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Construction and characterization of novel amperometric plant
tissue-based biosensors

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University of Hawaii, 1992
CONSTRUCTION AND CHARACTERIZATION OF NOVEL AMPEROMETRIC
PLANT TISSUE-BASED BIOSENSORS

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MY PARENTS
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ABSTRACT

A biosensor is an analytical device in which a biological material, capable of specific chemical recognition, is in intimate contact with a physico-chemical transducer to give an electrical signal. In this research, plant tissue materials (as the molecular recognition element) are coupled to electrochemical transducers to construct selective analytical biosensors for the detection of important analytes.

The use of eggplant fruit as a source of polyphenol oxidase to construct an amperometric biosensor for catechol detection is demonstrated. Amperometric measurements are based on the detection of catechol quinone produced at the electrode surface. The characterization of the novel sensor with respect to biocatalytic loading, linear dynamic range, sensitivity, selectivity and lifetime is reported.

A use of a novel thin layer electrode design, with a plant tissue reactor and a sensing tin oxide collector electrode, is reported for amperometric detection in flow injection analysis and compared with previous carbon paste electrode designs. Eggplant fruit containing the enzyme, polyphenol oxidase is used as the selective biocatalyst. The new system, evaluated for the case of catechol
measurements, is shown to offer superior sensitivity, dynamic range and detection limits.

In vitro cultured tobacco callus tissue, which contains high activity of peroxidase, is used to construct an amperometric biosensor for the detection of hydrogen peroxide. The tissue is incorporated into a carbon paste matrix along with ferrocene, an electron mediator. The detection of hydrogen peroxide was accomplished through its enzymatic reduction mediated by ferrocene. Comparison studies conducted with normally grown types of tobacco tissues indicate that the callus tissue has the highest biocatalytic activity and the least variance between tissue preparations with respect to sensor response toward hydrogen peroxide. Additionally, the sensor exhibits a remarkably long lifetime of about five months. Other important characteristics of the sensor include fast response times, wide dynamic range, and very low detection limits.

The possibility of using genetically transformed (transgenic) plants; specifically, transgenic potato (Solanum tuberosum) and transgenic tobacco (Nicotiana tabacum), in the construction of an amperometric biosensor for the detection of phenyl-B-glucuronide was attempted as an application of recombinant DNA technology in biosensor construction.
TABLE OF CONTENTS

Acknowledgements ........................................ iv
Abstract ......................................................... v
List of Tables .................................................. xi
List of Figures .................................................. xii

Chapter 1: Introduction ........................................ 1
1.1 A biosensor ................................................ 1
1.2 Progress of biosensor development ....................... 2
1.3 Types of biosensors ....................................... 4
  1.3.1 Immunological biosensors ......................... 4
  1.3.2 Intact chemoreceptor based biosensors .......... 6
  1.3.3 Isolated receptor based biosensors ............. 7
1.4 Development of tissue-based biosensors ................. 8
1.5 Plant tissue-based biosensors ........................... 12
1.6 Response characteristics of tissue based biosensors utilizing gas sensors .......... 14
1.7 Recent developments of plant tissue-based biosensors ............. 15
  1.7.1 Voltammetry ...................................... 15
  1.7.2 Amperometry .................................... 16
1.8 Electrode materials in biosensor construction ........... 17
1.9 Amperometric detection in flow injection analysis .... 18
1.10 Amperometric detection in liquid chromatography .......... 19
1.11 Mediated amperometric biosensors ..................... 21
1.12 Enzyme kinetics and amperometric analysis .......... 24
1.13 Mixed plant tissue-carbon paste bioelectrodes .......... 27
1.14 Research objectives .................................. 30
# Chapter 2: Eggplant Based Bioamperometric Sensor for the Detection of Catechol

## 2.1 Background

## 2.2 Quantitative analysis of catechol and related compounds

## 2.3 Experimental

- **2.3.1 Apparatus**
- **2.3.2 Reagents**
- **2.3.3 Construction of eggplant-modified carbon paste electrodes**
- **2.3.4 Procedure**

## 2.4 Results and discussion

- **2.4.1 Calibration of the eggplant sensor**
- **2.4.2 Selectivity of the eggplant biosensor**
- **2.4.3 Response times of the eggplant sensor**
- **2.4.4 Biocatalytic loading of the eggplant sensor**
- **2.4.5 Variation of the biocatalytic activity and the time stability upon the source of eggplant**

## 2.5 Conclusions

---

# Chapter 3: Use of Tin Oxide Electrodes in Flow Injection Analysis with Application to Plant Tissue Based Biosensors

## 3.1 Background

## 3.2 Surface modified electrodes for detection in flowing streams

## 3.3 Tin oxide electrodes

## 3.4 Tin oxide electrode-based biosensors

## 3.5 Experimental section

- **3.5.1 Apparatus**
- **3.5.2 Materials and methods**
- **3.5.3 Construction of working electrodes**
- **3.5.4 Procedure**

## 3.6 Results and discussion

- **3.6.1 Calibration of the sensor**
- **3.6.2 Bioanalytical performances of thin layer configurations**
Chapter 4: Application of Plant Tissue Cultures in Construction of Tissue Based Biosensors

4.1 Background
4.2 Plant tissue culture
4.3 Biochemistry of peroxidases
4.4 Quantitative measurements of hydrogen peroxide
4.5 Operation of tobacco callus tissue-based $H_2O_2$ sensor
4.6 Experimental
4.6.1 Apparatus
4.6.2 Reagents
4.6.3 Callus cultures of tobacco
4.6.4 Preparation of working electrodes
4.6.5 Optimization of pH and operational potential of the sensor
4.6.6 Composition of ferrocene and biocatalytic loading
4.6.7 Calibration of tobacco callus sensor
4.6.8 Variation of biosensor response and biocatalytic activity of different tissue preparations
4.6.9 Time stability studies of tobacco callus sensor
4.6.10 Response of tobacco callus sensor toward important hydrogen donors
4.6.11 Subculture and peroxidase activity of tobacco callus tissues

4.7 Results and discussion
4.8 Conclusions

Chapter 5: Use of Genetically Engineered (Transgenic) Plant Tissues in Biosensor Construction

5.1 Background
5.2 Glucuronides and their importance
5.3 Genetic transformations of higher plants to express GUS activity.........................98

5.4 Glucuronidase enzyme assay in animal tissues.....99

5.5 Assay of glucuronidase activity in genetically transformed plants....................100

5.6 Transgenic plant tissue-based biosensors........101

5.7 Experimental........................................103
  5.7.1 Apparatus........................................103
  5.7.2 Reagents.........................................104
  5.7.3 Blank determinations.............................104
  5.7.4 Enzyme electrode preparation......................104
  5.7.5 pH and temperature optimization of the enzyme electrode..........................105
  5.7.6 Calibration of the enzyme electrode..............105
  5.7.7 Lifetime of the enzyme electrode...............105
  5.7.8 Use of genetically transformed plant tissues in biosensor construction.........106
  5.7.9 Source of genetically transformed plant tissues......................................106
  5.7.10 Staining of transgenic plant tissues with X-gluc.................................107
  5.7.11 Preparation of transgenic tobacco leaf-modified carbon paste....................111
  5.7.12 Electrochemical studies of tobacco leaf-modified carbon paste electrodes......111
  5.7.12.1 Steady-state amperometry......................111
  5.7.12.2 Flow injection analysis.......................111
  5.7.13 Homogeneous experiments with transgenic tobacco leaves...........................112
  5.7.14 Staining of transgenic potato tissues...113
  5.7.15 Preparation of transgenic potato tissue-modified carbon paste electrodes...113
  5.7.16 Steady state amperometric experiments with petiol tissue-modified carbon paste electrodes..............................................................117
  5.7.17 Flow injection analysis experiments with potato petiol tissues...............117
  5.7.18 Steady-state amperometric experiments in water-alcohol mixed solvent systems...118

5.8 Results and discussion.................................118

Chapter 6: Conclusions and Future Directions............130

Chapter 7: Literature Cited................................134

x
LIST OF TABLES

Table                                                                 Page
1. Comparison of eggplant sensor response at steady-state to structurally similar compounds..................45
2. Bioanalytical performances of thin layer configurations.................................................................65
3. Selectivity measurements of thin layer configurations......................................................................66
4. The basic nutrient constituents of Murashige and Skoog’s medium; formulation for one liter of the medium...............................................................77
5. Variance and response sensitivities of various types of individual tobacco tissues.........................89
6. Composition of GUS buffer.................................................................108
7. Staining of transgenic tobacco tissues.........................108
8. Staining of potato tissues with X-gluc.........................113
9. Results of the homogeneous experiment conducted with transgenic tobacco tissues..................127
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Schematic diagram of the first bioselective membrane electrode probe</td>
</tr>
<tr>
<td>2.</td>
<td>A single line flow injection analyzer system</td>
</tr>
<tr>
<td>3.</td>
<td>Schematic representation of a mediated electron transfer reaction</td>
</tr>
<tr>
<td>4.</td>
<td>Schematic diagram of eggplant electrode construction and biocatalytic reaction at the electrode surface</td>
</tr>
<tr>
<td>5.</td>
<td>Experimental set up for steady-state amperometry</td>
</tr>
<tr>
<td>6.</td>
<td>pH dependence of eggplant sensor at $1.5 \times 10^{-5}$ M catechol</td>
</tr>
<tr>
<td>7.</td>
<td>Steady-state amperometric responses of eggplant sensor for catechol</td>
</tr>
<tr>
<td>8.</td>
<td>Calibration curves obtained with the eggplant sensor in 0.1 M phosphate buffer (pH 7)</td>
</tr>
<tr>
<td>9.</td>
<td>Time stability of carbon paste modified with regularly grown eggplant</td>
</tr>
<tr>
<td>10.</td>
<td>Time stability of carbon paste modified with organically grown eggplant</td>
</tr>
<tr>
<td>11.</td>
<td>Schematic diagram of a tissue reactor/tin oxide collector thin layer cell</td>
</tr>
<tr>
<td>12.</td>
<td>A part of the flow injection peaks obtained with tissue reactor/tin oxide collector thin layer cell for catechol</td>
</tr>
<tr>
<td>13.</td>
<td>A calibration curve obtained with tissue reactor/tin oxide collector thin layer cell for catechol</td>
</tr>
<tr>
<td>14.</td>
<td>Flow injection peaks of tissue reactor/carbon paste collector thin layer cell for catechol</td>
</tr>
<tr>
<td>15.</td>
<td>A calibration curve obtained with tissue reactor/carbon paste collector thin layer cell for catechol</td>
</tr>
</tbody>
</table>
16. Enzymatic reaction mechanism of peroxidase........71
17. Electron transfer scheme of tobacco callus
sensor..................................................74
18. A photograph of tobacco callus tissue.................78
19. pH dependence of tobacco callus sensor at
1 X 10^-5 M H2O2 in 0.1 M phosphate
buffer..................................................83
20. Effect of tobacco callus tissue composition upon
the sensor response to H2O2.............................85
21. Steady-state amperometric responses of tobacco
callus sensor............................................86
22. Steady-state current response of each sensor
type under optimized experimental conditions.........88
23. Time stability of tobacco callus sensor; current
response to 1 x 10^-5 M H2O2..........................90
24. Response of tobacco callus sensor toward
important hydrogen donors...............................93
25. Dependence of peroxidase activity of tobacco
callus tissue upon subculture..........................94
26. 5-Bromo-4-chloro-3-indolyl-β-D-glucuronide
(X-gluc)..................................................102
27. Enzymatic reaction responsible for staining.........109
28. A photograph of tobacco leaf section stained
with X-gluc..............................................110
29. A photograph of a potato leaf stained with
X-gluc....................................................114
30. A photograph of a cross section of a potato-
petiole stained with X-gluc............................115
31. A photograph of a cross section of a potato-
petiole (at a higher magnification) stained with
X-glue to illustrate the localization of the
enzyme in vascular bundle.............................116

xiii
32. Enzymatic hydrolysis of phenyl glucuronide.......119
33. pH dependence of glucuronidase enzyme electrode in 0.1 M phosphate buffer..................120
34. Temperature dependence of glucuronidase enzyme electrode in 0.1 M phosphate buffer........121
35. Steady-state amperometric responses of glucuronidase enzyme electrode......................122
36. Calibration curve for phenyl glucuronide obtained with glucuronidase enzyme electrode.....123
37. Time stability of glucuronidase enzyme electrode......................................................125
38. Lineweaver-Burk type plot for glucuronidase enzyme electrode......................................126
CHAPTER 1

INTRODUCTION

1.1 A BIOSENSOR

A biosensor is a device incorporating a "biological sensing element" either intimately connected to or integrated within a transducer\textsuperscript{1}. The biological component is considered as a molecular recognition element as it recognizes a particular analyte. The transducer is a physico-chemical device which converts chemical information resulting from molecular recognition into a measurable signal. The output of the transducer is mainly electrical but other type of signals such as optical, thermal and electro-mechanical are currently in use. Thus biosensors can be categorized into electrochemical, optical, thermal and piezoelectric based on the transducing element. Alternatively, since biocatalytic components such as isolated enzymes, animal and plant tissues, antibodies, isolated receptors and chemoreceptive structures can function as the molecular recognition element, biosensors can also be categorized into biocatalytic, immunological and chemoreceptive types.

Biosensor technology combines the specificity and the sensitivity of biological systems with modern chemical(analytical) instrumentation. This emerging technology crosses many traditional academic disciplines.
At the very least, it brings together analytical chemistry and biochemistry; but areas such as electrical engineering, computer science, molecular biology and materials science may play an expanding role. Biosensor research promises a powerful new tool which may revolutionize the field of analytical science. The possible applications of biosensors as an analytical tool will be in the area of medicine, environmental and industrial process monitoring, fermentation industry and, in the area of agricultural and food chemistry. Another large research endeavor involving biosensors is in the military, focusing on specialized needs such as biological and chemical defense against toxic nerve gases and pathogenic organisms in the field of combat.

1.2 PROGRESS OF BIOSENSOR DEVELOPMENT

Biosensors were first described in 1962 at a New York Academy of Sciences symposium by Clark and Lyons. Early devices employed enzymes as molecular recognition elements coupled to polarographic or potentiometric devices to make electrochemical biosensors. These devices were specific for certain substrates and operated through the detection of an increase in product from an enzyme catalyzed reaction or a decrease in a substrate consumed in the reaction. A good example is the "glucose probe", which consisted of a Clark type oxygen electrode with glucose
oxidase immobilized at the electrode tip\textsuperscript{4}. The sensor detected the consumption of oxygen concentration when glucose was converted to gluconic acid and hydrogen peroxide. Many other substrates have subsequently been measured using oxidoreductases, including lactate, pyruvate, galactose, cholesterol, glycerol, hypoxanthine, xanthine, oxalate and fructose\textsuperscript{5}.

By the 1970s numerous research group had furthered the state of the art of enzyme-based sensors. These early attempts included the development of biosensors using immunoagents, cells and binding proteins, as the molecular recognition element. A special consideration was given to research on bioselective membrane electrodes in the early 1970s when stable and reliable potentiometric sensors for ammonia, carbon dioxide, hydrogen sulfide and other dissolved gases became commercially available\textsuperscript{6}. This approach combined the high selectivity of an enzymatic reaction with ion or gas sensing properties of the basic membrane electrode. Thus potentiometric devices became a key element in the development of the newer types of bioselective probes as they could replace destructive analytical techniques such as flame photometry. Subsequently, bacteria and other microorganisms were immobilized on such devices. The first potentiometric membrane electrode with living bacterial cells as the
biocatalyst in the construction of an arginine-selective bioprobe was described in 1976.7

1.3 TYPES OF BIOSENSORS

1.3.1 Immunological Biosensors

Immunological biosensors are based on the immunological interactions of antibodies and antigens. Such complementary binding systems can be used to construct biosensors if they can be coupled to sensing or transducing elements.

The discovery of the detection of plasma insulin by radioimmunoassay by Yalow and Berson in 1960 provided a revolutionary new method for the accurate and specific measurements of very low levels of biologically important compounds such as hormones, enzymes, drugs, viruses, and tumor antigens8. Immunoassays are fundamentally simple in concept and are based on the interaction of an analyte or ligand in question with its specific binding partner or antibody, as given below.

\[ \text{Ab} + \text{Ag} \rightleftharpoons \text{AbAg} \]

\[ K = \frac{[\text{AbAg}]}{[\text{Ag}][\text{Ab}]} \]

Where,

\( \text{Ab} = \text{Antibody} \)
\( \text{Ag} = \text{Antigen} \)
\( \text{AbAg} = \text{Antibody-Antigen complex} \)
\( K = \text{Binding constant} \)
Therefore, for a fixed concentration of antibody, the ratio of bound-to-free antigen, at equilibrium, is quantitatively related to the total amount of ligand present. This forms the basis of all immunoassays. Labelling agents such as radio isotopes, enzymes, fluorescent probes, chemiluminescent probes, and electrochemical tags are used in immunoassay procedures to measure antigen or antibody. Accordingly, there are several different types of immunoassay procedures such as the radioimmunoassay, enzyme immunoassay, fluorescence immunoassay, and electrochemical immunoassay. Immunoassay techniques can also be categorized into homogeneous or heterogeneous assays depending upon the need for a separation step to discriminate between the bound and unbound labelled antigen or antibodies.

In spite of the sensitivity and low detection limits of radio immunoassay techniques, there are significant disadvantages, mainly the handling and disposal of radio isotopes. Thus the above mentioned alternative immunoassay techniques have been developed extensively. With regard to biosensors, much of the research work has been aimed at linking immunoassays to electrochemical detection. Current published electrochemical immunoassays are: (a) amperometric immunoassays based on Clark type oxygen electrodes, (b) amperometric enzyme immunoassays that employ electrochemically detectable
products of an enzyme\textsuperscript{12}, (c) amperometric immunoassays that utilize either an antibody or antigen that is labelled with an electroactive species\textsuperscript{13}, (d) potentiometric immunoassays based on a change in the potential when antigen binds with antibody\textsuperscript{14}, and (e) potentiometric immunoassays based on conventional potentiometric devices such as carbon dioxide electrodes\textsuperscript{15}.

1.3.2 Intact Chemoreceptor-Based Biosensors

Intact chemoreceptor structures are being employed by bioanalytical chemists due to their unique binding properties. They are highly selective molecular recognition elements for certain compounds. The use of such intact chemoreceptive structures in construction of biosensors was first described by Belli and Rechnitz in 1986\textsuperscript{16}.

Receptor-based biosensors or "receptrodes" use intact chemosensing structures (antennules) of crustaceans such as crab\textsuperscript{17} and crayfish\textsuperscript{18}. This approach allows the receptors to remain in their native environment, which presumably has already been optimised by nature. The antennular chemosensing cells function not only as highly selective and sensitive molecular recognition elements but also as biological transducers. They convert chemical information into electrical impulses in a matter of milli-
seconds. Because of the high sensitivity and the fast response of these chemosensing structures to aqueous analytes they can be used in the development of neuronal biosensors.

The antennular receptrodes have many advantages such as extremely short response times, high degree of specificity, broad analytical range, and sensitivity. However, there are several disadvantages such as very short lifetime and technical difficulties with handling these chemoreceptive structures due to their fragility.

1.3.3 Isolated Receptor-Based Biosensors

In 1987, Hallowell and Rechnitz reported a novel approach for drug detection called Enzyme Amplified Receptor Assay (ERA). The fundamental principles involved in receptor assay are very similar to those of immunoassay. Isolated acetylcholine receptors from Torpedo nobilana were used as recognition elements for phencyclidine, a potent hallucinogen. This compound has a high affinity for the ion channel binding site of the receptor. This concept combined the use of receptor recognition with an enzyme amplification step to yield a simple and convenient assay procedure for drug detection^{19}.
1.4 DEVELOPMENT OF TISSUE-BASED BIOSENSORS

As described in section 1.2, biosensors originated with enzyme-based biocatalytic systems. However, a number of isolated enzyme-based sensors are limited due to poor stability, low levels of specific activity and the need for cofactors. In some cases it is possible to coimmobilize several enzymes or co-factors at a sensor tip. However in such cases, compromised experimental conditions are required because optimal operating conditions for each component are different.

In the middle of 1970, an alternative approach to isolated enzyme-based sensors was explored. Consequently, natural organelles such as mitochondria and peroxisomes were used to construct biocatalytic biosensors for the detection of amino acids and herbicides\textsuperscript{20}. F. Schubert et. al. reported the construction of an aniline sensor using rabbit liver microsomes containing cytochrome P-450 as the biocatalyst. The microsomes were fixed to a rotating glassy carbon electrode and the quantitation of para amino aniline was accomplished by monitoring the enzymatically produced quinoneimine at + 0.25 V vs SCE\textsuperscript{21}.

An elegant extension of the area of biocatalytic biosensors is the use of tissue sections from animal sources as the biocatalytic materials. Such tissues already contain all the necessary enzymes and co-factors in an environment optimised by nature. This approach has
the advantage of extending the biocatalytic concept to a wide range of organisms or materials. Furthermore, natural structural properties of tissues provide a more robust and coherent biocatalytic materials for the construction of biosensors. In 1978, the first bioselective membrane electrode (figure 1) was made with intact animal tissues by G.A. Rechnitz. It utilized both beef liver tissue and isolated urease enzyme to mediate the conversion of amino acid, arginine into ammonia as follows.

\[
\text{Arginine} \rightarrow \text{ornithine} + \text{Urea}
\]

\[
\text{Urea} \rightarrow \text{CO}_2 + \text{NH}_3
\]

The quantitation of arginine was based on the production of ammonia detected by ammonia electrode. In spite of the limited utility of this hybrid arrangement, it effectively demonstrated the concept of using intact tissue slices as biocatalysts in construction of biosensors.

In subsequent years, mammalian tissues such as porcine kidney, rabbit liver, rat intestine utilized in construction of biosensors. Perhaps the best studied biocatalytic system is the glutamine biosensor with different biocatalytic materials (all contain the enzyme; Glutaminase) such as isolated enzymes, bacteria, mitochondria and porcine kidney. From these studies,
Figure 1. Schematic diagram of the first bioselective membrane electrode probe: a, dialysis membrane; b, bovine liver tissue slice; c, uricase enzyme suspension; d, gas permeable membrane; e, internal electrolyte; f, combination pH electrode; g, plastic electrode body
the advantages of using tissues (in the natural environment) were clearly demonstrated in terms of low cost, superior mechanical and time stability and ease of biosensor construction for the case of glutamine measurements. Intact tissue electrodes are very advantageous in situations where isolated, purified enzymes are not readily available, are unstable, are too costly or require cofactors for proper biocatalytic activity.

The latest advance in the development of biocatalytic biosensors is the discovery that plant tissue materials can be utilized as effective biocatalysts. In 1981 Kuriyama and Rechnitz reported the first successful use of intact plant tissues (meso carp tissue of yellow squash) in the construction of a bioselective potentiometric membrane electrode for the detection of glutamate.

Plants have biochemical pathways which are different from those found in animal tissues. Due to this reason, broad range of analytes can be detected with plant tissue-based biosensors. This class of biosensors could be used not only as analytical sensors for exogenous analytes but also to study inherent plant biochemistry and physiology.
1.5 PLANT TISSUE BASED-BIOSSENSORS

After the discovery that the squash tissues can be utilized successfully in construction of a biosensor for the detection of glutamate, a vast amount of research work has been focused on this area. Reviews of such plant tissue based-biosensors have appeared recently\textsuperscript{25}. A few examples of plant tissue based-biosensors reported in the literature will be presented in the following section.

In 1984, Smith and Rechnitz reported a plant tissue-based biosensor for the detection of cysteine using cucumber leaves. For the sensor construction, cucumber leaves were held at potentiometric ammonia gas sensing electrode. The concentration of cysteine present in the sample was proportional to the production of ammonia at the electrode tip\textsuperscript{26}.

Many fruits and vegetables are rich in enzymes, hence they can be utilized in biosensor construction. Sidwell and Rechnitz reported the "bananatrode" for the detection of dopamine, using banana fruit as the biocatalyst\textsuperscript{27}. Banana is rich in the enzyme polyphenol oxidase, and catalyzes the conversion of dopamine into its quinone derivative, in a process which consumes oxygen. Thus by coupling a thin slice of banana pulp tissue with a Clark type oxygen electrode, they could construct a biosensor for the detection of dopamine where the biocatalytic consumption of the oxygen was detected by the oxygen
electrode and was directly proportional to the dopamine concentration in the solution.

Another commonly found enzyme in plants is ascorbic acid oxidase which catalyzes the oxidation of ascorbic acid into dehydro ascorbic acid. In 1985, Vincke and co-workers reported L-ascorbic acid membrane electrodes based upon the use of four types of biocatalytic materials immobilized at an oxygen electrode. Biocatalysts studied involved isolated ascorbic acid oxidase in soluble form and in a covalently bound matrix, as well as cucumber peel, and bacterial cells (Enterobacter agglomerans). The evaluation of these various biocatalysts indicated that the cucumber peel-based biosensor is more suitable for analytical determination of vitamin C.

In 1983, Schubert and co-workers reported sugar beet-tissue based biosensor for the detection of tyrosine utilizing an oxygen electrode. Tyrosine was detectable in the concentration range of 20 μM to 900 μM using this sensor.

When the natural biocatalytic activity does not involve a consumption or liberation of materials compatible with electrochemical detection, an auxiliary step may be introduced. Such an approach was taken by Schubert and co-workers to construct a biosensor for the detection of inorganic phosphates and fluorides where potato (Solanum tuberosum) tissue slice and immobilized
glucose oxidase were coupled with an oxygen electrode. The measurements were based on the inhibition of the acid phosphatase of potato tissue by fluoride and inorganic phosphates. This hybrid sensor was used for the detection of phosphate ions in fertilizer and urine.\textsuperscript{31}

1.6 RESPONSE CHARACTERISTICS OF TISSUE BASED BIOSENSORS UTILIZING GAS SENSORS

Most of the early tissue-based biosensors utilize gas sensing devices where the biocatalytic layer is physically retained on the sensor tip with a supportive membrane. In this arrangement (figure 1) the biocatalytic and sensing site are spatially separated. Furthermore, the optimum thickness of the tissue slice reflects a compromise between mechanical stability and response time. Therefore, the slow response time of these devices is a severe drawback. The electrode response of biocatalytic membrane electrodes is based upon the steady-state response where the influx of measurable products is exactly counter balanced by the efflux at the electrode surface. This will give rise to a constant potential or current. In 1983 Hameka and Rechnitz reported a theoretical treatment for the mechanism of tissue-based sensors with an emphasis on the time dependent approach to the steady state potential. They concluded that the biocatalytic layer thickness and the effective solution-
substrate diffusion constant are important factors in determining electrode response times\textsuperscript{32}. 

1.7 RECENT DEVELOPMENTS OF PLANT TISSUE-BASED BIOSENSORS

Although early tissue based biosensors were basically constructed by utilizing potentiometric and amperometric gas sensing devices, modern electroanalytical techniques such as steady-state and hydrodynamic amperometry (FIA), voltammetry, electrochemical detection in liquid chromatography (LCEC) have recently been utilized in plant tissue-based construction. Therefore, in the context of biosensors it is appropriate to provide some fundamental description of these electroanalytical techniques.

1.7.1 Voltammetry

Voltammetry involves the sampling of the faradaic current over a range of potentials using different potential programs (wave forms). Voltammetric techniques can be categorized or named on the basis of the potential program involved such as linear sweep, normal pulse, differential pulse and cyclic voltammetry, etc. A three electrode system together with a potentiostat is used to control the potential of the working electrode. A stable reference electrode such as Ag/AgCl or saturated calomel (SCE) electrode is required for the proper control of the applied potential. Voltammetric experiments are of primary importance as a mean of characterizing a
biosensing system with respect to optimum operational potential\textsuperscript{33}.

\subsection*{1.7.2 Amperometry}

Amperometric detection has found wide applications in measurements of biologically important compounds. Under favorable conditions, detection limits of $10^{-8}$ M to $10^{-9}$ M and a linear dynamic range of $10^{-3}$ M to $10^{-7}$ M are achievable. Amperometry measures the current generated due to a redox reaction at the working electrode where a controlled potential is applied between reference electrode and the working electrode using an auxiliary electrode. The potential of the working electrode is controlled by a potentiostat. The measured current is directly related to the rate of the electrochemical reaction occurring at the working electrode. The rate of heterogeneous electron transfer occurring at the electrode can be controlled through the applied potential\textsuperscript{35}.

Amperometric devices provide a linear relationship between the measured current and the concentration of an analyte as opposed to the logarithmic relationship (potential Vs Concentration) observed in potentiometry. Amperometric biosensors are superior to potentiometric devices in terms of linear dynamic range, fast response time and sensitivity. However, poor selectivity, and electrode fouling are inherent limitations of amperometric
detection. The selectivity problems of amperometry can be circumvented by coupling amperometry with high performance liquid chromatography.

1.8 ELECTRODE MATERIALS IN BIOSENSOR CONSTRUCTION

The selection of a suitable working electrode material is important in the monitoring of a desired electrochemical reaction in an amperometric or voltammetric experiment. The achievable potential range or limit (potential window) for an electrochemical reaction depends upon the nature of the electrode material as well as the solvent system. Different electrode materials are available for a variety of electrochemical applications.

Mercury electrodes have commonly been used for redox reactions involving cathodic potentials due to their high overpotential for hydrogen reduction. However analytical biosensors based on Hg electrodes are very rare due to cumbersome experimental conditions and incompatibility with biological materials. Solid electrodes for biosensor construction include carbon paste, glassy carbon and platinum, amongst other element.

Carbon paste electrodes exhibit a wide potential range from +1.0 V to -1.0 V in aqueous media, and have low background current especially in the oxidative direction. Carbon paste electrodes are made by mixing an appropriate proportions of carbon powder and dielectric binder such as
mineral oil and packing them at the end of an electrode body. The solubility of the binder in organic liquids and the irreproducibility of the electrode surface are the major drawbacks of carbon paste electrodes.

Glassy carbon electrodes have significant advantages over conventional carbon paste electrodes. The advantageous characteristics of glassy carbon electrode include low background current, surface reproducibility, high solvent resistance, wide potential window (-1.3 V to +1.5 V) and long term stability. A major drawback of the glassy carbon electrode is its sensitivity of the electron transfer rate to the surface condition of the electrode. Metallic electrodes such as platinum are not as widely accepted as carbon electrode for biosensor construction because these electrodes exhibit high background current due to the formation of surface oxides.

1.9 AMPEROMETRIC DETECTION IN FLOW INJECTION ANALYSIS

Flow injection analysis (FIA) is based on the concept of controlled dispersion which allows the reproducible development and subsequent chemical reaction on an injected sample zone. The sample plug on injection disperses into carrier stream which is propelled by an appropriate pump, with concomitant dispersion of the reagent into the sample zone. Through this mutual dispersion, a sample profile is developed which is unique
to the FIA system employed. The magnitude of the response curve for a sample is a linear function of the sample concentration. Figure 2 illustrates a single line FIA manifold. FIA is a simple, automated microchemical technique capable of having a high sample rate and a minimum reagent consumption\textsuperscript{36,37}.

When an electrochemical detector (amperometric) is used in conjunction with FIA (EC-FIA), it is an example of hydrodynamic amperometry where the current is measured at a fixed potential under stirred solution conditions. EC-FIA involves controlled or restricted diffusion because the electrolytic solution is confined to the electrode surface. EC-FIA often results in a peak representation in contrast to wave representation in steady-state amperometry due to rapid increase and decrease in current of a selected redox species\textsuperscript{38}.

1.10. AMPEROMETRIC DETECTION IN LIQUID CHROMATOGRAPHY

Liquid chromatography with electrochemical detection (LCEC) has become a very powerful and selective tool for the trace determination of electro active compounds. Modern interest for electrochemical detection in liquid chromatography was stimulated with the finding that LCEC could be used to analyze aromatic metabolites in the mammalian central nerve system. Liquid chromatography and hydrodynamic electrochemistry are very compatible
Figure 2. (a) A single line flow injection analyzer system: P, pump; D, flow cell; S, Injection port; W, waste; (b) Analog output
techniques. These are the two most widely used analytical tools, and both techniques involve heterogeneous process with mass transfer and hydrodynamics. Partitioning of a solute between stationary phase and mobile phase and, the restricted diffusion electrochemistry are fundamental principles involved in liquid chromatography and electrochemistry respectively\textsuperscript{39}. In contrast to other liquid chromatographic detectors, electrochemical detection involves a chemical transformation at the electrode surface. Since it involves electron transfer, the reaction can be monitored by measuring the current as a function of time. The measured current is directly proportional to analyte concentration. The resolution of the column provides much of the selectivity in LCEC. The most recent development of LCEC has been the exploration of amperometric detection with different surface modified electrodes\textsuperscript{40}. Biological surface tailoring of the electrodes for liquid chromatographic detection has been reported with isolated enzymes\textsuperscript{41} and plant tissue materials\textsuperscript{42} along with thin layer amperometric detection.

1.1.1 MEDIATED AMPEROMETRIC BIOSENSORS

Difficulties in achieving direct catalytic electron transfer between enzymes and electrodes have encouraged the use of electroactive mediators to enhance the rate of the electron transfer\textsuperscript{43}. The role of the mediator is to
shuttle electrons efficiently between electrode and enzyme as illustrated in figure 3. In recent years ferrocene, bis(5-cyclopentadienyl)iron (FeCP₂) and its derivatives have been used as mediators. These compounds in their oxidized form, ferricinium ions, will act as electron acceptors for a number of flavoproteins including oxidases and dehydrogenases\(^{44}\). The solubility and the redox potentials of ferrocenes are particularly advantageous for using them as mediators in amperometric biosensors. The most successful application of ferrocene as a mediator has been in an amperometric enzyme electrode for the detection of glucose\(^{45}\). This system is independant of the oxygen concentration since the ferricinium ion replaces oxygen as the cofactor for glucose oxidation by glucose oxidase. Such a replacement approach has several advantages. First of all, it can overcome the problems associated with variable response characteristics of the sensor resulting from the variation of oxygen tension. Secondly, Due to the low redox potential of the mediator, a selected sensor can be operated at very low positive potentials. Therefore, electrochemical interferents commonly encountered in clinical samples, such as uric acid, can be minimized or eliminated. The detection of hydrogen peroxide has been achieved using ferrocene monocarboxylic acid as a mediator together with the enzyme peroxidase at a much lower positive potential than that required for the
Figure 3. Schematic representation of a mediated electron transfer reaction. $E^{(\text{ox/red})}$, redox forms of the enzyme; $\text{MED}^{(\text{ox/red})}$, redox forms of the mediator.
direct electrochemical detection of hydrogen peroxide. The use of alternative mediators other than ferrocene and its derivatives has been reported to enhance the electron transfer between enzymes and electrodes. These include benzoquinone, hexacyanoferrate and phenazine ethosulfate. However, in general these species do not have the versatility and favorable characteristics offered by ferrocene.

1.12 ENZYME KINETICS AND AMPEROMETRIC ANALYSIS

The general principles of chemical-reaction kinetics apply to the enzyme-catalyzed reactions. But the distinctive feature of enzymatic catalysis in contrast to noncatalytic reaction is substrate saturation. All enzymes exhibit the property of saturation but they vary widely with respect to the substrate concentration required to produce saturation. This saturation effect led some early investigators, particularly A.J. Brown and also V. Henri, to the hypothesis that the enzyme and substrate react reversibly to form a complex, as an essential step in the catalyzed reaction.

In 1913 L. Michaelis and M.N. Menten developed a general theory of enzyme action and kinetics. This theory provides the basis for the quantitative analysis of all aspects of enzyme kinetic and inhibition.
The Michaelis-Menten equation is the rate equation for a one-substrate enzyme-catalyzed reaction;

$$V_0 = V_{\text{max}} [S]/K_M + [S]$$

Where,

- $V_0$ = Initial reaction velocity
- $V_{\text{max}}$ = Maximum initial velocity
- $K_M$ = Michaelis-Menten constant
- $[S]$ = Substrate concentration

The Michaelis-Menten constant is equal to the substrate concentration at which the initial reaction velocity is half maximal. $K_M$ has the dimensions moles per liter and, is independent of the enzyme concentration. $K_M$ is not a fixed value but may vary with the structure of the substrate, with pH and with temperature.

The Michaelis-Menten equation can be algebraically transformed into experimentally more useful forms. One of these forms is the Line Weaver-Burk equation;

$$1/V_0 = (K_M/V_{\text{max}}) (1/[S]) + 1/V_{\text{max}}$$

(All the terms include in this equation are as same as those of Michaelis-Menten equation.)

Kamine and Wilson reported a model to explain the kinetic performances and the characteristics of the immobilized enzyme layer (glucose oxidase) using rotating ring disk voltammetry where the enzyme glucose oxidase was immobilized at the disk surface. An ideal situation for investigating surface bound biocatalysis requires that the
rate of the mass transport of a substrate to the catalytic surface be reproducible and well defined. Rotating disk voltammetry fulfills these requirements and can be used to study the kinetic behavior of an immobilized enzyme.

Kamin and Wilson's model assumes that the immobilized enzyme layer on the rotating disk surface obeys Michaelis-Menten kinetics. If the rate of the enzymatic reaction is catalytic controlled, apparent Michaelis-Menten constant \( K'_M \) can be determined amperometrically by using the following "Lineweaver-Burk type" equation:

\[
\frac{1}{i_{ss}} = \left( \frac{K'_M}{i_{max}} \right) \frac{1}{C} + \frac{1}{i_{max}}
\]

Where,

- \( i_{ss} \) = Steady-state current
- \( C \) = Concentration of the substrate
- \( i_{max} \) = Current at substrate saturation
- \( K'_M \) = Apparent Michaelis-Menten constant

since a plot of \( 1/i_{ss} \) vs \( 1/C \) results in a straight line with the slope equal to \( K'_M/i_{max} \) and intercept equal to \( 1/i_{max} \).

The kinetic parameters such as \( K_{cat} \) (turn over number) and \( K_M \) are obviously important in determining the properties of biocatalytic sensors. Michaelis constant \( K_M \) appears to affect linear range of biocatalytic biosensors. It is generally desirable to have relatively high specific constant value; \( K_{cat}/K_M \) for a particular analyte compared to the other potential substrates.
1.13 MIXED PLANT TISSUE-CARBON PASTE BIOELECTRODES

In 1988 J. Wang and M.S. Lin reported a novel approach for the construction of plant tissue-based biosensors by employing the new concept of coupling biocatalytic sensing with the technology of chemically modified electrodes\(^{49}\). The sensor was fabricated by incorporating the biocatalyst, banana pulp into the conventionally prepared carbon paste matrix. In this design, the biocatalyst was an integral part of the sensing element. Thus it eliminates the long diffusion path commonly encountered with early tissue electrodes. Consequently, this novel approach provides very fast response times. At the carbon paste electrode surface, dopamine was converted into dopaquinone by banana polyphenol oxidase. Amperometric reduction of quinone was monitored using a flow injection analysis system. The response time was as fast as 12 seconds.

A Yeast-based carbon paste bioelectrode for the detection of ethanol was reported by Kubiak and Wang. The response mechanism was based on the activity of alcohol dehydrogenase present in yeast\(^{50}\). The enzymatically produced NADH was monitored via mediated electron transfer with hexacyanoferrate(III) ions. The resulting amperometric current was quantitatively related to the concentration of ethanol present in the solution. The applicability of the sensor to ethanol detection in
alcoholic beverages was demonstrated. They further used the same biocatalytic system to construct a polishable and robust bioelectrode surface using epoxy-bonded graphite where yeast was immobilized\textsuperscript{51}.

Wang et. al. further reported a series-flow-thin layer dual electrode system consisting of a tissue reactor and a carbon paste collector electrode\textsuperscript{52}. The tissue reactor generates electroactive species which are hydrodynamically swept and detected amperometrically at the carbon paste collector electrode placed downstream. This novel concept was demonstrated using horse radish root, banana, and mushrooms as biocatalysts for the detection of hydrogen peroxide, dopamine and catechol respectively. Mottolo and co-workers further extended the use of series dual electrode approach for the detection of phenol using a tissue reactor and a collector electrode. A carbon paste electrode modified with hexacyanoferrate (a redox mediator) functioned as the amperometric collector electrode and the enzyme reactor contained the mushroom tissue as the biocatalyst\textsuperscript{53}.

In 1990, M.P. Connor et. al. reported a novel electrochemical detection scheme for liquid chromatography (LCEC), utilizing rapid responding plant tissue-based carbon paste electrodes\textsuperscript{54}. This approach adds a new dimension of selectivity to liquid chromatographic separation based on the substrate specificity of the
enzyme present in the tissue in question. This novel concept was demonstrated using banana, mushroom and yeast-modified carbon paste electrodes for the detection of dopamine, phenols and ethanol respectively. The applicability of this novel LCEC system for the analysis of dopamine and ethanol in urine was also demonstrated. The compatibility of tissue materials as well as electrode materials with the chromatographic mobile phase should be carefully considered when such a detection scheme is designed. However, it is well established that some enzymes such as tyrosinases, are capable of catalyzing reactions in organic solvents. For such instances, plant tissue-based electrochemical detection for liquid chromatographic separation is a very attractive approach.

An application of tissue-based electrodes aimed at eliminating interferences from co-existing electroactive components was described by Wang et. al. Zucchini tissue, which contains ascorbic acid oxidase, modified carbon paste electrodes ("eliminator electrode") were employed for the effective depletion of ascorbic acid from electrode surface. This approach resulted in a selective detection of dopamine at the electrode surface without any ascorbic acid interferences.
1.14. RESEARCH OBJECTIVES

The objective of the first project (chapter two) is to construct a novel amperometric biosensor for the detection of catechol. An electrochemical technique used in the sensor construction is steady-state amperometry and the eggplant fruit functions as the selective biocatalyst. Eggplant (fruit) is capable of converting catechol into catechol quinone. This is due to the presence of enzyme, polyphenol oxidase. Electrochemical signals result from the reduction of catechol quinone produced at the electrode surface. This study demonstrates how biological components could be incorporated intimately into a sensing site. This is in contrast with the early tissue based-biosensors where tissue slices were physically retained on a gas permeable membrane of a gas sensing device. Analytical features of the sensor are explored with respect to biocatalytic loading, linear dynamic range, sensitivity, selectivity, response time and time stability.

The possibility of using tin oxide electrodes as alternative electrode material for the construction of plant tissue-based biosensors is addressed in the second project (chapter 3). The biocatalytic system described in project one is used to construct an amperometric flow-through detector for flow injection analysis. The construction of the tin oxide flow-through detector and
its analytical performances with regard to sensitivity, selectivity, linear dynamic range and sample throughput are investigated for the case of catechol measurements. Furthermore, its bioanalytical performances are compared with those of tissue reactor/carbon paste collector dual electrode configuration.

The first use of in vitro cultured plant tissues in biosensor construction is described in the third project (chapter four). Specifically, in vitro cultured tobacco callus tissue is utilized as the biocatalyst to construct an amperometric biosensor for the detection of hydrogen peroxide. Peroxidase present in the callus tissue catalyses the reduction of hydrogen peroxide into water. The enzymatic catalysis is monitored electrochemically through a mediated electron transfer reaction using ferrocene. In this study tobacco callus tissue-based sensor operates at a potential of 0.0 V vs Ag/AgCl, a result of the low formal potential of the ferrocene/ferricinium couple. The isolation of biocatalytic preparations from plants with high reproducibility and activity for biosensor applications has been difficult in the past. The novel concept described in this project demonstrates that cultured plant tissues can improve the performances of tissue-based electrodes. Such an approach addresses early limitations of tissue-based biosensors and thus, provides a procedure
for development and implementation of selective biocatalytic material with good practical potential.

The possibility of using genetically transformed (transgenic) plants; specifically transgenic potato (Solanum tuberosum) and transgenic tobacco (Nicotiana tabacum), in the construction of an amperometric biosensor for the detection of phenyl-B-glucuronide is attempted in the fourth project (chapter five). This is a novel concept because the use of recombinant DNA technology in tissue-based biosensor construction has never been reported.
CHAPTER 2

EGGPLANT BASED BIOAMPEROMETRIC SENSOR FOR THE DETECTION OF CATECHOL

2.1 BACKGROUND

Catechol finds important uses in the manufacture of drugs, dyes and rubber and, as an antioxidant for lubricating oils\textsuperscript{57}. Catechols are also found in urine as well as in cigarette smoke. Furthermore, catechol related compounds such as catecholamine neurotransmitters play a vital role in central nerve system. The selective measurement of catecholamine neurotransmitters without any interferences from co-existing electroactive compounds in the brain extracellular fluid is a major research challenge in electroanalytical chemistry\textsuperscript{58}.

2.2 QUANTITATIVE ANALYSIS OF CATECHOL AND RELATED COMPOUNDS

Existing methods of catechol analysis are based on color reactions of phenolic compounds with a number of oxidants and complexing reagents\textsuperscript{59}. By their very nature, none of these methods is specific for catechol. Analysis thus requires a preceding separation of catechol from other phenolic compounds.

Biosensors provide a very selective detection method for catechol. Biosensors for the detection of catechol and related compounds has been reported based on the
isolated enzymes such as catechol 1,2-oxygenase [EC. 1. 13. 1. 1] 57. Construction of plant tissue-based biosensors for the detection of catechol and its related compounds has received much attention in the past. Most of the early plant tissue-based biosensors utilized a Clark type oxygen electrode 60,61. A new approach for the detection of this particular class of compounds involving the incorporation of biocatalytic materials into a carbon paste matrix was reported by Wang et. al. 49. Mottola and co-workers reported on compact bioamperometric phenol sensors incorporating the enzyme tyrosinase along with hexacyanoferrate ions (electrostatically immobilized on poly (4-vinylpyridine) into a carbon paste matrix 53. These new biosensor designs for the detection of catechol and related compounds are superior to early biosensors based on Clark-type oxygen electrodes.

The construction of a plant tissue-based amperometric biosensor for the detection of catechol based on eggplant (Solanum melangena) tissue as the selective biocatalyst will be presented in the following sections.

The eggplant tissue contains polyphenol oxidases 62,63. Phenol oxidases are copper proteins which catalyze the aerobic oxidation of certain phenolic substrates to quinones (figure 4). Although polyphenol oxidases are widely distributed among different plant tissues, each
individual enzyme tends to catalyse the oxidation of one particular phenolic substrate more readily than others. The eggplant polyphenol oxidases have a high activity toward catechol and catalyze the oxidation of catechol to its o-quinone as a primary product. The o-quinone produced can be monitored amperometrically and quantitatively related to the concentration of catechol (figure 4). This is the basis for the proposed sensor, which will be shown to exhibit high biocatalytic activity, selectivity, good stability, and fast response times.

2.3 EXPERIMENTAL

2.3.1 Apparatus

All amperometric measurements were made with a cyclic voltammograph (Bioanalytical systems, Inc.). An eggplant-modified carbon paste working electrode, a saturated calomel reference electrode (SCE) and a Pt wire counter electrode were used as the three-electrode system (figure 5). The amperometric responses were recorded on an Omniscribe series D 5000 strip chart recorder (Houston instruments). A Haake Model FS water bath circulator was employed to thermostat the experimental cell at 29 \( \pm \) 0.5°C. Amperometric detection of biocatalytically generated o-quinone was accomplished by applying a
Figure 4. Schematic diagram of eggplant electrode construction and biocatalytic reaction at the electrode surface
Figure 5. Experimental set up for steady-state amperometry
constant potential of -0.2 V (vs SCE) and allowing the background current to decay to a steady state value.

2.3.2 Reagents

All the solutions were prepared with distilled deionized water. All chemicals, including catechol and the chemicals used for interference studies, were purchased from Sigma Chemical Company. The stock solutions of catechol were prepared daily in distilled deionized water. The eggplants used for this study were purchased from a local grocery store and stored at 40 C until used.

2.3.3 Construction of Eggplant-Modified Carbon Paste Electrodes

The construction of the working electrode was carried out according to a previously described procedure49. The skin of the eggplant fruit (not the stem side) was peeled off and the immediate layer below the skin was used to make the electrode (Figure 4). A 0.2 g (9 % by weight) of the tissue was ground and mixed with 0.9 g (41 %) of mineral oil (Aldrich), then 1.1 g (50 %) of graphite powder (grade # 38 Fisher) was added and mixed thoroughly. This eggplant-modified carbon paste was packed at the end of an electrode body of 2.5 mm diameter. The electrical connection was provided by a copper wire.
2.3.4 Procedure

0.1 M phosphate buffer was used for pH optimization. The response of the eggplant sensor at 1.5 \times 10^{-5} \text{ M} catechol as a function of pH is shown in figure 6. The pH optimum of this sensor was found to be 7.0; consequently all the experiments were carried out in pH 7 buffer. The observed optimum pH was similar to that reported previously for the same enzyme in eggplant.\textsuperscript{65}

Calibration of the eggplant sensor was done with respect to catechol over the concentration range of 5.0 \times 10^{-6} \text{ M} to 5.5 \times 10^{-5} \text{ M}. Compounds having structural similarities to catechol were studied as interferences at a concentration of 1 \times 10^{-4} \text{ M} in the 0.1 \text{ M}, pH 7 phosphate buffer. The steady state current response of each compound was compared to that of catechol. Calibration curves were also constructed with the compounds which showed positive responses in the interference study.

The response time of the sensor to reach 90\% of maximum response (t-90) was calculated from the response-time curve at a concentration of 1 \times 10^{-4} \text{ M} catechol.

The same variety of eggplant (long eggplant) was obtained from both regular and organically grown sources. Parallel studies of sensors constructed with these two kinds of tissue were carried out with respect to biocatalytic activity and sensor lifetime.
Figure 6. pH dependence of eggplant sensor at $1.5 \times 10^{-5}$ M catechol
2.4 RESULTS AND DISCUSSION

2.4.1 Calibration of the Eggplant Sensor

The eggplant sensor produced a stable base line response after time (10-15 min) was allowed for the background current to decay. Steady-state responses of the sensor with successive injections of catechol (in 5 \( \mu \text{M} \) steps) are illustrated in figure 7. Furthermore, the responses of blank carbon paste electrodes (without eggplant tissue) were recorded over the same range of catechol concentrations. Current produced were found to be insignificant compared to those of the eggplant modified electrodes. The calibration plots (Figure 8) obtained for catechol with the eggplant sensor showed a linear response over a concentration range from \( 5.0 \times 10^{-6} \) M to \( 4.5 \times 10^{-5} \) M with a lower detection limit of \( 1 \times 10^{-6} \) M (based on a signal to noise ratio of 3). The slope of the calibration curve for catechol was \( 5.2 \times 10^3 \) \( \mu \text{A}/(\text{mol L}^{-1}) \) and is a measure of the sensitivity of the sensor for catechol. The relative standard deviation was 1.1 \% for seven measurements of \( 1.5 \times 10^{-5} \) M catechol. The sensor also exhibits a response for dopamine and L-dopa with linear ranges from \( 5.0 \times 10^{-5} \) M to \( 2.5 \times 10^{-4} \) M and from \( 1.0 \times 10^{-4} \) M to \( 6.0 \times 10^{-4} \) M respectively (Figure 8). The slopes of the calibration curves of dopamine and L-dopa were \( 1.05 \times 10^3 \) \( \mu \text{A}/(\text{mol L}^{-1}) \) and \( 1.95 \times 10^2 \) \( \mu \text{A}/(\text{mol L}^{-1}) \), respectively.
Figure 7. Steady-state amperometric responses of eggplant sensor for catechol. Applied potential; -0.2 V vs SCE, Electrolyte, 0.1 M phosphate buffer (pH 7)
Figure 8. Calibration curves obtained with the eggplant sensor in 0.1 M phosphate buffer (pH 7)
2.4.2 Selectivity of the Eggplant Sensor

Table 1 illustrates the selectivity of the proposed sensor for catechol where the steady-state current of the each interference over that of catechol, \( \frac{i_p(\text{int})}{i_p(\text{cate})} \), was used as a measure of the selectivity. The current responses observed for dopamine and L-dopa were smaller than that of catechol. L-dopa has been tested in addition to catechol as a substrate for eggplant polyphenol oxidase in an enzyme preparation and found to be active. Nevertheless, eggplant polyphenol oxidases are not active toward monophenols. It is interesting to note that some compounds, e.g. 3,4-dihydroxyphenyl acetic acid, 3,4-dihydroxyphenylglycol, serotonin, epinephrine norepinephrine, L-tyrosine and phenol, which have structural similarities to catechol, did not respond at all. This could be due to differences in \( K_m \) values. The reported average \( K_m \) values of catechol and L-dopa for the eggplant polyphenol oxidase are \( 4.0 \times 10^{-3} \) M and \( 5.6 \times 10^{-3} \) M respectively.

Interestingly, the sensor showed a response to \( 1 \times 10^{-4} \) M L-ascorbic acid (Table 1) but with a much lesser sensitivity than some of the other substrates (Figure 8).
Table 1. Comparison of Sensor Response at Steady-State to structurally Similar Compounds (all at $1 \times 10^{-4}$M)

<table>
<thead>
<tr>
<th>Compound</th>
<th>$i_p$ ($\mu$A)</th>
<th>$i_p$(int)/$i_p$(cate)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>catechol</td>
<td>0.15</td>
<td>1</td>
</tr>
<tr>
<td>dopamine</td>
<td>0.09</td>
<td>0.59</td>
</tr>
<tr>
<td>L-dopa</td>
<td>0.02</td>
<td>0.13</td>
</tr>
<tr>
<td>L-ascorbic acid</td>
<td>0.02</td>
<td>0.13</td>
</tr>
</tbody>
</table>

* $i_p$(int) = Steady-state current of each interference
  $i_p$(cate) = Steady-state current of catechol

Therefore ascorbic acid would not pose a significant interference for the detection of catechol with this sensor. However, in order to explore the effect of L-ascorbic acid, catechol (bulk concentration = $1 \times 10^{-4}$ M) was injected in a solution containing $1 \times 10^{-4}$ M L-ascorbic acid. L-ascorbic acid did not diminish the steady response of catechol to any significant extent. In order to further investigate the response to L-ascorbic acid, an amperometric experiment was carried out with a plain carbon paste electrode (without eggplant tissue) under similar experimental conditions but with commercially available tyrosinase [EC 1.14.18.1] dissolved (0.1 mg/ml) in the electrolyte. L-ascorbic acid did not give a response with tyrosinase as it did with the
eggplant tissue. In addition, cyclic voltammetry experiments were carried out with $1 \times 10^{-2}$ M L-ascorbic acid (in the working buffer) with both plain carbon paste and eggplant modified carbon paste electrodes. The potentials were scanned from +0.5 V to -0.5 V with the scan rates of 10 mV/s. The cyclic voltammetric behavior of ascorbic acid was essentially the same with both electrodes. This may be due to the lower sensitivity of the cyclic voltammetric technique. In any event, the small response to ascorbic acid is of negligible analytical consequence for catechol measurements with the proposed biosensor.

2.4.3 Response Time of the Eggplant Sensor

The steady-state response time (t-90) of this sensor was found to be approximately 18 seconds. This very rapid response is one of the major advantages for the proposed catechol sensor.

2.4.4 Biocatalytic Loading of the Eggplant Sensor

The effect of the composition of the tissue in the carbon paste upon the response toward catechol was tested by varying the percentage of the tissue from 6% to 15%. At 6% tissue, the sensor gave a negligible response. The sensor exhibited more or less similar response in the range of 9% to 15% tissue. Thus, 9% tissue in the carbon paste matrix represent a reasonable composition.
2.4.5 Variation of the Biocatalytic Activity and the Time Stability upon the Source of Eggplant

The biocatalytic activity of organically grown eggplant was lower but the lifetime was longer than those of the regularly grown eggplant. The lifetime studies of the eggplant modified carbon pastes are illustrated in Figure 9 and figure 10. The paste was active for at least twenty three days when stored at 4°C. However, the overall lifetime of the sensor is also related to the "freshness" of the eggplant used as the source of the tissue. Further, the moderate decrease in the response of the paste with time may be related to the aging effect of the tissue in the carbon paste upon storage.

2.5 CONCLUSIONS

The described eggplant-based bioamperometric sensor is very selective and sensitive toward catechol and showed a linear response over the concentration range from 5.0 x 10^-6 M to 4.5 x 10^-5 M with a lower detection limit of 1 x 10^-6 M. The eggplant modified carbon paste used in this study was stable for 23 days when it was stored at 4°C. The steady-state response time of the sensor was 18 seconds. The fast response time observed with the sensor can be attributed to the proximity between the biocatalytic site and the sensing site. The fast response time of this biosensing device clearly demonstrates the
Figure 9. Time stability of carbon paste modified with regularly grown eggplant.
Figure 10. Time stability of carbon paste modified with organically grown eggplant
potential use of this system in flow situations such as flow injection analysis. Such an approach is addressed in chapter three. Furthermore, this study clearly indicates the dependence of bioanalytical performances of tissue electrodes, (especially, sensitivity and lifetime) upon the source of the biocatalytic material. This is one of the inherent limitations of biocatalytic sensors based on plant tissues. One way of addressing this limitation, using in vitro cultured plant tissues, is presented in chapter four.
CHAPTER 3

USE OF TIN OXIDE ELECTRODES IN FLOW INJECTION ANALYSIS
WITH APPLICATION TO PLANT TISSUE BASED BIOSENSORS

3.1 BACKGROUND

The use of electrochemical detectors for monitoring electroactive species in flowing streams, such as flow injection analysis (FIA)\textsuperscript{66,67} and liquid chromatography\textsuperscript{39}, has received considerable attention in recent years. Such detection schemes offer excellent sensitivity, selectivity, improved stability, and wide dynamic range with relatively low cost for instrumentation. These detectors meet the requirements of applied laboratories where a large number of samples are efficiently analyzed.

3.2 SURFACE MODIFIED ELECTRODES FOR DETECTION IN FLOWING STREAMS

The use of surface modified electrodes as detectors in flowing streams is a very attractive approach because they provide the opportunity for tailoring the electrode surface to meet specific detection needs such as selectivity, sensitivity, and decreased electrode fouling. Most of the research work in this area has focused on electrodes modified with electro catalytic surfaces\textsuperscript{68} and permeselective coatings\textsuperscript{69}.
Electrodes modified with biological entities have been used as detectors for flow injection analysis. The amperometric detection of glucose using a flow injection system with a glucose oxidase-modified graphite paste electrode has been reported\textsuperscript{38}. Mixed plant tissue-carbon paste bioelectrodes have also been utilized in flow injection analysis. Thin layer electrode configurations used in these flow systems could be either single electrode designs\textsuperscript{49} or dual configurations consisting of a bioreactor and an electrode collector pair\textsuperscript{52,53}. Electrode designs containing plant tissue materials have been shown to be suitable for monitoring the dynamic changes in concentration that characterize the flow injection system. Furthermore, HPLC detection of amino acids has been reported by Yao et al. where an amino acid oxidase modified, Pt electrode was used as an amperometric detector\textsuperscript{41}. The utility of plant tissue materials (in a carbon paste matrix) as an integral part of an electrochemical detector in liquid chromatography (LCEC) for the analysis of biologically important compounds has been reported very recently\textsuperscript{54,70}. According to these reports, the presence of "class selective" enzymes in tissues is advantageous for LCEC operations.

52
The choice of electrode material is more critical in LCEC than for many electroanalytical experiments, primarily due to the requirement of mechanical ruggedness and long term stability. Although carbon paste still remains a popular choice as an electrode material for LCEC, it will neither stand up to most nonaqueous solvents, nor it will work well under the high flow rates used in recent chromatographic separations. Furthermore, carbon paste specifically adsorbs certain organic compounds\textsuperscript{71}. Therefore, its use can, in some instances, lead to non linear calibration curves due to the partitioning of an analyte or the electrochemical products of the analyte into the organic phase of the carbon paste\textsuperscript{72}.

3.3 TIN OXIDE ELECTRODES

Antimony (Sb) doped tin oxide (SnO\textsubscript{2}, Sn is in 4+ oxidation state) is an n-type semiconductor with the carrier density on the order of $10^{20} - 10^{21}$ electrons/cm\textsuperscript{3} and a resistance of $5 - 10\\Omega$. Tin oxide electrodes are prepared by spraying the substrate with SnCl\textsubscript{4} at high temperature (500 \textdegree{} C) followed by hydrolysis of the surface. The sensitivity of the electrochemical response of tin oxide electrodes depends on their electrical properties, chemical composition (impurities), and their bulk structure (degree of crystallization and grain size).
Tin oxide electrodes have positive potential limit (anodic direction) as high as +2.0 V. Since Sn is already in its highest oxidation state (Sn$^{4+}$), it does not show the same interfering background currents due to the formation of a surface oxide as Pt and Au electrodes do$^{73}$.

3.4 TIN OXIDE ELECTRODE-BASED BIOSENSORS

As an electrode material, tin oxide electrodes exhibit some favorable characteristics such as high electrical conductivity, chemical and electrochemical stability and ease of chemical surface modification. Furthermore, tin oxide electrodes are commercially available and relatively inexpensive and, hence, may be suitable as disposable electrochemical biosensors. In 1988, Watanabe et al. first demonstrated the use of tin oxide electrodes in the fabrication of an amperometric biosensor to detect glucose$^{74}$. In this research glucose oxidase was chemically immobilized on an SnO$_2$ electrode either using 2,4,6-trichloro-1,3,5-triazin (cyanuric chloride), 3-aminoprophyltriethoxysilane and glutaraldehyde (silane coupler) or via cross linkage of enzyme molecules by glutaraldehyde. The quantitation of glucose was accomplished by monitoring the enzymatically produced hydrogen peroxide at 0.90 V vs Ag/AgCl electrode. Tsuzuki et al. reported a novel glucose sensor using a glucose oxidase monolayer attached to tin oxide electrodes.
using the Langmuir-Blodgett technique. This is a very useful technique for molecular level tailoring of solid electrode surfaces since it can introduce functional groups on an electrode with controlled surface orientation and density\textsuperscript{75}. These electrodes have been utilized as detectors for flow injection glucose analyzers and high performance liquid chromatographic systems\textsuperscript{76,77}.

In the following sections, the utility of tin oxide electrodes containing plant tissues (biocatalysts) as a detector in flow injection analysis for catechol measurements will be presented.

The tissue-based tin oxide bioelectrode in these studies consists of a tissue reactor and an adjacent bare electrode surface which detects products of biocatalytic reaction. The analytical capabilities of this novel thin layer electrode design are demonstrated using the biocatalytic system used in the first project (chapter one), i.e eggplant tissue which has been shown to be rich in the enzyme polyphenol oxidase and highly selective toward catechol\textsuperscript{78}. Biocatalytically generated O-quinone is monitored amperometrically and quantitatively related to the concentration of catechol. The performance of this new electrode design was compared with that of a tissue reactor/carbon paste collector dual electrode configuration.

55
3.5 EXPERIMENTAL SECTION

3.5.1 Apparatus

All the hydrodynamic amperometric measurements were made using a model CV-1b cyclic voltammograph (Bioanalytical systems). Amperometric responses were recorded on a Omniscribe series D 500 Strip chart recorder. A single channel flow injection system consisted of a Rheodyne 7010 injection valve with a 50 uL stainless steel sample loop and a Rainin Rabbit peristaltic pump (Rainin Instrument Co. Inc.). The in-house designed thin layer electrochemical cell is in a 15 μL flow channel. All the components of the FIA system were sequentially connected using Teflon tubing.

3.5.2 Materials and Methods

Antimony doped tin oxide electrodes were purchased from Delta Technologies, Stillwater, Minnesota. All the solutions were prepared with distilled deionized water. All chemicals were purchased from Sigma Chemical Company. Standard solutions of catechol were prepared daily in the carrier solution (0.1 M Phosphate buffer pH 7). Eggplants were purchased from a local grocery store and stored at 4°C until used.
3.5.3 Construction of Working Electrodes

Antimony doped tin oxide electrodes, as received from Delta Technologies were cut into 3 cm x 0.8 cm pieces. The cut electrodes were boiled in hexane followed by concentrated hydrochloric acid. The electrodes were then rinsed thoroughly with distilled, deionized water and air dried. These cut and cleaned electrodes were used in a thin layer cell made of plexiglass. A cavity with the dimensions of 2.5 mm in diameter and 2 mm in depth was made on the tin oxide electrode surface to hold the tissue materials. The tissue containing cavity is upstream from the adjacent bare tin oxide electrode surface. In all cases, tin oxide electrodes were sandwiched between two plexiglass plates of the thin layer cell with the conductive side placed flush against the flow channel (Fig 11). The surface of the electrode in contact with plexiglass was lubricated with silicon grease to avoid any leakage. The tin oxide electrode in the thin layer cell was used as the working electrode. A Ag/AgCl micro reference electrode (model RE-1, Bioanalytical System) and a stainless steel auxiliary electrode were located downstream. For comparison purposes, a laboratory designed, carbon paste dual electrode thin layer cell was used where two circular cavities, each 2.5 mm in diameter and 2 mm deep, were positioned in series in the thin layer
Figure 11. Schematic diagram of a tissue reactor/tin oxide collector thin layer cell: (a) solution inlet; (b) solution outlet; (c) tissue reactor; (d) tin oxide electrode surface; (e) working electrode connection to potentiostat
channel. These were filled with a tissue slice (upstream) and carbon paste (downstream). The distance between the two cavities was 0.5 mm and the flow channel of the thin layer cell was 15 μl.

3.5.4 Procedure

The composition of the carbon paste used in this study was 45% mineral oil and 55% carbon powder (by weight). Amperometric detection of O-quinone was accomplished at a constant potential of -0.2 V vs Ag/AgCl at both tin oxide and carbon paste electrodes. The flow rate of the carrier solution (0.1 M phosphate buffer pH 7) in the FIA system was 1 ml/min.

3.6. RESULTS AND DISCUSSION

3.6.1 Calibration of the Sensor

The novel tin oxide thin layer electrode configuration was capable of rapidly detecting the o-quinone produced at the bioreactor surface. Amperometric measurements obtained at - 0.2 vs Ag/AgCl with the thin layer design were used to evaluate the analytical performance. Figure 12 illustrates a part of the flow injection peaks obtained at the tissue reactor/tin oxide collector pair for the concentration range of 5x 10^{-7} M to 9.5 x 10^{-6} M catechol. Figure 13 is the resulting calibrating plot. As a comparison, figure 14 shows the flow injection peaks obtained at the tissue reactor/carbon paste collector pair.
Figure 12. A part of the flow injection peaks obtained with tissue reactor/tin oxide collector thin layer cell for catechol. Applied potential, -0.2 V Vs Ag/AgCl
Figure 13. A calibration curve obtained with tissue reactor/tin oxide collector thin layer cell for catechol.
Figure 14. Flow injection peaks of tissue reactor/carbon paste collector thin layer cell for catechol. Applied potential, -0.2 V vs Ag/AgCl
Figure 15. A Calibration curve obtained with tissue reactor/carbon paste collector thin layer cell for catechol.
for the concentration range of $1 \times 10^{-5}$M to $2.5 \times 10^{-4}$ M catechol and the resulting calibration curve is given in figure 15.

3.6.2 Bioanalytical Performances of Thin Layer Configurations

Table 2 summarizes the bioanalytical performance of both thin layer electrode configurations; e.g. tissue reactor/tin oxide collector and tissue reactor/carbon paste collector under identical experimental conditions.

As is shown in table 2, the sensitivity and lower detection limit of the tissue reactor/tin oxide collector pair are better than those of the tissue reactor/carbon paste collector pair. Since the size of the tissue reactor is similar in both cases, the difference must be due to the collector electrode. In the case of tin oxide, electroactive molecules are exposed to a larger electrode surface area (approximately 60 mm$^2$) than for the carbon paste (approximately 5 mm$^2$). The difference in the electroactivity of 0-quinone at tin oxide vs carbon paste (which is presumably related to the heterogeneous electron transfer kinetics) as well as the uncharacterized flow patterns in thin layer configurations may also contribute to the observed difference. Nevertheless, it is obvious that the novel tin oxide electrode configuration can be used to obtain improved sensitivity, a wider dynamic range
and lower detection limits compared to analogous carbon paste thin layer configurations. It might also be used in situations where carbon paste cannot be successfully used.

Table 2. Bioanalytical Performance of Thin Layer Configurations.

<table>
<thead>
<tr>
<th>Detector</th>
<th>Sensitivity (µA Mol⁻¹ l⁻¹)</th>
<th>Linear range (Mol/l)</th>
<th>Det.limit (Mol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue/SnO₂</td>
<td>4.5x10³</td>
<td>5.0x10⁻⁷ - 9.5x10⁻⁶</td>
<td>2.5x10⁻⁷</td>
</tr>
<tr>
<td>Tissue/CP*</td>
<td>1.1x10²</td>
<td>1.0x10⁻⁵ - 2.5x10⁻⁴</td>
<td>5.0x10⁻⁶</td>
</tr>
</tbody>
</table>

* CP = Carbon paste

3.6.3 Selectivity Measurements of Thin Layer Configurations

Selectivity measurements made with both thin layer configurations for catechol with respect to similar substrates, such as dopamine and L-dopa, are given in table 3. These measurements are in agreement with the substrate specificity of eggplant polyphenol oxidase⁶³.

3.6.4 Response Times and Sample Throughput

The tin oxide thin layer electrode containing tissue reactor responded to changes in substrate concentration almost instantaneously. Sharp peaks with rapid increase and decrease of currents are observed (figure 12). The peak width at 90% of the maximum response is approximately 1.5 sec and the sample throughput is about seventy two injections per hour. The fast current decay indicates
rapid removal of o-quinone from the electrode surface. The response time characteristics of the novel thin layer design are comparable to those of the tissue reactor/carbon paste thin layer design.

Table 3. Selectivity measurements of thin layer configurations.*

<table>
<thead>
<tr>
<th>Compound</th>
<th>( \frac{i_p(int)}{i_p(cate)} ) [tissue/SnO(_2)]</th>
<th>( \frac{i_p(int)}{i_p(cate)} ) [tissue/CP*]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechol</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Dopamine</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td>L-dopa</td>
<td>0.5</td>
<td>0.25</td>
</tr>
</tbody>
</table>

* All the solutions are at 1 x 10^{-4} M.

\( i_p(int) \) = Current of interfering substrate

\( i_p(cate) \) = Current of catechol

CP = Carbon paste

3.6 CONCLUSIONS

The possibility of using tin oxide electrodes as an alternative electrode material for the construction of plant tissue based biosensors is demonstrated. The novel tissue reactor/tin oxide collector thin layer design can be used to obtain improved bioanalytical performance. Furthermore, the application of the novel thin layer design as an electrochemical detector for liquid chromatographic separation of catechol amines would be a logical extension for future research.
CHAPTER 4
APPLICATION OF PLANT TISSUE CULTURES IN CONSTRUCTION OF TISSUE BASED BIOSENSORS

4.1 BACKGROUND

In recent years, there has been a considerable interest in electrochemical sensors which use biocatalytic materials such as animal and plant tissues, i.e., in their natural environment, in replacement of isolated enzymes\textsuperscript{25}. Plant materials are chosen based on their intrinsic enzyme activities and their suitability for incorporation into analytical devices.

At present, biocatalytic tissue materials used in sensor construction are mostly obtained from commercial sources, although there are some exceptions. This poses some uncertainties relevant to biosensor response. For example, incomplete knowledge about the freshness and the state of ripeness of the biocatalytic material is problematic. In addition, the dependence of the biocatalytic activity of plant materials upon the growth conditions under which commercial samples are obtained is typically unknown. The seasonal availability of commercial plant samples can also be a serious constraint. Therefore, it is of prime importance to establish a well defined source of plant tissue materials in order to
obtain reproducible sensor responses and lifetimes as well as other analytical parameters.

The use of in vitro cultured plant tissues, as a very fresh and reproducible source of biocatalytic material with a well defined enzyme activity, for biosensor construction will be presented in the following section.

4.2 PLANT TISSUE CULTURE

Plant tissue culture technology involves the in vitro cultivation of all plant parts under aseptic conditions. These culturing procedures involve two fundamental steps; the isolation of explants from the rest of the plant body followed by the aseptic inoculation of explant in an appropriate nutrient medium in which it can express its intrinsic or induced potentials. Thus, the composition of the culture medium is one of the most important factors governing the growth of plant tissues in cultures. These culture media are generally made up of several basic components. This includes macronutrients (N, P, K, Ca, Mg, S), micronutrients (Mn, Zn, B, Cu, Mo), vitamins, amino acids and other nitrogen supplements, a carbon source, a solidifying agent and growth regulators. Although the term "plant tissue culture" is commonly used to include all types of aseptic plant cultures, it is sometimes preferable to use
more specific terms to distinguish various types of cultures such as plant cultures, embryo cultures, organ cultures, suspension or cell cultures, protoplast cultures, anther cultures and tissue or callus cultures.

Present day tissue culture technology involves interdisciplinary relationships between investigators in cellular and whole plant biology, plant physiology, horticulture and plant genetics. To date, in vitro cultured plant tissues have not been utilized in the construction of biosensors to our knowledge.

The advantages of using cultured plant tissues over regularly grown plants for the construction of biosensors involve several aspects. Most plant parts can be induced to form callus tissue under laboratory conditions rapidly, easily, and economically. Since the growth conditions and nutrients can be carefully controlled, reproducible and uniform growth of tissues can be obtained. Such plant tissues are very fresh and well nourished, so that enzymatic activity is expected to be high. The biosensor responses obtained with such tissues should be sensitive and very reproducible. Furthermore, cultured plant tissues are microorganism-free and the decaying process of tissue may be slower compared to regularly grown plants, resulting in a longer sensor lifetime. Plant tissue cultures are not subjected to climatic and seasonal
changes as regular plants, and can be obtained in any part of the world with the in vitro technique. In addition, cultured plant tissues can be maintained indefinitely by subculturing.

Specifically, the use of Tobacco (*Nicotiana tabacum*) callus tissues, which exhibit high peroxidase activity, for the construction of tissue-based biosensors is demonstrated in this research. Callus tissue is a disorganized proliferation of cells which arises from the explant of a plant organ in an appropriate nutrient medium. The Peroxidase activity of these cultures has been related to growth, differentiation and lignification and is intimately associated with activity of other enzymes. In this study, tobacco callus tissue is utilized as the biocatalyst to construct an amperometric biosensor for the detection of hydrogen peroxide.

### 4.3 BIOCHEMISTRY OF PEROXIDASES

Peroxidases are enzymes catalyzing the oxidation of a wide variety of organic and inorganic compounds by hydrogen peroxide or closely related compounds. They are heme proteins containing ferrisprotoporphyrin IX \([\text{Fe(III)}-\text{protoporphyrin IX}]\) as the prosthetic group, and play a vital role in plant and animal tissue metabolism. Peroxidases can be either membrane bound or soluble. In
animals, it is primarily involved in cellular defense and detoxification mechanisms. However, it also plays a major role in aging, carcinogenesis as well as biosynthesis of prostaglandins and thyroid hormones. Major roles of peroxidase enzymes in plant metabolism involve growth, morphogenesis and organogenesis, lignin biosynthesis, defense system, respiration and light mediated processes. Figure 16 illustrates the general catalytic mechanism of peroxidase enzymes.

Figure 16. Enzymatic reaction mechanism of peroxidase
4.4 QUANTITATIVE MEASUREMENTS OF HYDROGEN PEROXIDE

The quantitative determination of hydrogen peroxide is of importance in many areas ranging from industrial applications to clinical applications. It is used for waste water treatment, sterilization and as a source of oxygen. Hydrogen peroxide is a by-product of various oxidases (eg: glucose oxidase) operating in normal cellular metabolism. Many drugs and chemicals produce \( \text{H}_2\text{O}_2 \) through oxidative degradation in normal cellular metabolism. Therefore, many biologically significant compounds can be determined by monitoring the production of hydrogen peroxide.

A large number of analytical procedures have been developed for hydrogen peroxide based on a variety of detection methods. These include volumetric analysis\(^9^5\), spectrophotometry\(^9^6\) and chemiluminescence\(^9^7\). Major drawbacks of these techniques include interferences, long analysis times, and consumption of expensive reagents. Electroanalytical methods such as amperometry have been utilized for direct detection of hydrogen peroxide\(^9^8\). However, this requires a large positive over potential at which other electroactive compounds can also be oxidized.

Enzyme- and tissue-based biosensors provide a very selective alternative analytical procedure for detection of \( \text{H}_2\text{O}_2 \). Most of the early biosensing devices utilized a
Clark type oxygen electrode together with catalase, either the isolated enzyme or tissue such as bovine liver and grape tissues.

The use of mixed plant tissue-carbon paste amperometric bioelectrodes utilizing hydrogen donors and electron mediators for the detection of hydrogen peroxide minimizes the chances of electrochemical interferences since such devices can be operated at low potentials. Ferrocene and its derivatives have been very successfully employed as mediator due to their well defined electrochemistry.

4.5 OPERATION OF TOBACCO CALLUS TISSUE-BASED H₂O₂ SENSOR

The electron transfer scheme of the tobacco callus-modified, ferrocene based carbon paste electrode used in this study is given in figure 17. Peroxidase is oxidized by hydrogen peroxide and subsequently reduced by ferrocene. The net result is the formation of water through the oxidation of ferrocene to ferricinium. The rate at which the ferricinium ions are formed is monitored by its amperometric reduction at the electrode surface. This reduction current is quantitatively related to the concentration of hydrogen peroxide.
Figure 17. Electron transfer scheme of tobacco callus sensor
4.6 EXPERIMENTAL

4.6.1 Apparatus

Steady state amperometric measurements were made with a CV-1b cyclic voltammograph (Bioanalytical system, Inc). Tobacco callus-modified, ferrocene-based carbon paste working electrodes, Ag/AgCl reference electrode, and platinum wire counter electrodes were used in a three electrode arrangement. Amperometric responses were recorded on an Ominiscribe series D 5000 strip-chart recorder. Amperometric detection of ferricinium ions generated due to enzymatic oxidation at the electrode surface was accomplished by applying a constant potential of 0.0 V vs a Ag/AgCl reference electrode. All the measurements were taken at room temperature.

4.6.2 Reagents

Hydrogen peroxide (30% w/w), mineral oil and ferrocene were purchased from the Aldrich Chemical Company. Catechol, dopamine and L-dopa were purchased from Sigma Chemical Company while chloroform and graphite powder (#38) were purchased from Fisher Scientific. All the solutions were prepared with distilled deionized water. Stock solutions of hydrogen peroxide were prepared daily.
4.6.3 Callus Cultures of Tobacco

Callus cultures of tobacco were initiated with hypocotyl segments of aseptically grown seedlings of tobacco which were germinated on Murashige and Skoog medium (without growth regulators)supplemented with 15% v/v coconut water. The hypocotyl segments were aseptically transferred to Murashige and Skoog medium supplemented with growth regulators whose composition is given in table 1, and grown under a light intensity of 100 foot candles at room temperature. The callus cultures (figure 18) were harvested from solid agar medium on the third week of inoculation. Harvested tobacco callus cultures were washed and stored at 4°C until used.

4.6.4 Preparation of Working Electrodes

The composition of tobacco callus-modified, ferrocene-based carbon paste used in this study is 93.6% (by weight) in ferrocene modified carbon paste (3.6% ferrocene, 40% mineral oil, 56.4% carbon powder) and 6.4% in callus tissue. The tobacco callus-modified, ferrocene-based carbon paste was packed tightly at the end of an electrode body of 2.5 mm diameter and the electrode surface was smoothed on oil paper. The above mentioned tissue and ferrocene compositions were used throughout the study unless otherwise specified.
Table 4. The basic nutrient constituents of Murashige and Skoog's medium; Formulation for one liter of the medium*

<table>
<thead>
<tr>
<th>MINERAL SALTS</th>
<th>CONCENTRATION (mg l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium nitrate</td>
<td>1650</td>
</tr>
<tr>
<td>potassium nitrate</td>
<td>1900</td>
</tr>
<tr>
<td>potassium phosphate, dibasic</td>
<td>170</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>370</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>440</td>
</tr>
<tr>
<td>Manganous sulfate</td>
<td>22.3</td>
</tr>
<tr>
<td>Ferrous sulfate</td>
<td>27.8</td>
</tr>
<tr>
<td>Disodium ethylenediaminetetra-acetate</td>
<td>37.3</td>
</tr>
<tr>
<td>Zinc sulfate</td>
<td>8.6</td>
</tr>
<tr>
<td>Boric acid</td>
<td>6.2</td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>0.83</td>
</tr>
<tr>
<td>Cupric sulfate</td>
<td>0.025</td>
</tr>
<tr>
<td>Sodium molybdate</td>
<td>0.25</td>
</tr>
<tr>
<td>Cobalt chloride</td>
<td>0.025</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ORGANIC CONSTITUENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
</tr>
<tr>
<td>Glycine</td>
</tr>
<tr>
<td>Myo-inositol</td>
</tr>
<tr>
<td>Nicotinic acid</td>
</tr>
<tr>
<td>Pyridoxin.HCl</td>
</tr>
<tr>
<td>Thiamine.HCl</td>
</tr>
<tr>
<td>Agar</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GROWTH REGULATORS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinetin</td>
</tr>
<tr>
<td>Indoleacetic acid</td>
</tr>
</tbody>
</table>

*The pH of the medium was adjusted to 5.8 before the agar was added. Then the agar was melted and poured into vials (2.5 cm diameter) and autoclaved. The vials containing sterilized medium were used to grow tobacco callus tissue.
Figure 18. A photograph of tobacco callus tissue
4.6.5 Optimization of pH and Operational Potential of the Sensor

The pH dependence of the sensor response was monitored at 1 x 10^-5 M hydrogen peroxide concentration in a series of 0.1 M phosphate buffers, the pH of which varied from 5.0 to 8.0, at a constant potential of 0.0 V vs a Ag/AgCl reference electrode. The dependence of the sensor response upon the applied potential was studied in the potential range of +0.1 V to -0.2 V vs Ag/AgCl electrode in the same electrolyte (pH 6.5) at 1 x 10^-5 M hydrogen peroxide concentration.

4.6.6 Composition of Ferrocene and Biocatalytic Loading

The relative effect of ferrocene concentration in the carbon paste upon the sensor response to hydrogen peroxide was studied at three different loadings (e.g. 1.5%, 3.6% and 5%; by weight) with tissue composition being held constant (6.4%). Similarly, the effect of tissue loading on the sensor response was studied at 6.4%, 9% and 12% (by weight) of tissue at a fixed amount of ferrocene (3.6%).

4.6.7 Calibration of Tobacco Callus Sensor

Calibration of the sensor with hydrogen peroxide was performed over a concentration range of 5 x 10^-6 M - 1.10 x 10^-4 M. Sensor precision was evaluated with seven replicate measurements of hydrogen peroxide at 1 x 10^-5 M. The response time of the sensor was evaluated at a
concentration of $1 \times 10^{-5}$ M hydrogen peroxide. The time required to reach the 90% of the maximum response ($t_{90}$) was estimated.

4.6.8 Variance of Biosensor Response and Biocatalytic Activity of Different Tissue Preparations

In order to compare any variation in the sensor response toward hydrogen peroxide and the biocatalytic activity of each individual callus culture (grown under identical conditions) against those of agar-grown (same medium as callus tissue) and regularly grown (in a greenhouse on potting soil) tobacco plants, parallel experiments were carried out using ten separate sets of tobacco callus cultures (harvested on the third week of inoculation), ten individual regularly-grown tobacco plants (one month old), and ten individual agar-grown tobacco plants (one month old). In all three cases, the composition of the tissue in the carbon paste was 6.4%.

The sensor response was monitored for each case; tobacco callus culture at $1 \times 10^{-5}$ M $\text{H}_2\text{O}_2$, agar-grown plants at $1 \times 10^{-5}$ M $\text{H}_2\text{O}_2$ and regularly grown plants at $2 \times 10^{-4}$ M $\text{H}_2\text{O}_2$.

4.6.9 Time Stability Studies of Tobacco Callus Sensor

The tobacco callus tissue based sensor was calibrated for a period of five months with respect to hydrogen peroxide to determine the long term stability. The sensor
was stored in pH 6.5 phosphate buffer at 4°C when not in use.

4.6.10 Response of Tobacco Callus Sensor Toward Important Hydrogen Donors

The sensor response toward hydrogen donors such as catechol, dopamine and L-dopa was also examined using a carbon paste electrode containing 6.4% callus tissue and 93.6% carbon paste (with no ferrocene) under similar experimental conditions but with a constant concentration (2 x 10^-5 M) of hydrogen peroxide in the solution.

4.6.11 Subculture and Peroxidase Activity of Tobacco Callus Tissues

Tobacco callus cultures were maintained for approximately five months by subculturing them once every two weeks. Subculturing involves the aseptic transfer of a healthy and suitable size inoculum from a preceding culture to a fresh medium. In order to investigate the peroxidase activity of tissues after subculturing, the product of each subculture along with the ferrocene mediator was used to construct the working electrode. The sensor calibration was carried out with each working electrode over the concentration range of 5.0 x 10^-6 M to 6.5 x 10^-5 M hydrogen peroxide.
4.7 RESULTS AND DISCUSSION

The response of the tobacco callus sensor to a solution of $1 \times 10^{-5}$ M hydrogen peroxide as a function of pH is shown in figure 19. As illustrated in the figure, the optimum pH for operation of the sensor is 6.5, and all the subsequent experiments were conducted in pH 6.5 phosphate buffer (0.1 M). The optimum operating potential of the sensor was found to be 0.0 V vs. Ag/AgCl reference electrode. This potential is a compromise between sensitivity and background noise of this ferrocene mediated bioelectrode.

An increase in the ferrocene percentage in the carbon paste from 1.5% to 3.6% did not result in any significant change in the sensor response to $1 \times 10^{-5}$ M hydrogen peroxide. However, further increase in the ferrocene composition up to 5% resulted in a decrease of the sensor response by 25%. Fundamental studies regarding the decrease in the sensor response above 3.6% ferrocene are beyond the scope of this study and were not attempted. However, it was obvious that a ferrocene composition between 1.5% and 3.6% is suitable for the sensor construction and, 3.6% ferrocene was selected in this study.

Several controlled experiments were conducted in order to verify the response mechanism of the sensor toward
Figure 19. pH dependence of tobacco callus sensor at 1 x 10^{-5} M H_2O_2 in 0.1 M phosphate buffer
hydrogen peroxide. When plain carbon paste (without tissue and ferrocene) was used, the sensor did not give any response. Neither the 6.4% tissue containing carbon paste (without ferrocene) nor the ferrocene containing carbon paste (without tissue) gave any response to hydrogen peroxide, confirming the validity of the proposed mechanism.

The dependence of the sensor response upon the amount of callus tissue in the carbon paste matrix for the concentration range of $5 \times 10^{-6}$ M to $7.5 \times 10^{-5}$ M hydrogen peroxide indicates that the sensitivity of the sensor increases with increasing amounts of tissue (figure 20). The sensitivity (slopes of the calibration curves) of the sensor at 6.4%, 9% and 12% tissue are $1.12 \times 10^3$, $1.51 \times 10^3$ and $2.88 \times 10^3$ pA Mol$^{-1}$ l, respectively. However, the time period required to reach a stable base line was increased with increasing amounts of tissue in the carbon paste, probably due to back ground current effects. The time required to reach a stable baseline at 6.4%, 9% and 12% tissue are 5, 7, and 11 minutes, respectively. Thus, 6.4% was selected to be an appropriate value as it had an adequate response to $\text{H}_{2}\text{O}_2$ with a fast onset of a stable baseline.

An example of steady state current-time responses obtained using the tobacco-callus sensor under optimized
Figure 20. Effect of tobacco callus tissue composition upon the sensor response to hydrogen peroxide at, (□) 6% ; (●) 9% and, (■) 12% tobacco callus tissue
Figure 21. Steady-state amperometric responses of tobacco callus sensor. Electrolyte, 0.1 M phosphate buffer (pH 7); applied potential 0.0 V vs Ag/AgCl.
experimental conditions is illustrated in figure 21. The calibration plot obtained for hydrogen peroxide over the concentration range of $5 \times 10^{-6}$ M to $1.10 \times 10^{-4}$ M is shown by the inset in figure 21. The response sensitivity of the calibration curve (slope) is $1.43 \times 10^3$ $\mu$A Mol$^{-1}$ L. The lower detection limit of the sensor was found to be $7.5 \times 10^{-7}$ M at a signal-to-noise ratio of 3. The coefficient of variation of the sensor for the seven replicate measurements of $1 \times 10^{-5}$ M hydrogen peroxide was 4.1%.

Steady state current responses obtained with various kinds of tobacco tissues (callus, agar-grown and regularly grown plants) to given concentrations of hydrogen peroxide (figure 22) under similar experimental conditions indicate that the callus tissue has the highest biocatalytic activity and the least variance ($S$ or $\sigma^2$) (figure 22, table 5). The sensitivities given in the table are obtained from the calibration curves (not shown) with tobacco callus tissue, agar-grown and regularly-grown tobacco plants over the concentration ranges of $5 \times 10^{-6}$ M - $5.5 \times 10^{-5}$ M, $5 \times 10^{-6}$ - $5.5 \times 10^{-5}$ M and $1 \times 10^{-4}$ M - $6 \times 10^{-4}$ M hydrogen peroxide, respectively. The least variance and the highest biocatalytic activity observed with callus tissue preparations may be due to the well
Figure 22. Steady-state current response of each sensor type under optimized experimental conditions. (■) callus culture of tobacco to $1 \times 10^{-5}$ M H$_2$O$_2$, (♦) agar-grown tobacco plants to $1 \times 10^{-5}$ M H$_2$O$_2$, (▲) regularly-grown tobacco plants to $2 \times 10^{-4}$ M H$_2$O$_2$. 

Current (10$^{-3}$ μA)

Individual callus # or Plant #
Table 5. Variance and response sensitivities of various types of individual tobacco tissues

<table>
<thead>
<tr>
<th>Biocatalytic material</th>
<th>Variance ($10^{-6}$ μA$^2$)</th>
<th>Response sensitivity ($10^{-3}$ μA Mol$^{-1}$ l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco callus</td>
<td>2.178</td>
<td>1.12</td>
</tr>
<tr>
<td>Tobacco plants (Agar grown)</td>
<td>3.156</td>
<td>0.50</td>
</tr>
<tr>
<td>Tobacco plants (Regularly grown)</td>
<td>42.67</td>
<td>0.043</td>
</tr>
</tbody>
</table>

controlled experimental conditions under which the callus cultures are grown. In contrast to regular plant tissues (except root tip and apical meristem), the callus tissue consists of actively dividing cells containing high enzymatic activity.

The long term stability of the tobacco-callus sensor is illustrated in figure 23. As can be seen, the sensor exhibits a remarkable long term stability of about five months (without any significant decrease in the sensor response from the first day). However, it should also be noted that the sensor shows some enhancement in the magnitude of the response over a period of the first thirty days. Such an enhancement in electrode response has been observed with a previously reported tissue electrode$^{104}$ where it was speculated that the leaching of the mineral oil from the graphite paste with time would allow the graphite to compact and perhaps form a more
Figure 23. Time stability of tobacco callus sensor; current response to $1 \times 10^{-5}$ M $\text{H}_2\text{O}_2$
efficient electrode surface at which electron transfer can occur. Further investigation is required to explain the enhancement effects. Nevertheless, parallel time-stability studies conducted with regularly grown tobacco plants (in green house) indicated that the response of the regularly grown plant-based sensor is decreased by 25% (from the first day response) by the 40th day. Hence the time stability of the tobacco callus-tissue based sensor is much superior to that of the regular plant-based sensor. Such a long term stability may be due to the freshness and high enzymatic activity (callus has a fast metabolic rate compared to regular plants) of the callus tissue as well as the lack of microbial contamination (callus tissue is an aseptic preparation) in the tissue. Some physiological differences in the life cycle of cells in callus tissue vs whole plant may also contribute to the observed trend in long term stability.

The steady-state response time (t-90) of the sensor was estimated to be approximately 2 s. This fast response can be attributed to the intimate contact between the biocatalytic and sensing sites which results in rapid establishment of a steady state current due to low diffusion barriers within the sensor itself.

Calibration curves obtained using the tobacco callus sensor (without ferrocene) for catechol and dopamine over the concentration range of $1 \times 10^{-5}$ M to $5 \times 10^{-5}$ M in the
presence of $2 \times 10^{-5}$ M hydrogen peroxide are given in figure 24. Since these substrates can act as hydrogen donors in peroxidation reaction$^{105}$ and the product is electrochemically active, they can be detected by using tobacco-callus modified electrodes in the presence of hydrogen peroxide. Although L-dopa was also studied as a possible hydrogen donor, it did not give any response at the callus tissue modified electrode.

The change in the peroxidase activity from the callus initiation (mother culture) to the fourth subculture is illustrated in figure 25. As can be seen in the figure, peroxidase activity is drastically reduced on the second subculture and reached a constant level thereafter. The decrease in peroxidase activity may be due to the loss of vigor (healthy growth) of callus tissues. A carryover of toxic materials in the callus from one generation to the next may be another reason for such a behavior.

4.8 CONCLUSIONS

An amperometric biosensor, based on in vitro cultured tobacco callus tissue is constructed and characterized for the detection of hydrogen peroxide. The cultured tobacco callus tissue exhibits the highest biocatalytic activity and the least variance with respect to sensor response toward hydrogen peroxide compared to regularly-grown and agar-grown tobacco plants. In addition, the tobacco
Figure 24. Response of tobacco callus sensor toward important hydrogen donors. (●) dopamine, (□) catechol; applied potential 0.0 V vs Ag/AgCl; electrolyte; 0.1 M phosphate buffer
Figure 25. Dependence of peroxidase activity of tobacco callus tissue upon subculture
callus-based sensor showed a remarkable lifetime of more than five months. These favorable analytical features of the sensor may be due to the fact that the callus tissues are obtained under well controlled experimental conditions abundant in nutrients, resulting in rich enzymatic activity. Such controlled conditions cannot be expected from regularly grown plants. Presumably, other types of plant tissue cultures could easily be utilized to construct future biosensors in a similar manner. The proposed hydrogen peroxide sensor in this study may find applications not only in the area of bioanalysis and industrial testing, but also in plant tissue culture technology where it could provide a rapid screening test to investigate the peroxidase activity (or its changes during the growth) of cultures. Furthermore, it can be speculated that if commercial plant tissue-based biosensors were to be constructed, the use of in vitro cultured plant tissues would be a favorable approach to provide more precise and stable measurements for bioanalysis together with improvements in other desirable analytical parameters.
CHAPTER 5
USE OF GENETICALLY ENGINEERED (TRANSGENIC) PLANT TISSUES IN BIOSENSOR CONSTRUCTION

5.1 BACKGROUND

The major goal of this research project was to explore the possibility of using genetically engineered plants in the construction of biosensors. Specifically, genetically transformed Russet Burbank potato (Solanum tuberosum) and tobacco (Nicotiana tabacum) are attempted as possible biocatalysts in the construction of an amperometric biosensor for the detection of glucuronides. Glucuronides are clinically important because they are found in urine as detoxification products.

The mentioned transgenic plants contain the enzyme β-glucuronidase (GUS EC 3.2.1.31). It is a hydrolase which catalyzes in vitro hydrolysis of the β-D-glycosidic bond of certain glucuronides. The enzymatic reaction is given below.

\[ \beta-D\text{-glucuronide} + H_2O = \text{an alcohol} + \text{glucuronate} \]
The enzyme attacks all the natural β-D-glucuronides whether glycoside- or acylal-linked, aliphatic or aromatic. It does not show any activity on α-D-glucuronides or β-glucosides\textsuperscript{106}.

This enzyme is found in liver, spleen and certain tissues of the endocrine and reproductive systems. Rat seems to have a higher concentration of the enzyme compared to other mammals. The enzyme is also found in fish liver, snails, mollusks and in some insects. Most higher plants lack this enzyme. However, \textit{Escherichia coli} (a bacteria) contains a high activity of this enzyme. The enzyme β-glucuronidase is widely used in the determination of blood and urinary, steroid glucuronides\textsuperscript{107}.

Since most higher plants lack GUS, it is commonly used as a genetic marker. Alternatively, the expression of this enzyme can be used analytically to study the hydrolysis of glucuronides. This hydrolysis can lead to electroactive products and, it was the goal of this research to use this hydrolysis reaction to construct electrochemical biosensors.
5.2 GLUCURONIDES AND THEIR IMPORTANCE

Elimination of water between the anomeric hydroxyl group of glucuronic acid and the hydroxyl group of an alcohol yields a glucuronide.

\[
\begin{align*}
\text{COOH} & \quad \text{O} & \quad \text{H} \\
\text{OH} & \quad \text{O} & \quad \text{OH} \\
\text{OH} & \quad \text{OH} & \quad \text{OH}
\end{align*}
\]

\[\text{+ ROH} \quad \rightarrow \quad \text{COOH} + \text{O-R} + \text{H}_2\text{O}\]

where, \(R = \text{Aromatic or aliphatic group}\)

Glucuronides are formed in the liver to detoxify poisonous hydroxy-containing substances. The glucuronides present in the normal urine are those of phenol, cresole and indoxyl. After injection of poisons such as morphine, chloral hydrate and camphor, glucuronides are formed with the poison or its hydroxylated derivatives and excreted via urine\(^{108}\). Thus the detection of glucuronides has a clinical significance.

5.3 GENETIC TRANSFORMATIONS OF HIGHER PLANTS TO EXPRESS GUS ACTIVITY

Developments in recombinant DNA technology provide tools for both the localization and, the cloning of genes of interest and also for determining their roles in plant growth and development\(^{109}\). Genetic transformations of potato plants have been reported using \textit{Agrobacterium}
**tumefaciens** which carries a disarmed Ti plasmid pAL4404 and the binary vector PB1121. This vector carries two genes expressed in plant cells; β-glucuronidase (GUS) and neomycin phosphotransferase (which confers kanamycin resistance). The gene for β-glucuronidase is driven by the CaMV 35 S promoter. The GUS gene has been cloned and sequenced, and encodes stable glucuronidase enzyme that has desirable properties for the construction and analysis of gene fusion. Furthermore, tobacco (Nicotiana tabacum) plants have been transformed with Agrobacterium binary vectors which contain the transcriptional fusion domains of either the CaMV 35 S promoter or the tobacco rbcs promoter along with the coding region of glucuronidase. Many plants assayed today lack detectable glucuronidase activity. Therefore the assay for the chimeric gene expression of transformed plants can easily be accomplished without any interference from endogenous gene expressions.

5.4 GLUCURONIDASE ENZYME ASSAY IN ANIMAL TISSUES

The human liver-glucuronidase has been assayed using phenolphthalein-β-glucuronide as the substrate in pH 4.3 acetate buffer (0.1 M) at 37°C. The enzymatic reaction has been monitored spectrophotometrically with the addition of NaOH/NaHCO₃ buffer (pH = 10) at one hour of incubation. This buffer stops the hydrolysis reaction and
develops the pink phenolphthalein color. The apparent $K_M$ value obtained for phenolphthalein-$\beta$-glucuronide as the substrate was $4 \times 10^{-4}$ M$^{113}$. This is the most commonly used assay technique for glucuronidase. However, other substrates such as phenyl-$\beta$-glucuronide and nitrophenyl-$\beta$-glucuronide can also be used as substrates for enzyme assays.

5.5 ASSAY OF GLUCURONIDASE ACTIVITY IN GENETICALLY TRANSFORMED PLANTS

The enzyme, glucuronidase of transformed tobacco plants has been assayed in the extraction buffer (which contains NaH$_2$PO$_4$ [pH 7], EDTA, triton, sodium lauryl sarcosine and mercaptoethanol) at 37 °C using 4-methyl umbelliferyl-$\beta$-glucuronide (MUG). The progress of the reaction has been monitored by fluorometry (excitation and emission wave lengths are 365 nm and 455 nm respectively). The fluorescence was linear up to 1 nM to 5 - 10 $\mu$M MUG.

For these enzyme assays, different parts (leaf, root, stem) with various ages of transformed tobacco plants have been used. The expression of the GUS activity was dependent upon the type of transformant. Tobacco plants transformed with CaMV 35 S promoter expressed GUS activity in all plant parts. However, tobacco plants transformed with rbcS promoter expressed the GUS activity
in the decreasing order of older leaves (highest), young leaves, stem and roots (lowest).112

The assay of the glucuronidase enzyme of transformed potato plants has also been done according to the procedure developed for transformed tobacco plants. The substrate used for this assay was also 4-methyl umbelliferyl-β-glucuronide and the assay method was based on fluorometry. According to the assay performed with transformed potato leaves, glucuronidase activity was estimated to be $1.13 \pm 0.06 \mu\text{moles min}^{-1} \text{mg protein}^{-1}$.111

It has also been shown that the enzyme glucuronidase can be assayed histochemically (in vitro) to localize its activity in cells and in tissues by staining tissue sections with a indigogenic substrate, 5-bromo-4-chloro-3-indolyl-β-glucuronide (x-gluc, Figure 26). Untransformed plants never show staining with x-Gluc.

5.6 TRANSGENIC PLANT TISSUE-BASED BIOSENSORS

It is logical to use these transformed plant tissues which express the GUS activity, in construction of biosensors for the detection of glucuronides. The isolated enzyme, β-glucuronidase (EC. 3.2.1.31, E coli) is commercially available. However, it has never been used for analytical purposes such as biosensor construction. In this project the isolated enzyme was used to construct an amperometric biosensor (enzyme electrode) for the
Figure 26. 5-Bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc)
detection of phenyl glucuronide as a preliminary investigation prior to construction of biosensors with transgenic plant tissues. The characterization of enzyme electrodes with respect to analytical and kinetic parameters as well as the attempted experimental procedures utilized with transgenic plant tissues as biocatalytic materials will be presented in the following sections.

5.7 EXPERIMENTAL
5.7.1 Apparatus

Steady-state amperometric experiments were performed with a CV-1b cyclic voltamnograph (Bioanalytical Systems). Glucuronidase enzyme-modified and tissue-modified carbon paste working electrodes, Ag/AgCl reference electrodes and Pt wire counter electrodes were used in the three electrode system. Amperometric responses were recorded on an Ominiscribe series D 5000 strip chart recorder. Amperometric detection of phenols generated by enzymatic hydrolysis at the electrode surface was accomplished by applying a constant potential of + 0.75 V vs Ag/AgCl. A single channel flow injection analysis system consisting of Rheodyne 7010 injection valve with a 50 ul stainless steel sample loop and a Rainin Rabbit peristaltic pump (Rainin Instrument Co. Inc.) was used for hydrodynamic amperometric experiments.
5.7.2 Reagents

Phenyl-β-glucuronide, paranitrophenyl-β-glucuronide, paranitrophenyl-β-galacturonide, glucuronidase enzyme (EC. 3.2.1.31, E. coli) and 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-gluc) were purchased from Sigma Chemical Company. Graphite powder and mineral oil were purchased from Fisher Scientific and Aldrich Chemical Company respectively. All the experiments were conducted in 0.1 M phosphate buffer.

5.7.3 Blank Determinations

Cyclic voltammetric and steady-state amperometric experiments of the selected glucuronides were conducted at bare carbon paste electrodes to confirm the electroinactivity of these substrates in 0.1 M phosphate buffer (pH 7) at 0.750 V Vs Ag/AgCl electrode.

5.7.4 Enzyme Electrode Preparation

Glucuronidase-modified carbon paste (2% by weight) was prepared by incorporating the isolated enzyme into carbon paste matrix (55% carbon powder, 45% mineral oil). Glucuronidase-modified carbon paste was packed at the end of an electrode body of 2.5 mm diameter and polished on an oil paper.
5.7.5 pH and Temperature Optimization of the Enzyme Electrode

The dependence of the enzyme electrode response on the solution pH was studied by varying the pH from 5 to 7.5 (in 0.1 M phosphate buffer) at $1 \times 10^{-4}$ M phenyl-β-glucuronide. Similarly, the effect of temperature on the enzyme electrode response was monitored by varying the temperature from $25^0$ C to $45^0$ C at $1 \times 10^{-4}$ M phenyl-β-glucuronide.

5.7.6 Calibration of the Enzyme Electrode

The enzyme electrode was calibrated over the concentration range of $2.0 \times 10^{-5}$ M to $2.2 \times 10^{-4}$ M phenyl-β-glucuronide under optimized experimental conditions. The calibration curves were constructed. Furthermore, the steady-state current time responses obtained with the glucuronidase enzyme-electrode were used to construct the Lineweaver-Burk type plots. These plots were used to estimate the $K_M$ value of the enzyme. In addition, para nitrophenol-β-glucuronide and para nitrophenol-β-galacturonide were studied as interferents for the enzyme electrode.

5.7.7 Lifetime of the Enzyme Electrode

The time stability of the enzyme electrode was monitored over the period of three weeks at $2 \times 10^{-5}$ M
phenyl-β-glucuronide. The enzyme electrode was stored in 0.1 M phosphate buffer (pH 7) at 4°C when not in use.

5.7.8 Use of Genetically Transformed Plant Tissues in Biosensor Construction

Preliminary investigations conducted with the isolated enzyme, β-glucuronidase with respect to analytical and kinetic parameters encouraged the use of genetically transformed tissues containing glucuronidase enzyme to construct transgenic plant tissue-based biosensors for phenyl glucuronide. Enzyme electrode responded to the stepwise changes of phenyl-β-glucuronide rather rapidly with an acceptable linear dynamic range. Therefore it was expected that, provided with a sufficient specific activity, the transgenic plant tissues would respond the changes in the concentration of phenyl-β-glucuronide. The experiments conducted with these plant tissues will be presented in the following sections.

5.7.9 Source of Genetically Transformed Plant Tissues

Transformed potato tubers were obtained from the United States Department of Agriculture (Albany, California). Transgenic tobacco plants were obtained from the Department of Botany at the university of Hawaii at Manoa. Since the transgenic potato tubers had a very slow sprouting rate originally, small buds (eyes) of potato tubers were aseptically transferred to a tissue culture
medium (shoot forming) containing auxin and cytokinin to fasten the growth of potato shoots. The small shoots were then transferred to potting soil to obtain mature potato plants. Additionally, more cold treatments were provided to break the dormancy of potato tubers and allowed to sprout and, potato plants were also obtained on potting soil directly. Plant tissues were tested for GUS activity by staining them with X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide).

5.7.10 Staining of Transgenic Plant Tissues with X-gluc

X-gluc (1 mg dissolved in 20 ul of dimethyl formamide) was dissolved in 1 ml of GUS buffer whose composition is given in table 6. Aliquots of this solution (50 ul) were pipetted into eppendorf micro centrifuge tubes (1.5 ml). Each tissue section to be stained was placed separately in the tubes at room temperature. The extent of the staining for a constant concentration (2 x 10^{-3} M) of x-gluc was taken as a measure of the activity of GUS enzyme present in transgenic plant tissues (table 7, figure 28). The enzymatic reaction associated with the staining of tissues is given in figure 27.
Table 6. Composition of GUS buffer

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium phosphate (mono basic)</td>
<td>5.34</td>
</tr>
<tr>
<td>Sodium phosphate (dibasic)</td>
<td>8.70</td>
</tr>
<tr>
<td>Potassium ferrocyanide</td>
<td>0.22</td>
</tr>
<tr>
<td>Potassium ferricyanide</td>
<td>0.16</td>
</tr>
<tr>
<td>Ethylene diamine tetracetic acid</td>
<td>0.37</td>
</tr>
<tr>
<td>Triton-x 100 (1 ml/ l)</td>
<td></td>
</tr>
</tbody>
</table>

Table 7. Staining of transgenic tobacco tissues

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Extent of stain *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature leaves</td>
<td>++</td>
</tr>
<tr>
<td>Young leaves</td>
<td>+</td>
</tr>
<tr>
<td>Stem</td>
<td>1/2 +</td>
</tr>
<tr>
<td>Wild type leaves (untransformed)</td>
<td>-</td>
</tr>
</tbody>
</table>

* Number of pluses indicate the extent of staining
Figure 27. Enzymatic reaction responsible for staining
Figure 28. A photograph of tobacco leaf section stained with x-gluc
5.7.11 Preparation of Transgenic Tobacco-leaf Modified Carbon Paste

Mature tobacco leaves were selected to be the best biocatalytic material for electrochemical experiments among the stained tissue types. Carbon paste electrodes containing 5%, 10% and 15% of tobacco leaf tissue were prepared. These electrodes were used in steady state and hydrodynamic amperometric experiments at a constant potential of 0.750 V vs Ag/AgCl with Phenyl-B-glucuronide as the enzyme substrate.

5.7.12 Electrochemical Studies of Tobacco Leaf Tissue-Modified Carbon Paste Electrodes

5.7.12.1 Steady-state amperometry

Steady-state amperometric experiments were conducted with transgenic tobacco leaf-modified carbon paste electrodes (tissue composition 5%, 10%, 15% by weight) in 0.1 M phosphate buffer (at variable pH values from 5 to 8). Temperature of the electrochemical cell was also varied from 25°C to 45°C in the same buffer at pH 7.

5.7.12.2 Flow injection analysis

A single electrode carbon paste thin layer electrode containing 15% (by weight) tobacco tissue was used in the flow injection analysis system. The flow rate of the carrier stream (0.1 M phosphate buffer; pH = 7) in the FIA system was varied from 0.2 to 1.0 ml/min to allow
adequate time for the enzymatic reaction at the working electrode surface.

5.7.13 Homogeneous Experiments with Transgenic Tobacco Leaves

The steady-state and hydrodynamic amperometric experiments conducted with mature tobacco leaves indicated that the specific activity of the GUS enzyme was not sufficient to respond within the electrochemical time scale. Consequently, a homogeneous experiment was conducted to allow more time for the enzymatic reaction between the tissue and the substrate, phenyl-β-glucuronide. Pulverized tobacco leaves (0.5 g) were placed in a dialysis bag (Technicon, membrane type "C", molecular weight cut off 3500) and dipped in 1 x 10^{-4} M phenyl glucuronide (in 0.1 M phosphate buffer pH 7). The enzyme substrate as well as the products can diffuse through the dialysis membrane. The reaction mixture was allowed to react with continuous stirring for 30 minutes and for two hours in two separate experiments. The reaction mixtures were then injected to a flow injection analysis system with a plain carbon paste electrode poised at 0.75 V vs Ag/AgCl electrode to observe any response from the accumulated product (phenol).
5.7.14 Staining of Transgenic Potato Tissues

Staining of potato tissues was conducted according to the procedure described for transgenic tobacco tissues. The extent of staining was considered to be a measure of the glucuronidase enzyme activity of potato tissues (Table 8 and figure 29-31).

Table 8. Staining of potato tissues with X-gluc

<table>
<thead>
<tr>
<th>Type of potato tissue</th>
<th>extent of stain*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tuber</td>
<td>-</td>
</tr>
<tr>
<td>Buds (eyes)</td>
<td>+</td>
</tr>
<tr>
<td>Callus</td>
<td>1/2 +</td>
</tr>
<tr>
<td>Young leaves</td>
<td>+</td>
</tr>
<tr>
<td>Mature leaves</td>
<td>++</td>
</tr>
<tr>
<td>Young stem</td>
<td>+++</td>
</tr>
<tr>
<td>Young petiole</td>
<td>+++++</td>
</tr>
<tr>
<td>Mature petiole</td>
<td>+++</td>
</tr>
<tr>
<td>Mature stem</td>
<td>+</td>
</tr>
<tr>
<td>Roots</td>
<td>+</td>
</tr>
</tbody>
</table>

* The number of pluses indicate the extent of staining.

5.7.15 Preparation of Transgenic Potato Tissue-Modified Carbon Paste Electrodes

Young petioles (stem of leaves) of potato were selected to be the best biocatalytic material for electrochemical experiments among the tissue types stained. It was further identified that the stainings were concentrated on the vascular bundle of the petiole. Therefore, the petioles were dissected and only vascular
Figure 29. A photograph of a potato leaf stained with x-gluc
Figure 30. A photograph of a cross section of a potato-petiole stained with X-gluc
Figure 31. A photograph of a cross section of a potato-petiole (at a higher magnification) stained with X-gluc to illustrate the localization of the enzyme in vascular bundle.
tissues where the enzyme is highly expressed, were used to prepare carbon paste electrodes for electrochemical experiments.

5.7.16. Steady-State Amperometric Experiments with Petiole Tissue-Modified Carbon Paste Electrodes

Carbon paste electrodes containing 10%, 13% and 15% of vascular tissue (of potato petioles) were prepared. Steady state amperometric experiments were conducted with these electrodes in 0.1 M Phosphate buffer at variable pH (pH 5 to 8) and temperature values (25°C to 45°C).

5.7.17. Flow Injection Analysis Experiments with Potato Petiole Tissues

Tissue reactor/carbon paste collector dual electrode configuration was used for the flow injection analysis experiments. The cavity containing the tissue (potato petiole) is upstream from the adjacent bare carbon paste electrode surface. The dual electrode configuration was specially designed with large cavities. Each hole is 5 mm in diameter. The purpose of such a design is to provide a large number of catalytic sites (potato tissue surface) and large electrode surface area (sensing site) for detection of enzymatic products. The flow rate of the carrier stream (0.1 M phosphate buffer, pH = 7) in the FIA system was varied from 0.1 to 1 ml/min.
5.7.18 Steady-state Amperometric Experiments in Water-Alcohol Mixed Solvent Systems

There is a tendency to enhance the glucuronidase enzyme activity on certain glucuronidase substrates (with spectrophotometric assays) in the presence of aqueous-alcohol mixed solvent systems. Therefore, steady state amperometric experiments were conducted in the following solvent systems with glucuronidase enzyme-modified and potato-petiole tissue (dissected for vascular tissue) modified carbon paste working electrodes.

a. 10% ethanol and 90% 0.1 M phosphate buffer (pH = 7)

b. 10% propanol and 90% 0.1 M phosphate buffer (pH = 7)

c. 5% Butanol and 95% 0.1 M phosphate buffer (pH = 7)

5.8 RESULTS AND DISCUSSION

Cyclic voltammetric and steady-state amperometric experiments conducted at bare carbon paste electrodes indicated that phenyl glucuronide is not electroactive at 0.75 V vs Ag/AgCl. Therefore the proposed electrochemical detection scheme based on the detection of enzymatically produced phenol at 0.750 V (figure 32) was feasible without any electrochemical interferences from the enzyme substrate, phenyl glucuronide. Thus, the amperometric currents associated with the electrooxidation of phenol at the electrode surface were quantitatively related to the concentration of phenyl glucuronide present in the solution.
Figure 32. Enzymatic hydrolysis of phenyl glucuronide

The response of the glucuronidase enzyme electrode to a solution of $1 \times 10^{-4}$ M phenyl glucuronide as a function of pH is shown in figure 33. As illustrated, the optimum pH for the operation of the sensor is 7.0. Furthermore, the dependence of the enzyme electrode response as a function of temperature at $1 \times 10^{-4}$ M phenyl glucuronide indicated that the temperature optimum for the enzyme electrode is $40^0$ C (figure 34). The observed pH and temperature optima of the enzyme electrode were very similar to those of the isolated enzyme$^{114}$. 

Steady-state amperometric responses obtained at glucuronidase enzyme electrode with $2.0 \times 10^{-5}$ M increments of phenyl glucuronide are given in figure 35. Figure 36 illustrates the resulting calibration curve over the concentration range of $2.0 \times 10^{-5}$ M to $2.2 \times 10^{-4}$ M phenyl glucuronide. Paranitro phenyl glucuronide and para nitrophenyl galacturonide did not give any response at this potential and hence they do not pose any interference for the enzyme electrode response. The response time
Figure 33. pH dependence of glucuronidase enzyme electrode in 0.1 M phosphate buffer
Figure 34. Temperature dependence of glucuronidase enzyme electrode in 0.1 M phosphate buffer
Figure 35. Steady-state amperometric responses of glucuronidase enzyme electrode. applied potential, 750 mV Vs Ag/AgCl; electrolyte 0.1 M phosphate buffer
Figure 36. Calibration curve for phenyl glucuronide obtained with glucuronidase enzyme electrode
t-90) of the enzyme electrode was estimated to be approximately 2 sec. As it can be noticed in the figure, this enzyme electrode shows a time stability of three weeks without any significant decrease in the response from the first day (figure 37). This indicates the stability of the immobilized (in carbon paste) glucuronidase enzyme under storage conditions.

A Lineweaver-Burk type plot of $1/i_{ss}$ vs $1/C$ obtained with glucuronidase enzyme electrode is given in figure 38. Such plots are linear if the current is controlled by the catalytic rate rather than the mass transfer limitation. The linear relationship between $1/i_{ss}$ and $1/C$ made it possible to calculate the apparent Michaelis-Menten constant, $K_m'$. The apparent Michaelis-Menten constant thus calculated for phenyl glucuronide based on the slope of the figure 38 is $9.6 \times 10^{-5}$ M.

Transgenic tobacco leaves used in this project expressed the glucuronidase enzyme activity. It was confirmed by staining them with X-gluc as shown in figure 27. However, steady-state and hydrodynamic amperometric experiments conducted with tobacco leaves indicated that the specific activity of the enzyme is not sufficient to respond within the electrochemical time scale. The results of the homogeneous experiments conducted with tobacco leaves according to the procedure described in the experimental section (5.7.13) are summarized in table 9.
Figure 37. Time stability of glucuronidase enzyme electrode
Figure 38. Lineweaver-Burk type plot for glucuronidase enzyme electrode
As seen in table 9 there is no significant difference in currents when tobacco leaves were reacted with and without the substrate. Furthermore, there is no significant difference in currents when transformed and untransformed (wild type) tobacco leaves were reacted with phenyl glucuronide. Thus, homogeneous experiments did not lead to any conclusive results regarding the glucuronidase enzyme activity present in the transgenic tobacco leaves. This is probably due to the diffusion of endogenous plant phenols present in the transgenic tobacco tissue into the solution which may mask the enzymatic production (if any) of phenol from phenyl glucuronide.

Table 9. Results of the homogeneous experiment conducted with transgenic tobacco tissues

**AT TWO HOUR INCUBATION**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Current (FIA) / uA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trans: Toba: + no sub</td>
<td>0.32</td>
</tr>
<tr>
<td>Trans: Toba: + sub</td>
<td>0.36</td>
</tr>
<tr>
<td>Wild type + no sub</td>
<td>0.32</td>
</tr>
<tr>
<td>Wild type + sub</td>
<td>0.22</td>
</tr>
</tbody>
</table>

**AT HALF HOUR INCUBATION**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Current (FIA) / uA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trans: Toba: + no sub</td>
<td>0.38</td>
</tr>
<tr>
<td>Trans: Toba: + sub</td>
<td>0.40</td>
</tr>
</tbody>
</table>

* Sub = substrate, phenyl-β-glucuronide
Trans: Toba: = Transgenic tobacco
It is concluded that the expression of the β-glucuronidase enzyme of potato tissues is stronger than that of tobacco leaves by observing the extent of staining to a constant concentration of X-gluc. However, the activity of Glucuronidase enzyme in the potato-petiole vascular tissue (tissue with the highest expression of glucuronidase enzyme) was still not sufficient to respond within the electrochemical time scale. Therefore, any response was not observed with steady-state and flow injection analysis (at dual electrode; tissue reactor/carbon paste collector) experiments conducted with potato-petiole vascular tissue.

An enhancement of the glucuronidase enzyme activity on certain glucuronides including phenolphthalein glucuronide and paranitrophenyl glucuronide has been observed with aqueous-alcohol mixed solvent systems in spectrophotometric assay procedures. Such an enhancement was observed with monofunctional alcohol with two to five carbon atoms. However, the exact reasoning has been very controversial. It was suggested that some physicochemical properties of the enzyme is involved with the observed enhancement. Lovrein and co-workers have concluded that monofunctional alcohols have an effect on the rate of the catalysis observed on the reaction of phenolphthalein glucuronide. According to recent
reports, it appears that specific solvent interactions with substrate, product or enzyme are required for enhancement of reaction rate\textsuperscript{114}.

However, such an enhancement effect on the reaction of phenyl glucuronide was not observed in aqueous-alcohol mixed solvent systems with steady-state amperometric experiments at the glucuronidase enzyme- modified carbon paste electrode. This may be due to several reasons. There may be no enhancement effect at all, or even if there is an enhancement, it would not be seen within the fast electrochemical time scale in contrast to the long incubation times involved with spectrophotometric assays. Furthermore, the sluggish electrode kinetics associated with the solution uncompensated resistance of mixed aqueous solvents may reduce the actual amperometric currents.
CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTIONS

Plant tissue-based amperometric biosensors provide analytical detection schemes with very good selectivity and sensitivity. The most valuable feature of such devices is the fast response time resulting from the intimate incorporation of biological components with electrochemical transducing elements. In the future, such research projects could be extended in several directions.

The tissue reactor/tin oxide collector thin layer electrode configuration could be used as an amperometric detector for catechol and related compounds in eluents after liquid chromatographic separation of real biological samples. This particular configuration (as a detector for HPLC) will be more suitable than the tissue reactor/carbon paste collector electrode configuration because of the compatibility of tin oxide electrodes with organic solvents employed in liquid chromatographic separations. For the same reason, the tin oxide electrode configuration may be used to construct organic phase biosensors. The use of biocatalytic reactions in organic solvents is a novel research area in enzyme biotechnology and, only a few research reports have appeared on this subject. Operation of enzyme electrodes, based on polyphenol oxidase, in chloroform for the detection of para cresol$^{117}$
and, mediated amperometric-enzyme electrodes incorporating horseradish peroxidase for the determination of hydrogen peroxide in organic solvents such as dioxane, chloroform and chlorobenzene\textsuperscript{118} have been reported recently. Such an approach could be extended to plant tissue-based biosensors by coupling a suitable tissue material with tin oxide electrodes to perform bioanalysis in organic solvents.

The use of plant tissue cultures in biosensor construction will find many applications in future research. Since cultured plant tissues are fresh, economical, and reproducible sources of biocatalytic material, they would be suitable in construction of commercial and disposable biosensors. In addition to callus cultures used in this research, other types of cultures such as embryo cultures, suspension cultures, anther cultures and protoplast cultures could be utilized in future biosensor constructions. Careful investigation of physiology and biochemistry of enzymes present in such cultures will be required prior to construction of biosensors. It has already been recognized that genetic manipulations can be accomplished with protoplast cultures\textsuperscript{119}. Therefore, it may be possible to perform genetic transformations with protoplast cultures to meet biosensing requirements (i.e. to enhance the required
enzyme activity or to suppress the undesirable enzyme activities).

Plant tissue cultures are very rich in secondary metabolites such as alkaloids and terpenoids. These compounds are produced via several enzymatic reaction pathways which may not be found in regularly grown plants. These unusual enzymatic reactions may be used to construct biosensors for the detection of biologically important secondary metabolites.

Cultured plant tissue-based biosensors may also be used as a tool for monitoring enzyme activity of plant tissue cultures and their gradual changes during the growth period, and hence, may find many applications in plant sciences such as horticulture and plant physiology.

The use of genetically engineered plants in biosensor construction is a very novel and exciting research area. Transgenic plants used in this research expressed glucuronidase enzyme activity. However, its expression was insufficient to be utilized in the construction of electrochemical biosensors.

In order to obtain a biosensor response with transgenic plant tissues, it will be necessary to enhance the desired enzymatic activity of transgenic plant tissues as well as the sensitivity of the transducing elements.
One of the latest advances in the use of T DNA (transfer DNA) as a vector for introducing genes into plants is the use of specific plant promoters to express the transferred genes. The promoter region controls the expression of a gene of interest. Therefore, in order to obtain an enhancement in the activity of a desired enzyme it may be necessary to use strong promoters to maximize the gene expression. Furthermore, the gene of interest may be cloned into a plasmid such that it is very close to a strong promoter. Thus, the resulting expression vector with a strong promoter permits efficient transcription and translation which will lead to greater production of the protein (i.e. enzyme of interest). If such an enhancement is accomplished through the use of current genetic engineering procedures (A number of plasmids have been engineered such that they contain sites for restriction enzymes which allow insertion of foreign DNA "downstream" of an efficient promoter), the specific activity of the enzyme of interest would be sufficient to give a response in a biosensing device.

Even if the enzyme of interest is weakly expressed in transgenic plant tissues, they may still be utilized in biosensor construction by coupling with highly sensitive transducers. The use of fiber optic devices with fluorescence and chemiluminescence detection would be a possible approach in this endeavor.
CHAPTER 7

LITERATURE CITED


134


