Chapter 6

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

6.1 Introduction

Enzyme inhibition is perhaps the one key point that has the most tangible relevance to everyday life. Biosensors based on the principle of enzyme inhibition have by now been applied for a wide range of significant toxic analytes that inhibits the normal enzyme function [1]. These toxic analytes include organophosphorous (OP) pesticides [2-6], organochlorine pesticides [7], derivatives of insecticides, heavy metals [8] and glycoalkaloids [9-11] etc. In general, the development of these biosensing systems is based on a quantitative measurement of the enzyme activity before and after exposure to a target inhibitor [12-15].

The study of inhibition is often a vital point in medicinal field also, because some drugs are based on the inhibition of particular enzymes of biological pathways [16,17].

Enzyme inhibitors may interact with enzymes and/or enzyme-substrate complexes in several different ways to make the activity of an enzyme futile to carry an enzyme-catalyzed reaction. The enzyme inhibition reactions follow certain rules. An enzyme interacts and then binds with substrate in a specific ratio at the active site in the form of a lock-key 3D arrangement. Inhibitors compete with substrates for enzyme active site or allosteric catalytic site in a synergistic manner or first come first preference, to make enzyme activity inefficient. These interactions depend on active free energy loss and thermodynamic principles [18].

Inhibition arises in a number of ways, but can broadly be classified into two types, reversible and irreversible inhibition. Irreversible inhibition is marked by a covalent link between the inhibitor and the active site of the enzyme. Consequently, the enzyme became permanently inactive. Many irreversible inhibitors are therefore considered as potent toxins [19]. For example, organophosphorus compounds inhibit acetylcholinesterase (AChE) activity by reacting covalently with an important serine residue present within the active site of the enzyme. The physiological effect of AChE deactivation is neurotransmitter inactivation at the synapses of nerves [20-23]. In contrast to this, reversible inhibition allows the inhibited enzyme to recover its original activity by a simple wash with buffer or water. Knowledge of inhibition type helps in selection of suitable reactivator during medical treatment against the poisoning effect of

any toxicant, specially the pesticides. Various kinds of reversible inhibition have been reported in literature: the inhibitor may link with the free enzyme (competitive inhibition), enzyme-substrate complex (uncompetitive inhibition), both free and enzyme–substrate complex with the same affinity (non competitive inhibition) or with different affinity (mixed inhibition) [17, 24-27]. A brief introduction to different modes of inhibition has already been comprised in chapter 1. For each mode of inhibition, it is possible to calculate an inhibitor dissociation constant, K_i, which reflects the potential interaction between the enzyme and the inhibitor. It can be said that K_i for an inhibitor is analogous to K_m for a substrate; a small K_i value exhibits tight binding of an inhibitor to an enzyme, whereas a larger K_i value shows weaker binding [28].

Thus, in order to acquire knowledge on the field of applicability as well as reusability of inhibition based biosensors, it is important to have a thorough study of the inhibition kinetics of different types of pesticides.

6.2 Objectives of this chapter

- To study the mode of inhibition of different pesticides belonging to different classes on the catalytic activity of GST enzyme.
- To find out the value of dissociation constant (K_i) for each pesticide in order to evaluate their strength of binding with the enzyme or enzyme-substrate complex.

6.3 Experimental

6.3.1 Cyclic voltammetry measurements

Cyclic voltammetric experiments were conducted employing three electrode configurations. Pt electrode was used as working, a Pt wire as counter and Ag/AgCl refilled with 0.1 M KCl as the reference electrode. The total volume of the working solution in the electrochemical cell was 3 mL and prepared by mixing together 1.5 mL of 2 mM GSH in PB with 1.5 mL of 2 mM CDNB in 50% methanol. An amount of 20 μL of GST enzyme was used to carry out the analyses. CV measurements were done with the potential sweeping from − 0.4 V to 1 V at scan rate 20 mV/ s.

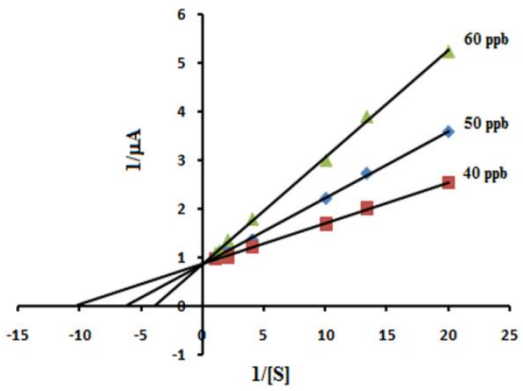
6.4 Results and Discussion

6.4.1 Kinetics of Inhibition

Taking fenobucarb, temephos, dimethoate, DDT, cypermethrin, chlorpyrifos, carbendazim, dinocap and ethion as the effectors, all belonging to different classes of pesticides, we probed the effects of these compounds on the activity of GST enzyme while catalyzing the conjugation of GSH to CDNB through the double-reciprocal Lineweaver–Burk plots. Detail calculations are shown in Appendix A(IV) Chapter 6.

Fenobucarb, DDT and cypermethrin were found to be competitive inhibitors since increasing their concentration resulted in a family of lines with a common intercept on the 1/*V* axis and hence a constant V_{max} but with increasing K_m (Figure 6.1). In this type of inhibition, because of their molecular similarity, the inhibitor competes with the substrate for an active site on the enzyme. As a result, the rate of catalysis depends on the relative concentrations of the inhibitor and the substrate. In the presence of a competitive inhibitor, the V_{max} for an enzyme should be the same as for the uninhibited case. However, the apparent K_m should be larger in the presence of the inhibitor K_m^{app}. The equilibrium constant for inhibitor binding with free enzyme, K_i, was obtained from a plot of K_m^{app}/ K_m versus the inhibitor concentration $[I_0]$ which is linear, the slope of the line giving K_i value which is 10.30 mM, 21.27 mM and 7.46 mM for fenobucarb, DDT and cypermethrin respectively.

A



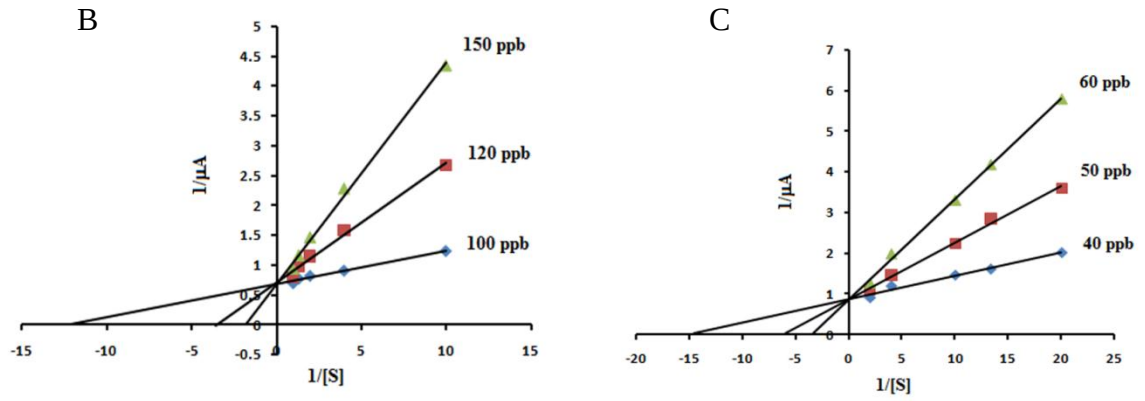


Figure 6.1. Lineweaver-Burk plots showing the effect of different concentrations of A. fenobucarb, B. DDT and C. cypermethrin on the kinetics of GST catalyzed GSH-CDNB reaction.

Table 6.1. Kinetic parameters of GST catalyzed GSH-CDNB reaction in absence and presence of competitive inhibitors:

Name of Pesticide (inhibitors)	V_{max}	K_m	K_i (mM)
GSH-CDNB reaction	1.14 (RSD 0.61%)	0.08 (RSD 0.49%)	
Fenobucarb	1.14 (RSD 0.48%)	i.0.25 (RSD 0.66%) (60 ppb) ii 0.15 (RSD 0.26%) (50 ppb) iii. 0.09 (RSD 0.79%) (40 ppb)	10.30
DDT	1.14 (RSD 0.41%)	i. 0.33 (RSD 0.23%) (150 ppb) ii. 0.23 (RSD 0.74%) (120 ppb) iii. 0.14 (RSD 0.71%) (100ppb)	21.27
Cypermethrin	1.14 (RSD 0.58%)	i. 0.28 (RSD 0.58%) (60 ppb) ii. 0.15 (RSD 0.66%) (50 ppb) iii. 0.10 (RSD 0.39%) (40 ppb)	7.46

The kinetic behaviour of temephos, ethion and chlorpyrifos showed a non-competitive mechanism. The Lineweaver–Burk plots yielded a family of straight lines with different slopes and with a common intercept on the *x*-axis. The results are shown in Figure 6.2, indicating that these inhibitors can decrease the apparent value of V_{max} with no effect on K_m. So, it is a case of non-competitive inhibition in which inhibitors do not compete for the same binding site. A non-competitive inhibitor usually binds at a location other than the active site but is able to change the conformation of the active site in such a way that the enzyme is no longer in the optimal arrangement to efficiently catalyze the reaction. The V'_{max} for an enzyme in the presence of a non-competitive inhibitor will be less than the one observed under uninhibited conditions V_{max}. The magnitude of this decrease will reflect the strength of the interaction between the enzyme and the inhibitor. However, there will be no change in the K_m. The value of inhibition constant can be obtained from a plot of the vertical intercept ($1/V'_{max}$) versus the inhibitor concentration [I_0]. The slope of ($1/V'_{max}$) *vs* [I_0] gives $K_{i,}$ which is linear.

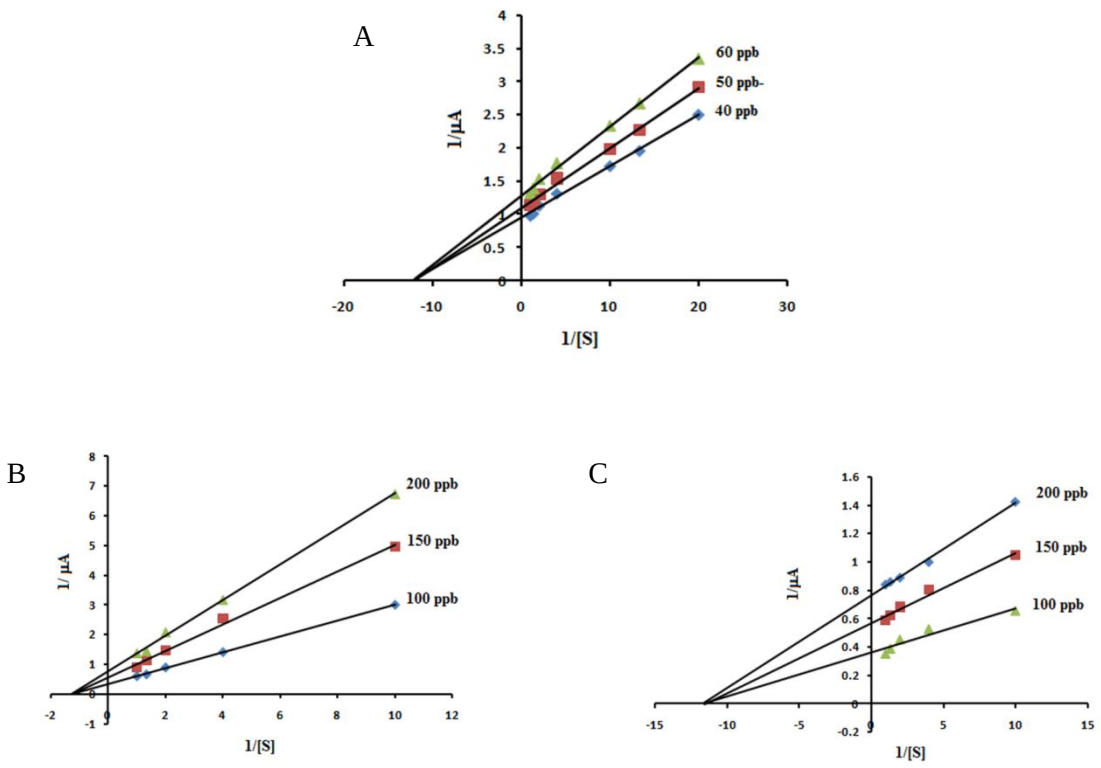


Figure 6.2. Lineweaver-Burk plots showing the effect of different concentrations of A. temephos, B. ethion and C. chlorpyrifos on the kinetics of GST catalyzed GSH-CDNB reaction.

Table 6.2. Kinetic parameters of GST catalyzed GSH-CDNB reaction in absence and presence of non-competitive inhibitors:

Name of Pesticide (inhibitors)	V_{max}	K_m	K_i (mM)
GSH-CDNB reaction	1.14 (RSD 0.61%)	0.08 (RSD 0.49%)	
Temephos	i. 0.78 (RSD 0.16%) (60 ppb) ii. 0.91 (RSD 0.32%) (50 ppb) iii.1.05 (RSD 0.43%) (40 ppb)	0.08 (RSD 0.34%)	50.82
Ethion	i. 0.30 (RSD 0.42%) (200 ppb) ii. 0.41 (RSD 0.38%) (150 ppb) iii. 0.62 (RSD 0.59%) (100 ppb)	0.08 (RSD 0.73%)	54.82
Chlorpyrifos	i. 1.31 (RSD 0.54%) (150 ppb) ii. 1.75 (RSD 0.81%) (120 ppb) iii. 2.74 (RSD 0.28%) (100 ppb)	0.08 (RSD 0.39%)	219.29

The other major type of inhibition occurs when the inhibitor is capable of binding to both the free enzyme and to the enzyme-substrate complex. Here, dimethoate, carbendazim and dinocap show this type of inhibition (Figure 6.3). In this case, the inhibitor can bind to both E and ES; but with different affinities. It is not possible to calculate a single K_i value for this type of inhibition as dissociation constant for binding the free enzyme may differ from the dissociation constant for binding the enzyme-substrate complex.

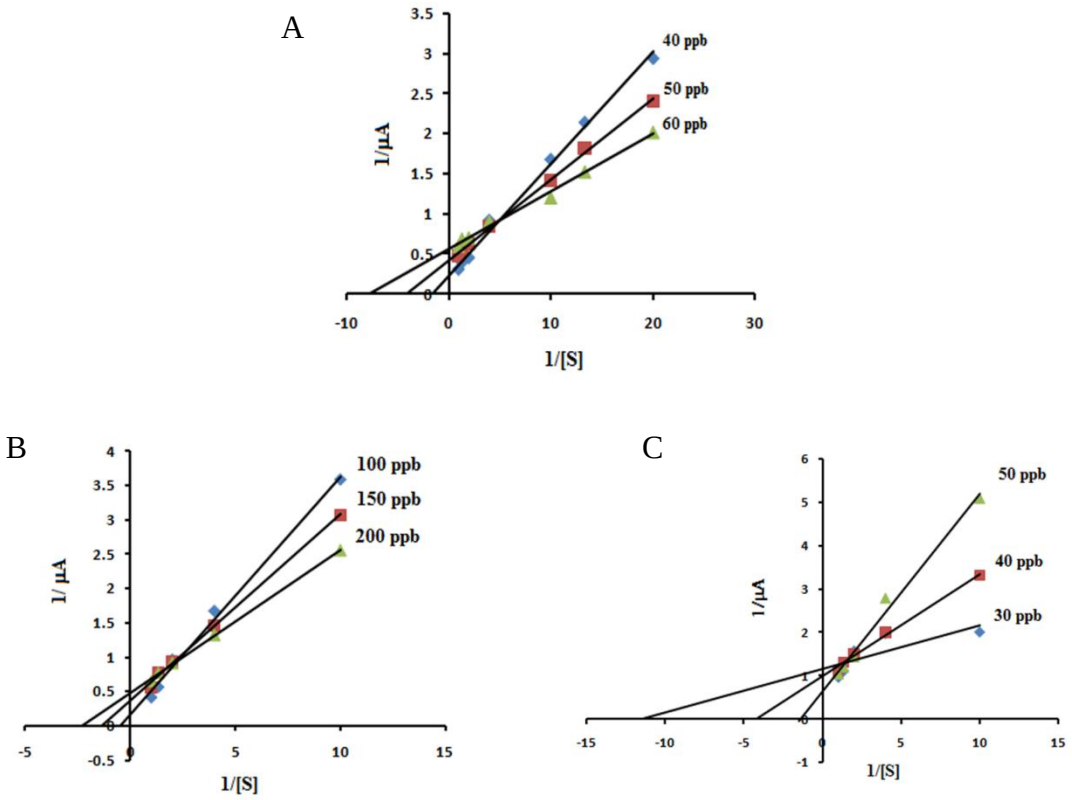


Figure 6.3. Lineweaver-Burk plots showing the effect of different concentrations of A. dimethoate B. dinocap and C. carbendazim on the kinetics of GST catalyzed GSH-CDNB reaction.

Table 6.3. Kinetic parameters of GST catalyzed GSH-CDNB reaction in absence and presence of mixed type of inhibitors:

Name of Pesticide (inhibitors)	V_{max}	K_m	K_i (mM)
GSH-CDNB reaction	1.14 (RSD 0.61%)	0.08 (RSD 0.49%)	
Dimethoate	i. 1.77 (RSD 0.82%) (60 ppb) ii. 2.38 (RSD 0.77%) (50 ppb)	i. 0.12 (RSD 0.95%) (60 ppb) ii. 0.23 (RSD 0.68%) (50 ppb)	-

	iii. 4.32 (RSD 0.72%) (40 ppb)	iii.0.60 (RSD 0.85%) (40 ppb)	
Carbendazim	i. 0.086 (RSD 0.28%) (60 ppb) ii. 1.00 (RSD 0.62%) (50 ppb) iii.1.55 (RSD 0.74%) (40 ppb)	i. 0.086 (RSD 0.69%) (60 ppb) ii. 0.23 (RSD 0.48%) (50 ppb) iii. 0.70 (RSD 0.81%) (40 ppb)	-
Dinocap	i. 2.06 (RSD 0.11%) (200 ppb) ii. 2.68 (RSD 0.68%) (150 ppb) iii. 5.64 (RSD (0.44%) (100 ppb)	i. 0.42 (RSD 0.36%) (200 ppb) ii. 0.72 (RSD 0.90%) (150 ppb) iii. 1.94 (RSD 0.51%) (100 ppb)	-

6.5 Conclusion

Cypermethrin shows highest binding affinity to GST (with K_i value 7.46) among the studied pesticides. It was also observed that inhibition of GST activity by different members of the same group differs. This is attributed to structural influence on binding site selectivity and/or on the kinetics. Thus temephos showed non-competitive inhibition while dimethoate showed a mixed type of inhibition although both belong to the same organothiophosphate group. This shows the possibility of theoretical study of the inhibitor-enzyme's binding site correlation.

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