Abstract

Development of Novel methods for Detection and Quantification of some common Pesticides

Preamble

This thesis focuses on development of novel methods for detection and quantification of different classes of pesticides using Glutathione S-transferase enzyme as analytical probe.

Background

The Green Revolution which brought together improved varieties of food, increased use of fertilizer, irrigation and synthetic pesticides, is credited with the recent sufficiency in global food production proportional to the population. The world agricultural productivity has been improved due to the use of chemical pesticides. Despite the wide ranging benefits of using pesticides in agriculture, several incorrect applications can result in high and undesirable levels of these compounds in the produce that reaches consumers. These include inappropriate selection of pesticides used on foodstuffs, over use of pesticides and harvesting the crops before the residues have washed off after application. It has been estimated that less than 0.1% of the pesticides applied to crops actually reaches the target pest. The rest enters the environment gratuitously, which may directly pollute the environment after application, and then enters into the food chain. Pesticides are one of the most hazardous contaminants of the environment. They can be carcinogenic and cytotoxic. They can also produce bone marrow and nerve disorders, infertility and immunological and respiratory diseases. Inappropriate application of pesticides affects the whole ecosystem by entering the residues in the food chain and polluting the soil, air and ground and surface waters. In recent years the public has become more concerned about the extensive use of pesticides and their effects on the environment on a global scale.

Historically, it was the persistent organochlorine pesticides (DDT, dieldrin, etc.) that drawn the first focus of attention, mainly because they were very persistent in the

environment and so were found far from the sites where they had been used to control local pests. These chemicals have been used not only in agriculture but also to control the vectors disease; their use is continuing in developing countries where the problems of insect pests and insect transmitted disease are severe and alternative methods of control are too expensive or insufficiently developed. Organophosphorus compounds and carbamates with high toxicity are the most extensively used pesticides, whose overuse has generated a serious environmental concern and public health risk. It is estimated that more than 200000 people in developing countries die from organophosphate and carbamate pesticide poisoning every year. In general, organophosphate pesticides are much less persistent than organochlorine ones, but many of them exhibit mammalian toxicity. Other classes of pesticide include benzimidazole, phenolic and synthetic pyrethroid classes. Benzimidazole pesticides are systemic pesticides and playing an ever increasing part in the control of fungal diseases. Phenolic pesticides are applied as herbicides all over the world as post-emergent herbicides.

Detection of these pesticides down to the lowest level as recommended by the Environmental Protection Agency (EPA) and World Health Organization (WHO) remains as a challenge. Chromatographic methods coupled to selective detectors have been traditionally used for pesticide analysis due to their sensitivity, reliability and efficiency. Nevertheless, they present strong drawbacks such as they are time-consuming and laborious and require expensive equipments and highly-trained technicians. These issues turn out to be a major problem when rapid and sensitive measurements are needed in order to take the necessary corrective actions in a timely fashion.

In recent years, enzyme-linked immunosorbent assay (ELISA) method has grown rapidly as a tool for pesticide measurement. However, it has certain limitations: generally the immunoassays allow the detection of one pesticide or a limited set of structurally similar compounds, due to the high specificity of the method; the technique is expensive and it is necessary to have a prior knowledge on the type of pesticide present in the samples.

To meet the requirements of rapid warning and field deployment, more-compact low-cost instruments, coupled to smaller sensing probes, are highly desirable for facilitating the task of on-site monitoring of pesticide compounds. Methods in combination with electrochemistry and biological processes *in-vitro* have been developed in the past two

decades as the most promising idea for direct monitoring of pesticides, which is commonly based on quantification of their inhibition in the presence of pesticides. A biosensor is a self-contained device that integrates an immobilized biological element (e.g. enzyme, DNA probe, antibody) that recognizes the analyte (e.g. enzyme substrate, complementary DNA, antigen) and a transduction element used to convert the (bio) chemical signal resulting from the interaction of the analyte with the bioreceptor into an electronic one. Electrochemical enzyme sensors with high sensitivity, long term stability and low cost detection of specific biological binding events have extensively reduced sampling and testing times in pesticide determinations.

Though many enzyme biosensors have been developed for pesticide detection, there still remains some limitations in their practical utilization. Two obvious limitations are 1. their class specificity and 2. inability to operate in organic solvents. The enzymes used in pesticide biosensors are class specific, e.g., organophosphorus hydrolase works for organophosphate pesticides (OP), acetylcholinesterase for OPs and carbamates, polyphenyloxidase (PPO) works for phenolic classes and so on. With the emergence of diverse classes of pesticides to the market day by day, it is quite challenging to come up with different enzyme or bio-receptor molecules to make the biosensing technology a dependable one. On the other hand, the inhibition based enzyme biosensors cannot operate in organic solvents. Few workers have reported use of 5% acetonitrile as the solvent but this causes excessive dilution of the sample and also affects the reusability of the biosensor. Attempting to address these two problems, we have chosen glutathione S-transferase (GST) enzyme for development of new biosensor with the presumption that since it is a detoxification catalyst capable of binding with many hydrophobic compounds, it may bind with the pesticides also, thus affecting the catalytic action of itself and thereby triggering a biochemical signal. The GST based detoxification reactions rely on the catalysis of the conjugation between a xenobiotic and reduced glutathione (GSH) forming a conjugate compound which in turn is further metabolized inside our body or excreted in a subsequent step. Different substrates are in use for *in vitro* study of the GST catalyzed conjugation with GSH, the most commonly used one among them is 1-chloro-2,4-dinitrobenzene (CDNB). Conjugation of GSH with CDNB produces a yellow colored complex that absorbs at 335 nm. Presence of certain

pesticides in the reaction mixture dampens the UV peak through binding with GST and hence decreasing its catalytic action towards the conjugation reaction.

So, the thesis work was aimed at development of a biosensor for different classes of pesticides using direct use of the enzyme GST and also through physical entrapment of GST in a suitable immobilization matrix, optimization of parameters for stability, longevity and real samples analysis, so as to contribute some efforts towards food safety and quality control.

Contents and layout of the thesis

The main contents of this thesis have been divided into seven chapters. **Chapter 1** includes the introduction part and **Chapter 2** describes the details of materials, experimental methods and characterization techniques used in the entire work. The main experimental segments along with results and discussion thereof have been described in **Chapter 3**, **Chapter 4**, **Chapter 5** and **Chapter 6**. Conclusions from each chapters and significant of the research work along with the future scopes are covered in **Chapter 7**.

Chapter 1: Introduction

Chapter 1 deals with the general introduction of pesticides, biosensors and enzymes. This chapter gives insight into the different classes of pesticides and a discussion on the problems associated with uncontrolled use of pesticides, their toxic effects on the environment and the human health. The conventional methods those were used to detect these pesticides and their limitations are discussed. Elaborate description on biosensor and its different components has been provided along with discussion of advantages of biosensors over conventional methods. Brief review on various enzymes employed in biosensor preparation and also diverse ways of immobilization of these enzymes have been discussed. Moreover, role of GST enzyme in pesticide detection and quantification is explored in brief. Finally the chapter defines the overall objective of the current study and a highlight on the methodology adopted to achieve the final goal of developing biosensors.

Chapter 2: Materials and methods

This chapter provides the details of material that are used in the entire study. It also contains the details of different analytical tools and techniques that have been used in this work. The experimental procedure for the synthesis of graphene oxide is also described. Chemical structures of some important molecules used in the study are also included.

Chapter 3: Electrochemical study of GSH-CDNB reaction in methanol and ethanol and the influence of GST and pesticides on it

Chapter 3 highlights the cyclic voltammetric study of GSH-CDNB reaction in phosphate buffer and two other different solvents namely methanol and ethanol. Observed effect of GST enzyme on the said reaction is discussed. UV-visible spectroscopic study is also shown to corroborate the results of CV analysis. Results of cross reactivity checking of different components present in the reaction mixture are also elaborated. The course of electrochemical behaviour of GSH - CDNB mixture was found to be different in the two solvents methanol and ethanol. A plausible mechanism behind such behavior has been proposed based on the CV, UV-visible and FTIR analysis. Various factors influencing the reaction such as enzyme loading, substrate concentration, time of reaction between the enzyme and the inhibitor (incubation time), pH etc. are discussed in this chapter. Finally, influence of typical pyrethroid pesticide, cypermethrin, on the GSH-CDNB reaction has been observed.

Chapter 4: Application of GST catalyzed GSH-CDNB reaction in methanol as a new method for detection of selected pesticides belonging to different classes

This chapter describes the use of newly developed bioelectrochemical method based on GST catalyzed detoxification reaction between GSH and CDNB, taking methanol as electrolyte, for detection and quantification of different classes of pesticides. Typical examples of dimethoate and temephos from organophosphate class and fenobucarb from carbamate class have been selected for the study. Limit of detection and linear ranges of each class of pesticides have been discussed herein.

Chapter 5: Biosensor fabrication by immobilizing GST enzyme on platinum electrode using graphene oxide mediator

Chapter 5 includes a method for easy and effective fabrication of a GST biosensor probe using graphene oxide (GO) as immobilization matrix. It acts as transducer in improving sensitivity and amplifying the response generated. GO sheets having a large specific surface area and bonded functional groups is an ideal substrate for enzyme immobilization. So in this chapter, we have shown the use of graphene oxide-gelatin paste as immobilization matrix followed by use of cross linking agent gluteraldehyde for stability enhancement of the fabricated electrode. Scanning Electron Microscopic (SEM) study to verify the immobilization, study of the electrochemical behavior of immobilized electrode, study of optimum operational conditions for maximum signal output of the sensor, suitable reactivation mechanism etc. are included in this chapter.

Chapter 6: Study of inhibition kinetics of selected pesticides belonging to different classes by using GST catalyzed GSH-CDNB reaction

In chapter 6, the study of enzyme inhibition has been discussed, owing not only to its importance in providing useful information on the nature of enzyme catalysis but also to its implications in pharmacology and toxicology. Three important kinds of inhibitions namely competitive, non-competitive and mixed type have been encountered while studying the inhibition kinetics of those selected pesticides. Typical example of DDT from organochlorine class; cypermethrin from pyrethroid class; ethion, dimethoate and chlorpyrifos from organophosphate class; fenobucarb from carbamate class; carbendazim from benzimidazole class and dinocap from phenolic class have been chosen for the study.

Chapter 7: Conclusions and future scope

The final chapter of the thesis summarizes the major findings of the studied areas with significance of the work and also describes the overall conclusions. The current study is concluded with a discussion of future prospects in the field of biosensor development for pesticide detection.