed with 18 μl 10% acetic acid (buffer), 12 μl 50% methanol (quencher), and 6 μl 10% sulfuric acid (catalyst) in an eppendorf tube in a volume ratio of 5:3:2:1 respectively. The solution was then quickly mixed with an appropriate amount of 1.2% glutaraldehyde (e.g. 34.1 μl for cross-linking ratio of 0.06) and stirred using vortexer. Solution must be used immediately before it polymerized.

3.2.1.2 Membrane Preparation

In another eppendorf tube, 90 μl of the cross-linking solution was mixed and stirred with 15 μl of DAO solution in a volume ratio of 6:1. 75 μl of the mixture solution was pipetted on a glass slide, air dried for 1 hour and covered with another glass slide to prevent the membrane from contracting. Membrane thickness was controlled using aluminium spacer tape (e.g. average thickness = 0.39 mm, membrane average diameter = 8 mm). Both glass slides were clamped together and left for 24 hours at $\pm 25^{o}C$. The glass slides was then soaked and unclamped in 35 ml potassium phosphate buffer pH 7.0 for a prescribed amount of time. The cross-linked PVA-DAO membrane was gently stripped out from the glass slide. The washing buffer was collected and analyzed for enzyme activity assay purpose (enzyme leakage). The PVA-DAO membrane was soaked in 5 ml of the same buffer in a universal bottle at $4^{o}C$. The membrane was then transferred to another universal bottle containing 5 ml of the same buffer for every 24 hours. Each buffer was collected and analyzed for enzyme activity (enzyme leakage). After the membrane

was stable and no further enzyme leakage, the membrane was tested every 3 or 5 days to determine its apparent enzyme activity.

CHAPTER 4

RESULTS AND DISCUSSION

Recently, some researchers such as Tombelli *et al.*, (1998), Niculescu *et al.*, (2001), Wimmerova *et al.*, (1999) and Frebort *et al.*, (2000) have found that diamine oxidase (DAO) extracted and purified from pea seedling shows higher activity than the commercial DAO from porcine kidney. Pea seedling DAO has wide substrate specificity, oxidizing preferably natural diamines and polyamines. This can improve the sensitivity of the biosensor.

4.1   Purification of Pea Seedling Amine Oxidase

In this work, DAO from pea seedling will be used to develop a biogenic amines biosensor. However, since pea seedling DAO is not commercially available, it has to be extracted and purified. The type of pea used in this work is *Cicer arietinum* (chick pea). The purification methods followed the methods given by Merek Sebela *et al.,* (1998) with some modifications. The chick pea diamine oxidase (CPAO) was purified using ammonium sulfate precipitation and dialysis, followed

by DEAE-Cellulose, hydroxyapatite (Bio-Gel HTP) and Sephacryl S-300 HR chromatography.

4.1.1   Sample Preparation

Seedlings of chick pea were germinated in the dark for 7 days. Maximum amine oxidase activity was usually found to be present between 5 – 7 days of germination and declined thereafter. After the homogenization of the germinated seedlings (30 g, minus roots) in 3 weight volumes (v/w) of 0.1 M potassium phosphate buffer pH 7.0, the crude extract was fractionated with 30% ammonium sulfate to remove the suspended solid and some contaminating proteins. The amine oxidases were precipitated completely with 70% ammonium sulfate. The precipitate was then re-dissolved in 3 volumes of 0.1 M potassium phosphate buffer pH 7.0, and dialyzed overnight against 20 mM potassium phosphate buffer pH 7.0 at $4^{o}C$ to remove salt and low molecular weight contaminating proteins. During the heat treatment process, after a short heating at $60^{o}C$ for 5 minutes, the total protein content decreased significantly. The CPAO were further purified by DEAE-Cellulose, Bio-Gel HTP and Sephacryl S-300 HR chromatography.

CPAO activity was measured colorimetrically using a coupled reaction with horseradish peroxidase (HRP) and chromogen solution utilizing o-dianisidine as the dye. Figure 4.1 shows the hydrogen peroxides standard curve, which was used to measure the CPAO activity. Protein concentration was determined according to bicinchoninic acid (BCA) assay with BSA as a standard. Figure 4.2 shows the BSA standard curve used to determine the protein concentration.
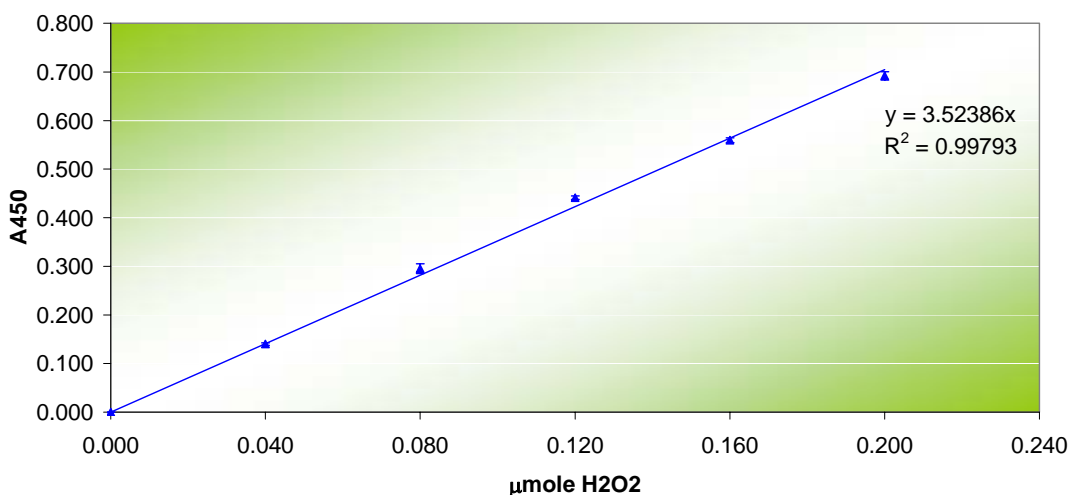
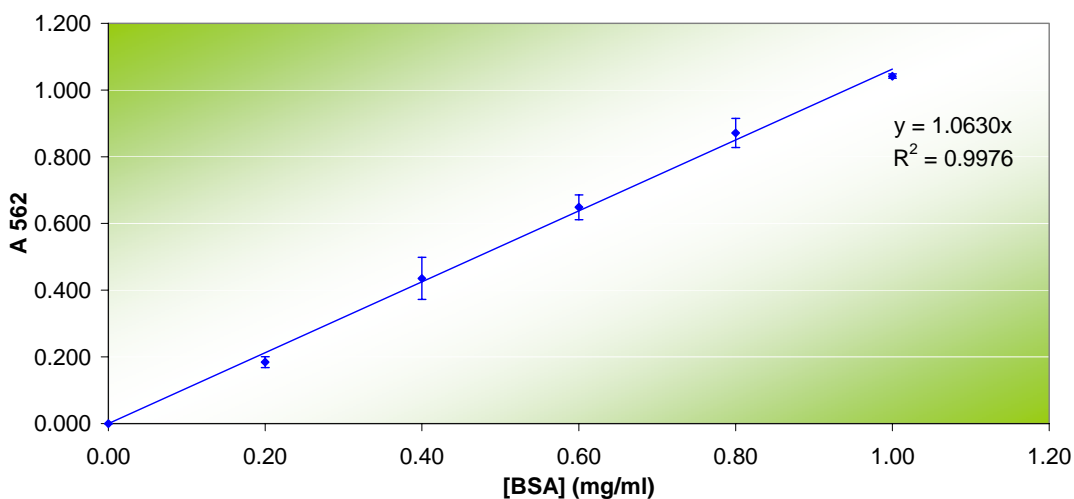**Figure 4.1** : Hydrogen peroxides (H_2O_2) standard curve.

Figure 4.2 : Protein concentration standard curve (BSA standard curve).

4.1.2   Anion Exchange Chromatography (DEAE-Cellulose)

The ion exchange principle permits the protein to bind even when a large buffer volume is applied, making this method useful for an initial purification step from a crude extract. Ion exchange chromatography separates proteins based on their net charges. Negatively or positively charged functional groups are covalently bound

to a solid matrix, yielding either a cation or anion exchanger, respectively. When charged proteins is applied to an exchanger of opposite charge, it is absorbed, while proteins that are neutral or have the same charge with the matrix are eluted in the void volume of the column. Binding of the charged proteins are reversible and absorbed proteins are commonly eluted with a salt, pH gradient or increased in buffer concentration.

CPAO from the previous steps was further purified by an anion exchange (DEAE-Cellulose) chromatography. DEAE-Cellulose has a positively charged group and defined as an anion exchange matrix. After the CPAO was applied, the column was equilibrated with 20 mM potassium phosphate buffer pH 7.0. Figure 4.3 shows the protein elution profile for the DEAE-Cellulose chromatography.
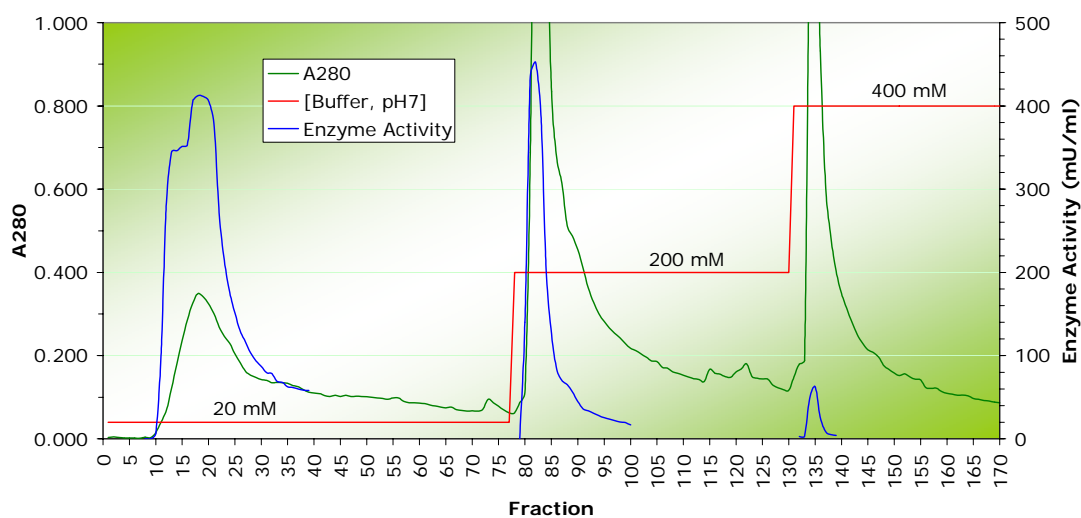

Figure 4.3 : Protein elution profile for the DEAE-Cellulose chromatography.

From the figure, most of CPAO did not bind to the DEAE-Cellulose column equilibrated with 20 mM potassium phosphate buffer pH 7.0 and was collected in the initial fraction (first peak). This suggested that the isoelectric point of CPAO might be higher than 7.0. Isoelectric point (pI) is defined as the pH at which the positive charges equal the negative charges (in other words, the net charge of the protein is

zero). At a pH above its pI, the protein of interest will be negatively charged, and at a pH below its pI, the protein will be positively charged. However, some CPAO were also present in the second and third peak, after the column was eluted with the 200 mM and 400 mM potassium phosphate buffer of the same pH, respectively. Its total activity is lower than in the initial fraction/peak. Therefore, only the active fractions from the first peak were pooled and prepared for further purification.

4.1.3   Hydroxyapatite Chromatography (Bio-Gel HTP)

Hydroxyapatite, $(Ca_5(PO_4)_3OH)_2$ is a crystalline form of calcium phosphate that is widely used in preparative biochemistry, having proven itself a unique tool for the fractionation and purification of monoclonal antibodies and other proteins, enzymes and nucleic acids. Hydroxyapatite is useful for preparative work in column or batch modes, and for quantitative analysis of proteins or nucleic acids. Hydroxyapatite chromatography can be utilized at any stage in a process from initial capture to final polishing. Hydroxyapatite has unique separation properties and unparalleled selectivity and resolution. It often separates proteins that appear to be homogeneous by other chromatographic and electrophoretic techniques.

Hydroxyapatite has unique selectivity. Since molecular separation on hydroxyapatite is not primarily dependent on molecular weight, molecular size, charge density or isoelectric point, hydroxyapatite chromatography is a valuable complement to other separations techniques. Hydroxyapatite has a high capacity for nucleic acids and proteins. Its surface area is about 50 m^2. Non-specific adsorption of hydrophobic substances is minimized by the inorganic crystalline matrix of hydroxyapatite. Hydroxyapatite displays negligible adsorptive capacity for low molecular weight substances such as mononucleotides, salts and amino acids. Hydroxyapatite is chemically and thermally stable. It is compatible with wide range

of aqueous and organic solvents and can be sanitized in sodium hydroxide. It has a pH tolerance greater than 5.5 and is autoclavable.

The active fractions from the DEAE-Cellulose column was loaded directly onto the hydroxyapatite column, equilibrated with 20 mM potassium phosphate buffer pH 7.0 and the CPAO was eluted with 200 mM potassium phosphate buffer pH 7.0. The active fractions were pooled, dialyzed overnight against 20 mM potassium phosphate buffer pH 7.0 and concentrated using ultrafiltration. Figure 4.4 shows the protein elution profile for the hydroxyapatite (Bio-Gel HTP) chromatography.
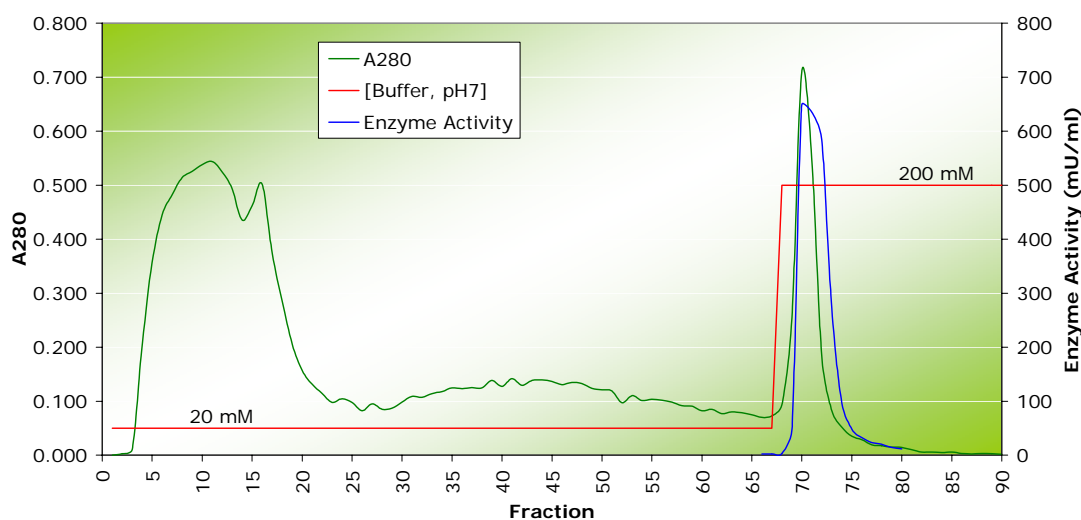

Figure 4.4 : Protein elution profile for the hydroxyapatite (Bio-Gel HTP) chromatography.

4.1.4 Size-Exclusion Chromatography (Sephacryl S-300 HR)

The remaining impurities, mainly proteins of lower molecular weight, were removed in the final polishing step using size-exclusion (Sephacryl S-300 HR) chromatography. The protein elution profile for the Sephacryl S-300 HR

chromatography is shown in Figure 4.5. The overall purification results of chick pea diamine oxidase are shown in Table 4.1 and 4.2.

The final preparation of CPAO had a specific activity of 12,702 mU/mg (putrescine as a substrate), 11,695 mU/mg (cadaverine as a substrate) and 452 mU/mg (histamine as substrate). The final purification fold was 53.4, 66.1 and 35.0-fold for putrescine, cadaverine and histamine as substrate, respectively. However, the final recovery (yield) of CPAO for each substrates was very low, less than 20% of the total CPAO activity in the crude extract.
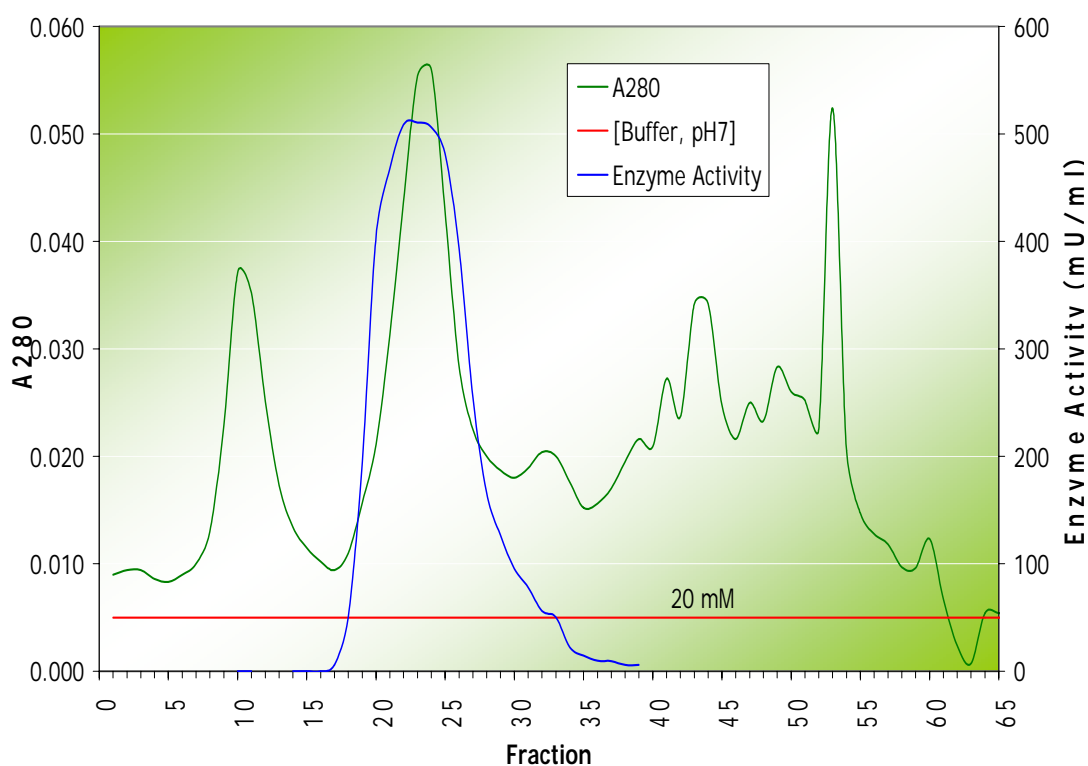

Figure 4.5 : Protein elution profile for Sephacryl S-300 HR chromatography

Table 4.1 : The purification of chick pea diamine oxidase (CPAO).

Steps	Volume (ml)	CPAO Activity (mU/ml)			Total CPAO Activity (mU)			Total CPAO Recovery (%)		
		Putrescine	Cadaverine	Histamine	Putrescine	Cadaverine	Histamine	Putrescine	Cadaverine	Histamine
Crude Extract	100.0	3,315	2,468	180	331,467	246,816	18,003	100%	100%	100%
Ammonium sulfate Precipitation (30%), Supernatant	98.0	3,081	2,393	180	301,970	234,481	17,643	91%	95%	98%
Ammonium sulfate Precipitation (70%), Precipitate, Dialysis	40.0	6,802	4,803	331	272,090	192,110	13,241	82%	78%	74%
Heat Treatment, Dialysis	42.0	4,648	3,540	247	195,196	148,665	10,366	59%	60%	58%
Anion Exchange Chromatography (DEAE-Cellulose)	38.0	2,606	1,727	75	99,040	65,642	2,858	30%	27%	16%
Hydroxyapatite Chromatography (Bio-Gel HTP)	6.2	10,689	10,411	438	66,271	64,550	2,718	20%	26%	15%
Size-Exclusion Chromatography (Sephacryl S-300 HR)	18.3	2,426	2,234	86	44,373	40,857	1,577	13%	17%	9%

Note : 1000 mU = 16.67 nkat

Table 4.2 : Specific activity and purification fold of chick pea diamine oxidase (CPAO).

Steps	Total Protein (mg)	Protein Concentration (mg/ml)	CPAO Specific Activity (mU/mg)			Purification Fold		
			Putrescine	Cadaverine	Histamine	Putrescine	Cadaverine	Histamine
Crude Extract	1394.2	13.942	238	177	13	1.0	1.0	1.0
Ammonium sulfate Precipitation 30%, Supernatant	1194.8	12.192	253	196	15	1.1	1.1	1.1
Ammonium sulfate Precipitation 70%, Precipitate, Dialysis	483.9	12.098	562	397	27	2.4	2.2	2.1
Heat Treatment, Dialysis	294.5	7.013	663	505	35	2.8	2.9	2.7
Anion Exchange Chromatography (DEAE-Cellulose)	29.6	0.778	3,350	2,220	97	14.1	12.5	7.5
Hydroxyapatite Chromatography (Bio-Gel HTP)	10.7	1.718	6,222	6,060	255	26.2	34.2	19.8
Size-Exclusion Chromatography (Sephacryl S300 HR)	3.5	0.191	12,702	11,695	452	53.4	66.1	35.0

Note : 1000 mU = 16.67 nkat

4.1.5 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE of the final purified CPAO showed a single band corresponding to the relative molecular weight of about 73 kDa (Figure 4.6 and 4.7). This value is in agreement with those of other pea seedling amine oxidases such as 72 kDa for both from *Lathyrus odoratus* (sweet pea) and *Lathyrus sativus* (grass pea) (Sebela *et al*., 1998), 75 kDa from *Trigonella foenum-graecum* (fenugreek) (Sebela *et al*., 1997) and 68 kDa from *Vigna radiata* (mungbean) (Choudary *et al*., 1999). Some of the molecular properties of the enzyme have been determined using an improved purification method which gave a homogeneous enzyme (Padiglia *et al*., 1991). The molecular mass of 150 kDa determined by gel permeation chromatography and of 72 kDa found by SDS-PAGE confirmed the enzyme dimeric structure (Sebela *et al*., 1998). Similar properties were found for the amine oxidase from seedlings of *Lathyrus cicera* (Cogoni *et al*., 1989).
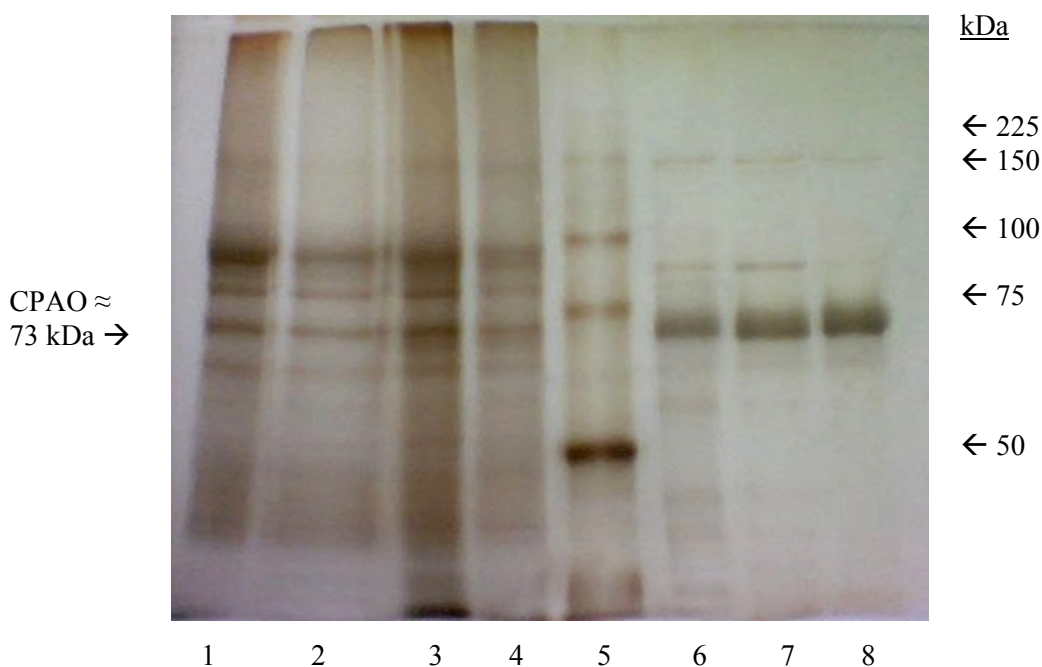

Figure 4.6 : SDS-PAGE of CPAO; Lane 1 (crude extract), Lane 2 (30% ammonium sulfate precipitation), Lane 3 (70% ammonium sulfate precipitation and dialysis),

Lane 4 (heat treatment and dialysis), Lane 5 (protein marker), Lane 6 (DEAE-Cellulose chromatography), Lane 7 (Hydroxyapatite chromatography), Lane 8 (Sephacryl S-300 HR chromatography)
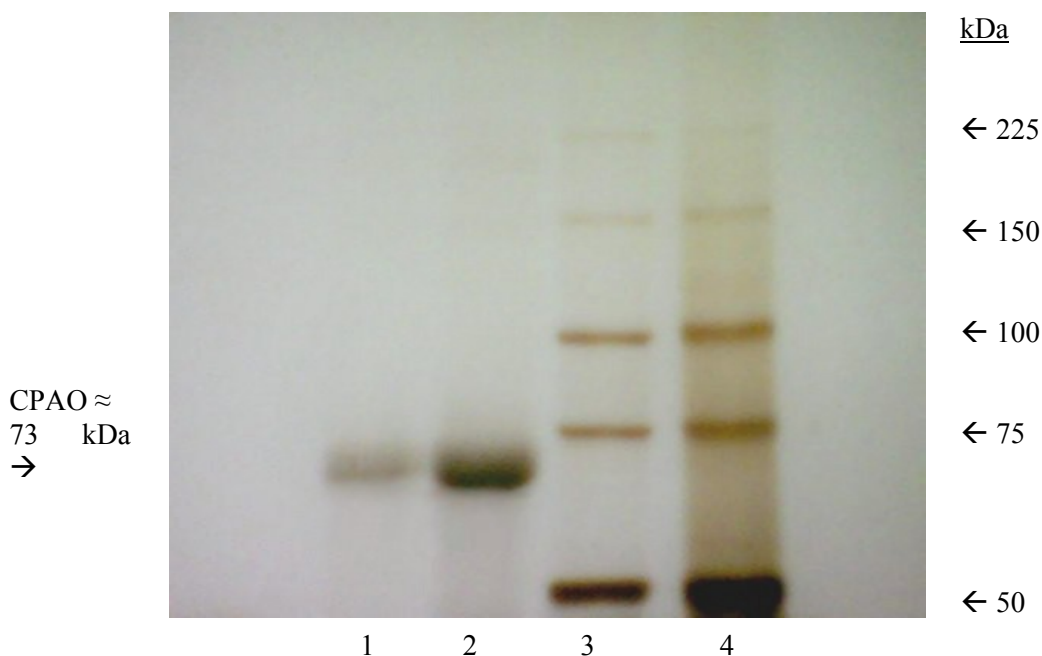

Figure 4.7 : SDS-PAGE of CPAO; Lane 1 (Sephacryl S300 HR chromatography), Lane 2 (Sephacryl S300 HR chromatography, concentrated), Lane 3 (protein marker), Lane 4 (protein marker, concentrated)

4.2 Immobilization of Diamine Oxidase (Cross-linking Method)

In addition to purification, immobilization of DAO in cross-linked poly(vinyl alcohol) (PVA) has also been performed. Initial works were done using commercial porcine kidney diamine oxidase (PKAO) to determine the optimum parameter for immobilization. PVA concentration was varied between 5 – 15% while the cross-linking ratio, CR (ratio of moles glutaraldehyde to moles of PVA repeat unit) was fixed at 0.06. Then the cross-linking ratio was varied between 0.02 – 0.12 while the

PVA concentration was fixed at 10%. Initial result suggested that the optimum parameters for DAO immobilization were at PVA concentration of 10% and cross-linking ratio of 0.06. PKAO immobilized at 10% PVA with cross-linking ratio of 0.06 gives the highest apparent activity compared to others. Figure 4.8 shows PKAO-PVA membranes apparent activity at 10% PVA concentration with the cross-linking ratio varied. Figure 4.9 shows PKAO-PVA membranes apparent activity at cross-linking ratio of 0.06 with the PVA concentration varied.

Figure 4.8 : PKAO-PVA membranes apparent activity at 10% PVA concentration with the cross-linking ratio varied.

Figure 4.9 : PKAO-PVA membranes apparent activity at cross-linking ratio of 0.06 with the PVA concentration varied.
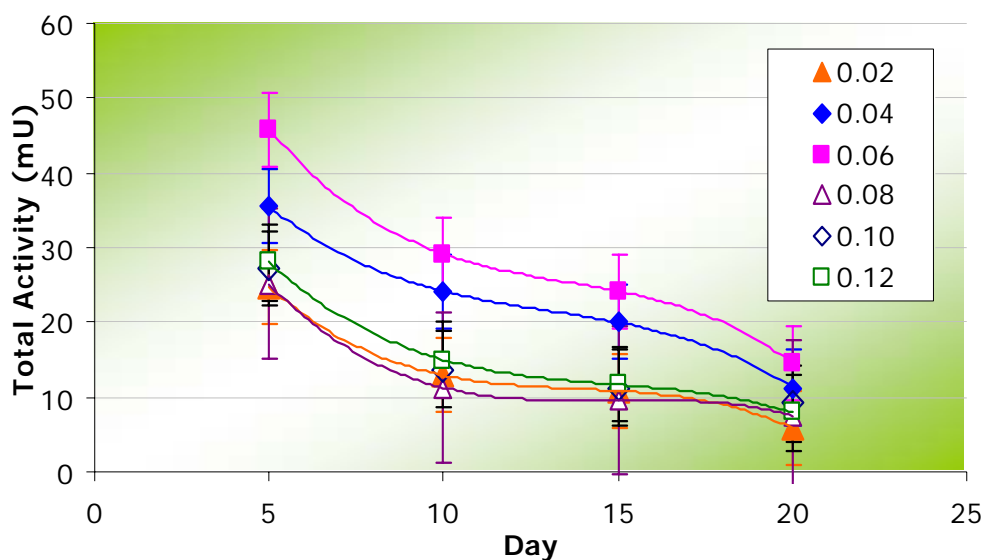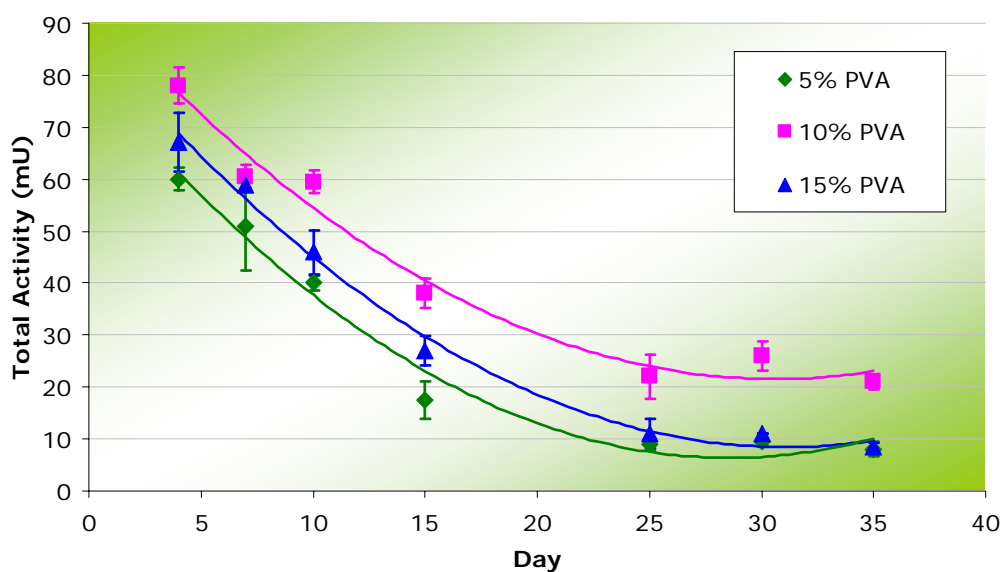
Immobilization of partially purified CPAO has also been done. Initial result showed that at an equal enzyme loading (45 mU), CPAO-PVA membranes gives a higher apparent activity compared to the commercial PKAO-PVA membranes. After 40 days, CPAO-PVA remained about 40% of its apparent activity compared to 20% for PKAO-PVA membranes. Comparison of CPAO-PVA and PKAO-PVA membranes apparent activity at an equal enzyme loading was shown in Figure 4.10. The results showed that CPAO-PVA membranes are promising as the potential bio-active element in a biogenic amines biosensor.
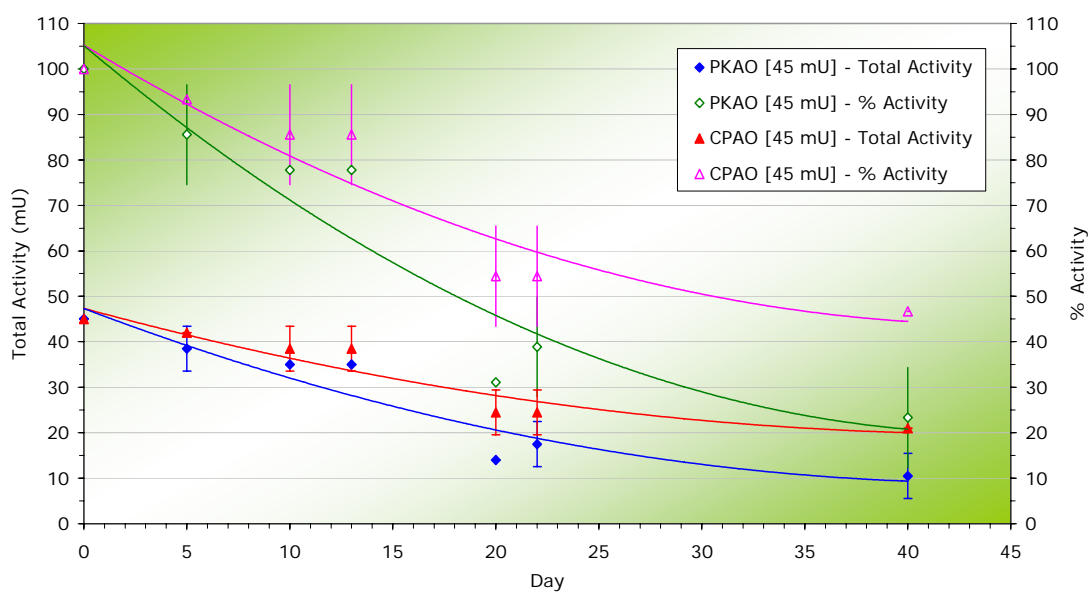
Figure 4.10 : Comparison of CPAO-PVA and PKAO-PVA membranes apparent activity at an equal enzyme loading.

CHAPTER 5

SUMMARY AND RECOMMENDATION

5.1 Summary

Pure amine oxidase has been successfully isolated from the *Cicer arietinum* seedlings, following three chromatographic steps. The molecular weight of the purified chick pea amine oxidase (CPAO) was 73 kDa, as shown by a single band determined by SDS-PAGE. The pure CPAO gave a specific activity of 12.7, 11.7 and 0.45 U/mg with putrescine, cadaverine and histamine as substrate, respectively. The purification fold was about 53-fold for putrescine as a substrate, 66-fold for cadaverine and 35-fold for histamine. Although the pure CPAO has been successfully obtained, the total recoveries of the enzymes were quite low, which were about 13%, 17% and 9% with putrescine, cadaverine and histamine as substrate, respectively. The substrate selectivity relative to putrescine (assuming 100% for putrescine) was 92% for cadaverine and 4% for histamine.

The optimum parameters for CPAO immobilization were PVA concentration of 10% and cross-linking ratio of 0.06, determined earlier by the immobilization of PKAO in cross-linked poly (vinyl alcohol) (PVA). Result for immobilized partially purified CPAO showed that at an equal enzyme loading, CPAO-PVA membranes

gave higher apparent activities compared to the commercial PKAO-PVA membranes. CPAO-PVA membranes retained about 40% of its apparent activity compared to 20% for PKAO-PVA membranes, after 40 days of storage.

5.2 Recommendations

Although pure CPAO had been successfully isolated from the chick pea seedlings, however enzyme recovery was still very low. The type of anion exchange chromatography used in this research resulted in a significant loss of the total enzymes (recovery of only about 30-50%, while typical recovery is 70-90%). Thus, it is suggested that other type of anion exchange matrix available in the market is used. Other types of chromatography system can also be used, such as absorption chromatography.

The success of an enzymatic biosensor partly depends on the enzyme immobilization technique. Poor technique will result in significant loss of enzyme activity and short operational life. Since the reusability and the recovery of the free enzymes are limited, immobilization of enzymes is very attractive. The advantages of immobilized enzymes are that they can be used either in batch or continuous systems, they can be removed easily from the reaction medium and they can be used for the controlled production. Immobilized enzyme systems do also have limitations, such as loss of activity due to the immobilization technique and decrease in the apparent activity due to mass transfer resistance towards the substrate and product. An optimal support material should provide large surface area per unit volume (or mass) of the carrier for effective immobilization of the desired amount of enzyme and should allow substrate and product transport with the least diffusional resistance. The immobilization technique should also lead to high immobilization and activity yields.

In this research, CPAO was immobilized by entrapment and cross-linking of the enzyme in a poly (vinyl alcohol) (PVA) membrane, cross-linked with glutaraldehyde. The disadvantages of using the cross-linking approach are there is some diffusion limitation and there can be damage to the biomaterial. Diffusion of solutes is determined by the cross-linking density of the membrane, or mesh size and the degrees of swelling. The `pores' are the open spaces between the cross-link points and are not fixed. Alternatively, a physically cross-linked PVA through freeze-thawing method may be a good choice for immobilizing the enzymes. It can minimize the problems associate with chemically cross-linked PVA while maintaining the good properties of PVA. The exposition of aqueous PVA solution to several freeze-thawing cycles leads to reinforced gels. Fine crystallites are formed after the freeze-thawing process, due to the slow heat treatment.

Addition of protein stabilizing agent, such as bovine serum albumin (BSA) into the PVA mixture for immobilization might be very helpful to increase the membranes performance. With the existence of BSA, less enzymes leak out from the membranes, thus more enzymes can be retained inside the membranes. An appropriate amount of BSA must be calculated, because too much BSA will not necessarily correlate well with enzymes activity. Too much BSA might block the active sites of the enzymes, thus it becomes hard for the substrate to diffuse into the membranes, resulting in low reaction rates. The thickness of the membranes must be well controlled. The thickness of the membranes affects the diffusion rate and permeability. The thicker the membranes, the longer the response time is. So, the possibility of casting thinner enzymes membranes that can still retain the sensitivity to the substrate should be examined.

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