5
Interaction of OP and Their Metabolites with Trypsin: An NMR Case Study

5.1 INTRODUCTION

Analysis of organophosphate pesticides (OP) interaction with digestive proteins is of particular interest since these proteins could be the indirect binding target for OP. Trypsin is a digestive enzyme that also plays key roles in different other physiological processes. Due to the importance of trypsin in the digestive system, it has been considered as a model binding protein to investigate the effect of ligands on the structure and conformation [Wang et al, 2020]. Interaction analysis of ligand-trypsin adducts and inhibition kinetics of trypsin due to such interactions have been addressed in literature by employing multi-spectroscopic methods. A number of research articles are available that reported the effect of small molecules such as bis henols [Wang and Zhang, 2014] tannin [Gonçalves et al, 2011], psoralen [Liu et al, 2015], 8-Methoxypsoralen [Liu et al, 2017], procyanidin [Gonçalves et al, 2010], Dimethyl phthalate [Wang et al, 2015], acteoside [Wu et al, 2018] bisphenol S [Wang and Zhang, 2014] Ligupurpuroside A [Wu et al, 2017], Folic acid [Shi et al, 2017], gold nanoparticle [Zhang et al, 2014] curcumin [Wang et al, 2016] on the structure and function, especially the enzymatic activity of trypsin. It should be noted that studies related to pesticides-trypsin interaction are limited. There are some occasions where in one case, organic pollutants such as polyphenols are used to monitor the interaction with trypsin using Fluorescence and UV-vis spectrophotometric methods while in another studies *in vitro* interaction of trypsin [Saadati & Mirzaei, 2016] has been reported with pesticides of other origins [Liu et al, 2012; Wang and Zhang, 2014; Wang et al, 2015]. The literature survey clearly indicated that the molecular interaction of OP-trypsin and OP metabolites-trypsin have not been addressed yet. Therefore, the effect of OP on the activity of proteases needs to be examined thoroughly.

Hence, the present Chapter aims to bring the insights on the binding behavior of CPF, DZN, and their metabolites 3,5,6-trichloro-2-pyridinol (TCPy), and 2-isopropyl-6-methyl-4-pyrimidinol (IMP) with trypsin by integrating computational and *in vitro* experimental methods, namely ^{1}H NMR chemical shift changes, Saturation Transfer Difference (STD) NMR, and fluorescence spectroscopy. The effect of OP-trypsin interaction in terms of the alteration of enzymatic activity of trypsin has been highlighted by employing ^{1}H NMR chemical shift changes for N_{α}-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE) [Fang et al, 2017; Gonçalves et al, 2010; Gonçalves et al., 2011; Liu et al, 2017; Ren et al. 2019]. BAEE is the molecule of choice to detect enzymatic activity of digestive proteins. The standard literature protocol to monitor the alteration of enzymatic activity is in general by employing UV-vis spectrophotometric methods detecting changes of the UV-vis spectrum of BAEE in the presence of the digestive protein and the test molecule interacting with the protein [Chi et al, 2010; Gonçalves et al, 2011; Shuai et al, 2014; Wang and Zhang, 2014; Wang et al, 2015]. However, it must be mentioned here that solution-state NMR methods have also been applied to investigate the enzyme activity of proteins with various macromolecular systems of interest [Limtiaco et al, 2011; Swainson et al, 2019]. Similar report on NMR data is not available in case of trypsin using BAEE. Therefore, the present work reports the application of solution-state small molecule-based NMR chemical shift change as the method to determine the alteration of enzymatic activity of trypsin for the first time.

This study will be helpful in providing the effects of OP to digestive enzymes activity in case of accidental exposure and may contribute to explain the effect of OP on the digestive system. The graphical representation of the current study is illustrated in Figure 5.1.

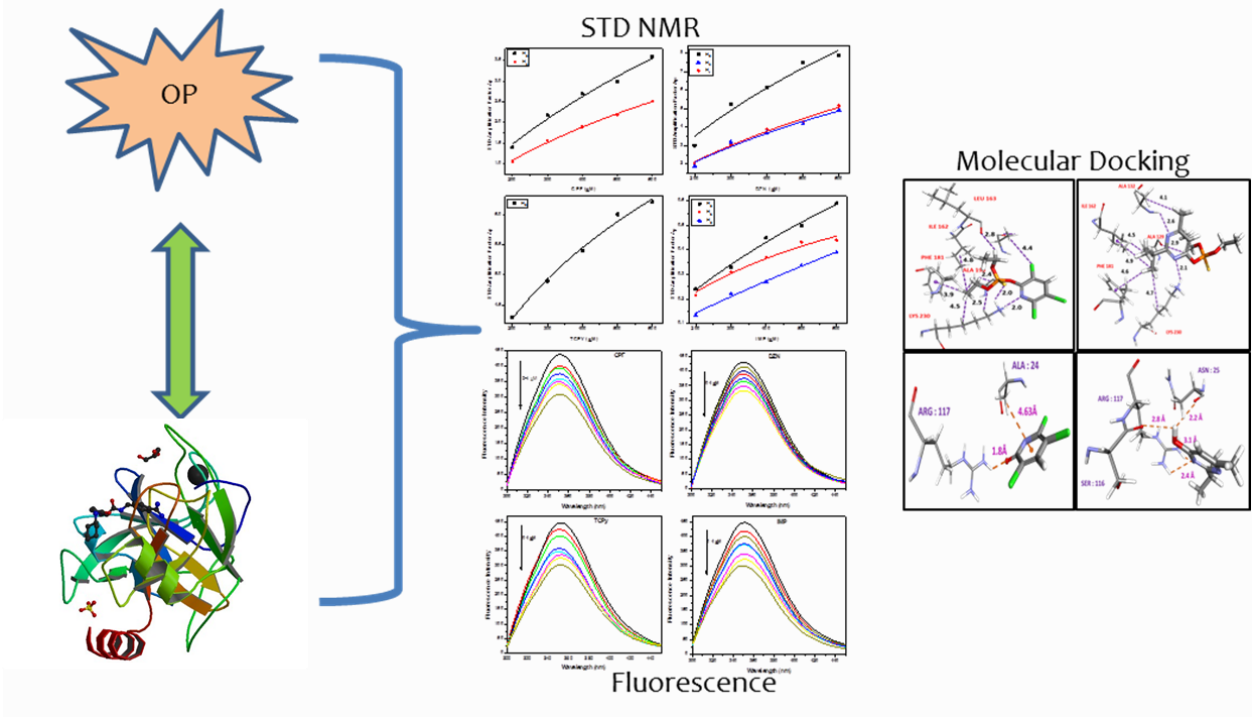

Figure 5.1 : Schematic representation of OP-trypsin interaction as analyzed by ^{1}H NMR, Fluorescence quenching and molecular docking studies.

5.2 SAMPLE PREPARATION

For STD NMR, the 200 µM stock solution of trypsin was prepared in deuterated phosphate buffer (0.05 M) of pH 7.4 and stored at -20 °C. Due to low solubility of OP in buffer, the stock solutions of OP and their metabolites were made in DMSO d_6. The dilutions contain 3:1 D_2O: DMSO ratio. BAEE was dissolved in DMSO to prepare the 10 mM stock solution.

For fluorescence measurements, the 200 µM stock solution of trypsin was prepared in PB. 1 µM trypsin concentration was used for quenching analysis. The test ligands stocks were prepared in DSMO. 10% DMSO was used in final sample dilutions.

5.3 EXPERIMENTAL DETAILS

5.3.1 STD NMR Experiments

For the STD NMR experiments, the parameters given in Chapter 3 section 3.3.1 were used. Table 5.1 summarizes all the experimental parameters used for the STD NMR experiment.

Table 5.1 : Experimental parameters used to obtain the STD NMR spectrum.

Experimental parameters	Values
Pulse program	STDDIFFESGP.3
Number of scans	2048
Saturation time	2 s (K_D determination)
Shaped pulse for saturation	50 ms Gaussian pulses
On-resonance frequency	0 ppm
Off-resonance frequency	40 ppm
Spin lock time	10 ms
Shaped pulse for solvent presaturation	Squa100.1000

5.3.2. Molecular Docking Details

The 3D X-ray crystal structure of trypsin was obtained from Protein Data Bank (PDB ID: 2ZQ1). Discovery studio 4.0 was used to identify the potential interacting residues between OP and OP metabolites with trypsin. The structures for the ligand molecules were obtained from PubChem with PDB Ids CID: 2730 for CPF, CID: 3017 for DZN, CID: 23017 for TCPy and CID: 135444498 for IMP. All the other details are the same as given in Chapter 3 (section 3.3.2).

5.3.3 Fluorescence Measurements

All the experimental details regarding fluorescence measurements are given in Chapter 4 (4.3.2). All the experiments were measured at two different temperatures 298 K and 313 K.

5.3.4 Trypsin Activity Measurement

Trypsin activity is, in general, measured through UV-vis spectrophotometry. In the present case, ^{1}H NMR chemical shift changes of BAEE were monitored [Fonseca et al, 2010]. Trypsin catalyzed BAEE into N-benzoyl-L-arginine (BA) [Saadati and Mirzaei, 2016; Wang et al, 2020] (Figure 5.2). The ^{1}H NMR spectra of BAEE was acquired before and after the addition of trypsin. Further, five different sets of OP-trypsin samples containing (100-500 µM ligands and 10 µM trypsin) in the presence of BAEE were acquired to determine the trypsin activity before and after addition of mentioned OP. The BA spectrum was used as a reference, and then the relative effect of test ligands was analyzed.

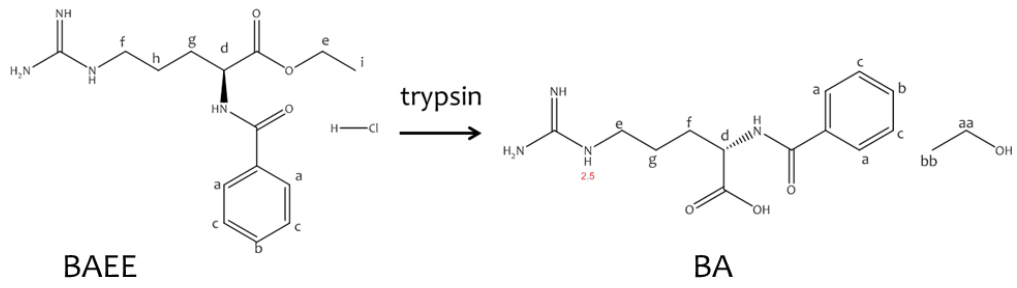

Figure 5.2 : The enzyme catalysis of BAEE to BA in the presence of trypsin.

5.4 RESULTS AND DISCUSSION

5.4.1 Enzyme Activity of Trypsin

The OP/OP metabolite-trypsin interactions are analyzed, and the conformational changes in trypsin activity have been investigated using ^{1}H NMR spectroscopy. Two sets of experiments are performed in order to check the possibility of OP-trypsin adduct formation affecting the enzymatic activity of trypsin: (i) The digestion of BAEE into BA by trypsin in the absence of aforementioned ligands; (ii) The alteration of enzyme activity of trypsin in the presence of OP and their metabolites with increasing concentration of test ligands. The former set has been labeled as a reference, while the latter set has been analyzed in comparison to the reference. Figure 5.3 represents a stack plot of ^{1}H NMR spectra recorded for BAEE in the absence and in the presence of trypsin, revealing the digestion of BAEE into BA [Fonseca et al, 2010].

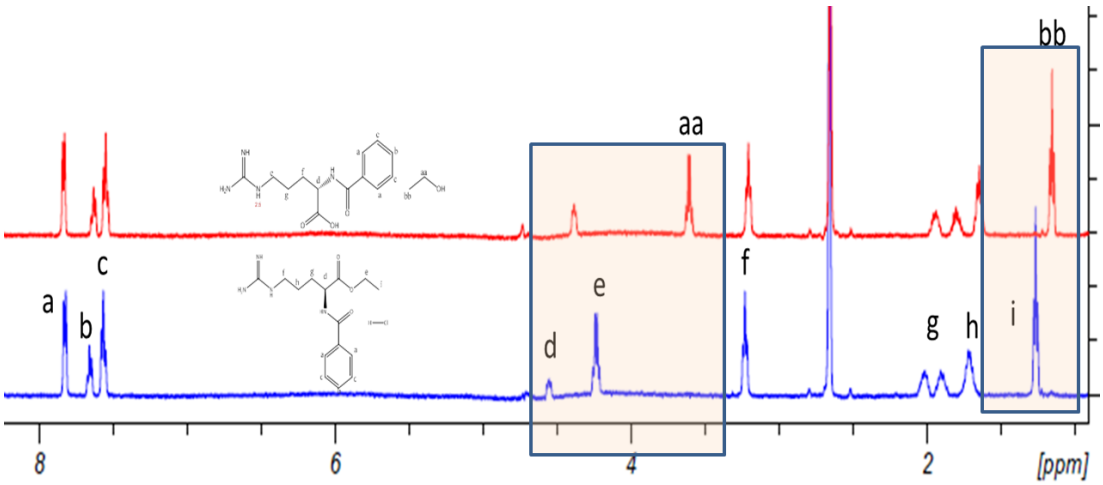

Figure 5.3 : The ^{1}H NMR spectra of BAEE (before the addition of trypsin) and BA (after addition of trypsin). *T* = 298 K, pH = 7.40.

The traditional method of investigating enzyme activity of trypsin considers recording of UV-vis spectrum of BAEE in the absence and in the presence of trypsin. The hyperchromic shift in the UV absorption band of BAEE at 253 nm is monitored after 30 s to 10 mins from the time of addition of trypsin. The digestion of BAEE in to BA is the reason for the hyperchromic shift [Gonçalves et al, 2010; Gonçalves et al, 2011; Liu et al, 2017; Wang and Zhang, 2014]. To

understand the inhibition of trypsin enzymatic activity in the presence of a ligand, a similar set of UV measurements can be carried out as a function of ligand concentration. Due to technical constraints of measuring NMR spectra of BAEE right after a 30 s of the addition of trypsin, a different method has been followed in the present case. Initially, the ^{1}H NMR spectrum of 1 mM BAEE is recorded. A constant concentration of trypsin (10 μM) is added to the sample, and the ^{1}H NMR spectrum is recorded once again. The process of addition of trypsin followed by mixing and routine NMR set up generally takes ca. 10 mins. Therefore, the spectrum of BAEE has been recorded only after 10 mins of the addition of trypsin. During the initial 10 mins BAEE has been digested by trypsin to produce BA. Hence, the NMR spectrum of BA has been recorded and used as further reference for a series of spectra recorded with increasing concentration of OP in the presence of a constant concentration of trypsin and BAEE. The NMR peaks at 1.15 and 3.6 ppm are set as reference peaks for BA.

In the present case, a solution of OP and trypsin has been used with OP concentrations changed from 100-500 μM keeping protein concentration fixed at 10 μM. To this solution, BAEE is added to monitor the alteration in trypsin activity in the presence of these test ligands by measuring the spectral integrals of the two BA ^{1}H peaks. Further, the integrals are compared with that of the reference BA spectrum that is recorded in the absence of OP and right after 10 mins of the addition of 10 μM of trypsin to 1 mM BAEE solution as described in the previous paragraph. Figure 5.4 represents the stack plot of ^{1}H NMR spectra of BA in four different OP and OP metabolite solutions. It is clearly seen that all the OP considered here are able to modify peak integrals of BA at 1.15 and 3.6 ppm indicating alteration of the trypsin activity. A closer inspection of Figure 5.4 shows that in the case of CPF, the intensity of the BA peaks continuously increased with an increase of CPF concentration except in case of 400 μM. This indicates that the increase in CPF concentration leads to increase in trypsin activity. This can be probably due to the entrance of CPF into trypsin's active site cavity, as mentioned in literature for other ligands. On the other hand, the relative activity of trypsin decreases with the increase in the concentration of DZN, TCPy, and IMP. The maximum decrease in activity is observed in the case of TCPy, indicating the highest inhibitory effect of TCPy amongst all the other OP. In the case of DZN and IMP, the inhibitory effect of DZN is far greater compared to IMP. In the case of IMP, the impact of inhibition increases with increasing concentration of IMP till 400 μM. Both TCPy and DZN show no particular concentration effect on trypsin activity.

Further, Figure 5.5 exhibits the % relative activity of trypsin with increasing ligand concentrations. The % relative activity has been calculated by comparing the BA spectral integral at 1.15ppm in the presence and in the absence of OP. In the case of CPF, a *ca*. 56% increment in trypsin activity is found at 500 μM. On the other hand, the trypsin activity is reduced to a *ca*. 67% in case of 500 μM DZN and 61% and 37% decrease in the case of TCPy and IMP, respectively. A trend in the reduction of trypsin activity can now be written for the test OP as TCPy>DZN>>IMP. CPF on the other hand exhibits a completely reverse effect. The reason for such alteration of trypsin activity can be due to conformation changes of the enzyme structure after addition of ligands. The results demonstrate OP-trypsin interaction without any doubt. The possible binding sites on trypsin are (i) site contains three catalytic residues His 57, Asp 102 and Ser195 and (ii) contains hydrophobic cavity consists of residues 189–195, 214–220 and 225–228, and known as S_1 binding pocket which is responsible for primary substrate-binding site. In the following section, the OP-trypsin interaction has been quantified using ligand-based STD NMR.

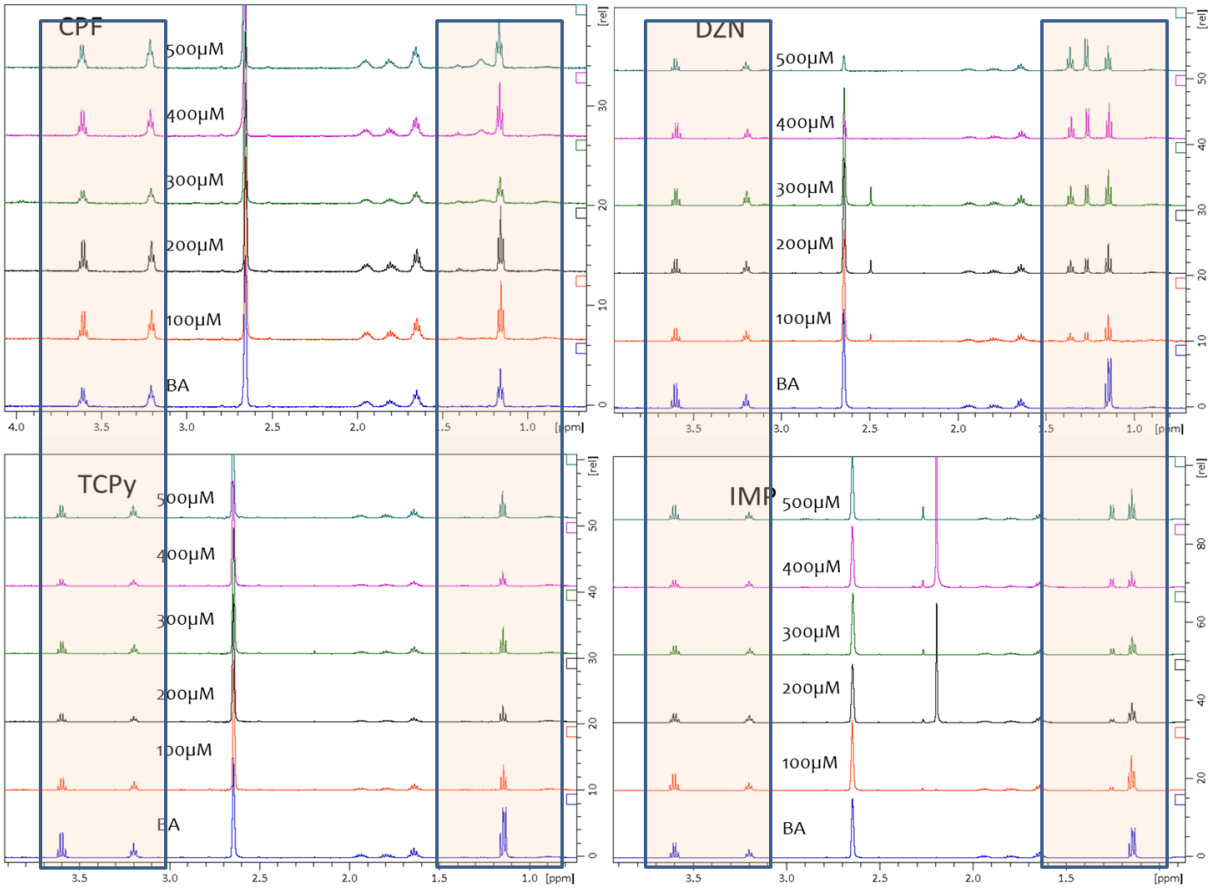

Figure 5.4 : ^{1}H spectra shows the relative effect of CPF, DZN, TCPy, and IMP on trypsin activity with increasing concentration from 100-500 µM. (peak at 1.15 ppm is considered for further analysis of the residual activity of trypsin).

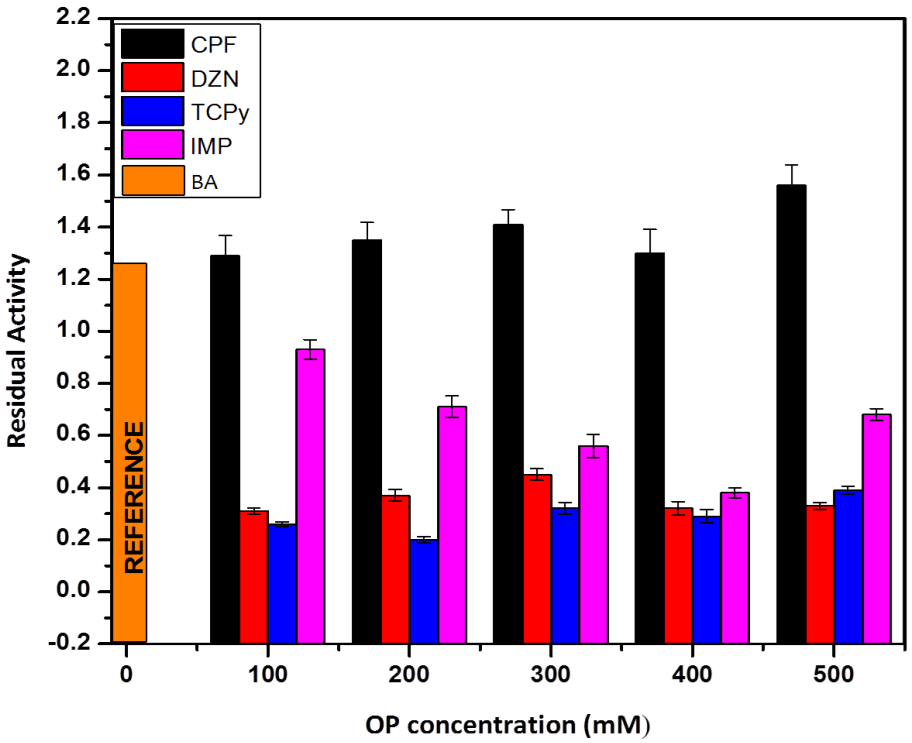

Figure 5.5 : The activities of trypsin in the different concentrations of CPF, DZN, TCPy, and IMP.

5.4.2 Identification of OP-BSA Binding by STD NMR

STD NMR is acquired with CPF, DZN, TCPy, and IMP with trypsin to gain insight into their interaction in solution-state. The STD effect is observed for all the test OP and confirms their binding with trypsin, as shown in Figure 5.6.

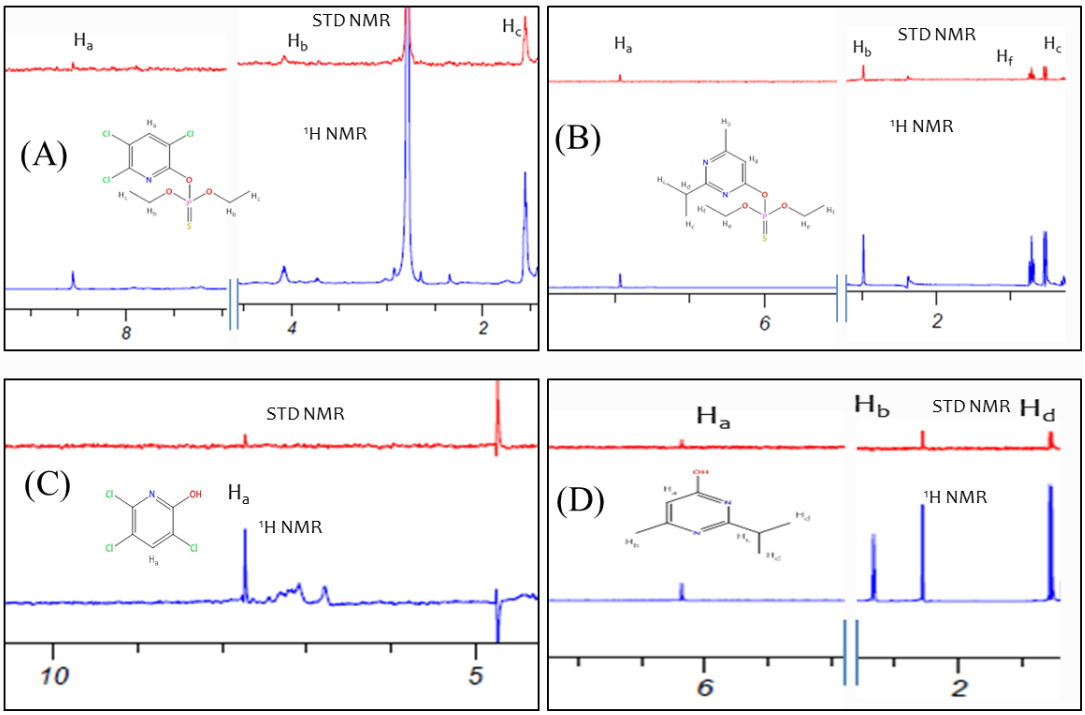

Figure 5.6 : (A) ^{1}H NMR spectrum and ^{1}H STD NMR spectrum of CPF in the presence of trypsin in 40:1 ratio; (B) ^{1}H NMR spectrum and ^{1}H STD NMR spectrum of DZN in the presence of trypsin in 40:1 ratio; (C) ^{1}H NMR spectrum and ^{1}H STD NMR spectrum of TCPy in the presence of trypsin in 40:1 ratio; (D) (C) ^{1}H NMR spectrum and ^{1}H STD NMR spectrum of IMP in the presence of trypsin in 40:1 ratio; Spectra are taken at 298 K on a 500 MHz spectrometer at 298 K. NS=2048, TD = 32 K.

Figure 5.7 shows the Group Epitope Mapping (GEM) for the aforementioned OP and their metabolites in the presence of trypsin by calculating the relative STD (%) (R_{STD}). The relative STD effect is calculated for each molecule, and the values reflect the relative amount of saturation transfer from trypsin to the ligand ^{1}H. The proton which receives the highest STD value is assumed to be in closest proximity with the protein surface than others [Angulo et al, 2010; Mayer and Meyer, 2001; Tanoli et al, 2018]. The various R_{STD} values obtained for all the OP ^{1}Hs are tabulated in Table 5.2. The OP and their metabolites show similar binding modes to trypsin, where the ring moiety of each OP participated in the binding event. In each case, the ring proton gets the highest saturation, which confirms that this proton is in close contact with the protein binding site.

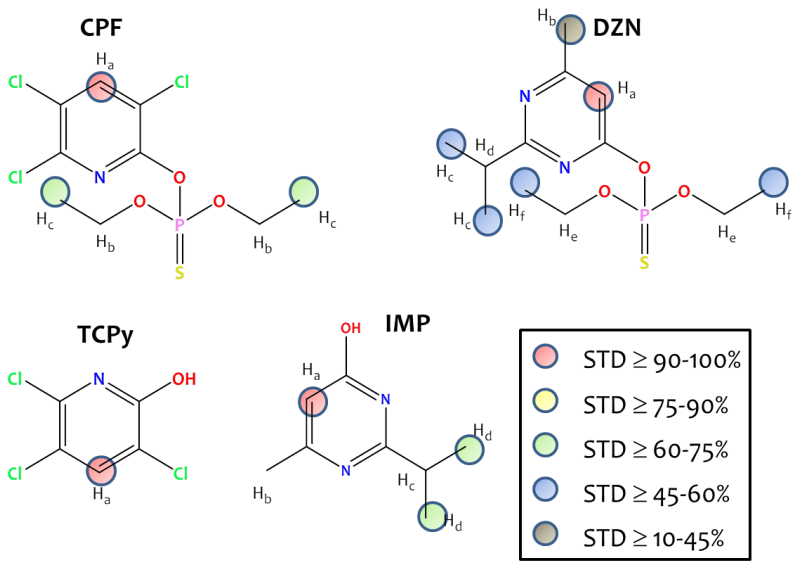

Figure 5.7 : The representation of GEM for CPF, DZN, TCPy, and IMP**.** The relative degree of saturation of hydrogen is mapped for CPF, DZN, TCPy, and IMP. (Color code represents the relative STD %).

Table 5.2 : R_{STD} (%) value for CPF-BSA, DZN-BSA and PA-BSA complex. The symbol * denotes proton could not be identified, and # represents proton does not exist.

OP	Chlorpyrifos R_{STD} (%)	Diazinon R_{STD} (%)	TCPy R_{STD} (%)	IMP R_{STD} (%)
H_a	100	100	100	100
H_b	*	41.7	#	*
H_c	69	55.7	#	*
H_d	#	*	#	67.4
H_e	#	*	#	#
H_f	#	56	#	#

Figure 5.7 represents the STD amplification factor. The STD build-up experiments are performed with varying protein saturation time ranging from 0.5-4 s for the test OP at a ligand to protein ratio 40: 1. The STD_{max} (maximal STD intensity) and k_{sat} (saturation rate constant) are obtained for CPF, DZN, TCPy, and IMP by using Eq.(2.4) as the fitting Equation.

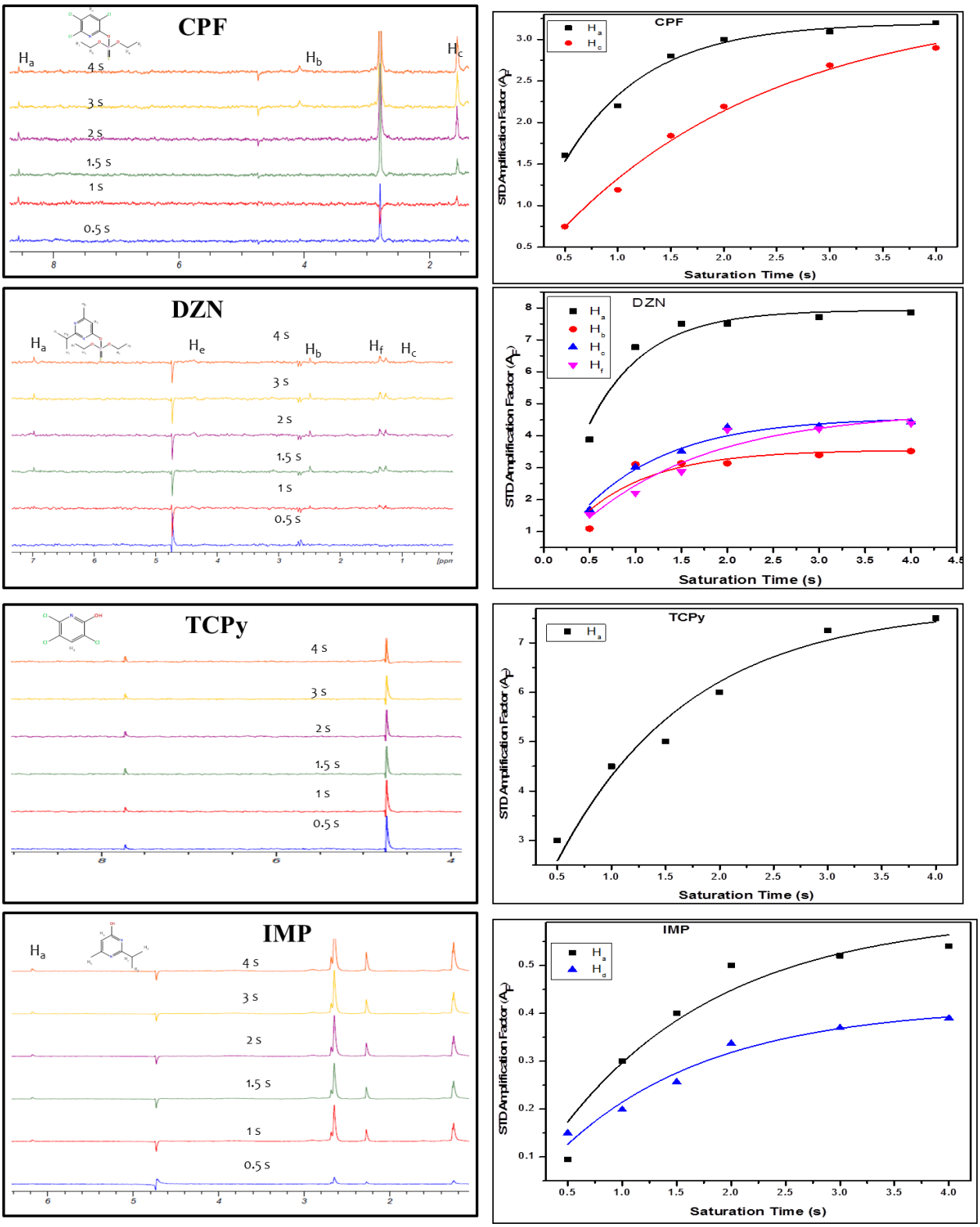

Figure 5.8 : STD amplification factor of CPF-trypsin, DZN- trypsin, TCPy- trypsin, and IMP-trypsin, as a function of saturation time.

5.4.3 K_D Determination

To calculate the binding strength of OP-trypsin, STD titration experiments with varying ligand concentrations (0.2 to 0.6 mM) and constant protein concentration (10 µM) is performed. The dissociation constant K_D is calculated for each OP and metabolites using equation Eq.(2.5) (Chapter 2), by plotting the A_F (STD amplification factor) against ligand concentration (Figure 5.8) . The K_D and average K_D for OP-trypsin is given in Table 5.3 and 5.4. Direct comparison of experimentally determined K_D values for all the ligands clearly indicate (1) the metabolite TCPy shows the highest binding affinity towards trypsin than other ligands (2) all the rest OP exhibit similar binding affinity probably due to structural similarity. Further, by comparing the binding of OP-trypsin and metabolite-trypsin with OP-BSA, and OP metabolite-BSA it is clear that the binding of OP and their metabolites is much stronger in the case of serum protein. This is in complete agreement with the literature where it is already reported that OP interaction with serum protein is stronger compared to the OP-enzyme interaction. These results provide the significance of the role of structure in binding with protein. Moreover, trypsin does not exhibit any enzymatic effect on these OP.

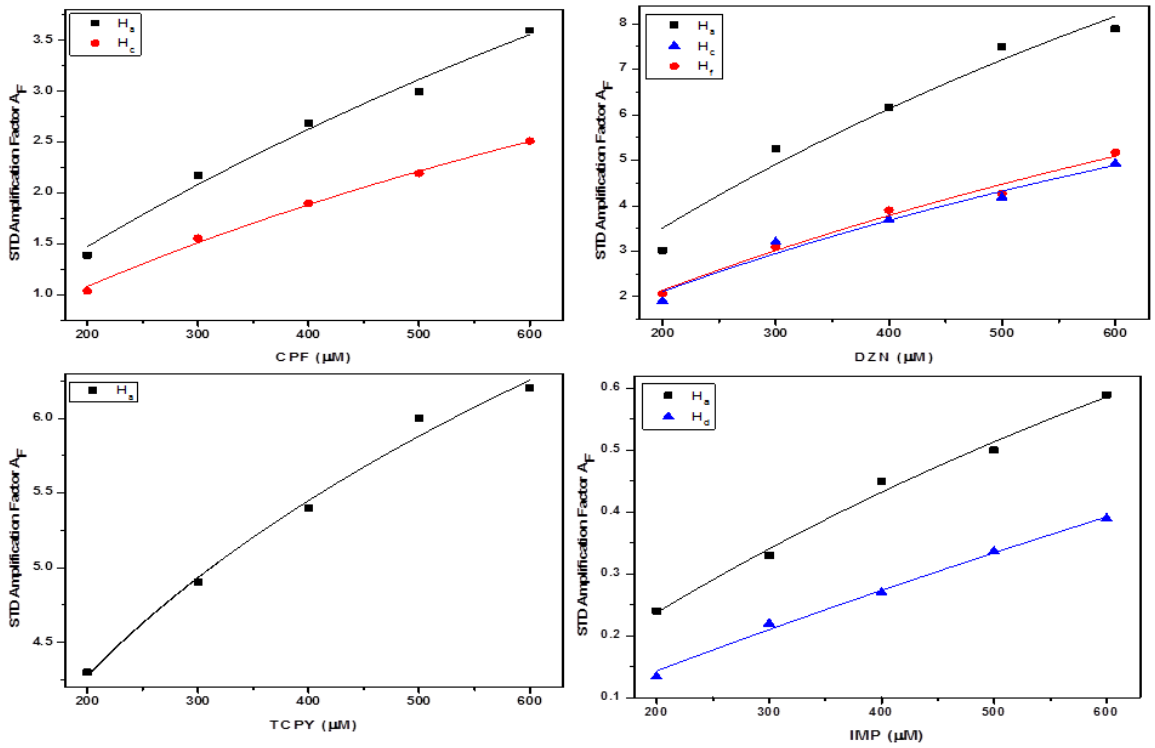

Figure 5.9 : STD amplification factor (A_F) curves with varying ligand concentration (CPF, DZN, TCPy, and IMP) from 200 µM -600 µM, keeping BSA concentration constant (10 µM). 2 s saturation time is used at 298 K temperature on 500 MHz NMR.

Table 5.3 : K_D value for CPF-trypsin, DZN-trypsin, TCPy-trypsin, and IMP-trypsin.

CPF K_D **(M)** $\times 10^{-3}$ R^2 =0.988		**DZN** K_D **(M)** $\times 10^{-3}$ R^2 =0.988			**TCPy** K_D **(M)** $\times 10^{-4}$ R^2 =0. 0.988	**IMP** K_D **(M)** $\times 10^{-3}$ R^2 =0.988	
H_a	H_c	H_a	H_c	H_f	H_a	H_a	H_d
1.46 ±0.50	1.16 ±0.18	1.18 ±0.62	1.33 ±0.46	1.16 ±0.49	1.85 ±0.24	1.59 ±0.49	3.95 ±0.21

Table 5.4 : Average Dissociation Constant (K_D) Values for CPF-trypsin, DZN-trypsin, TCPy-trypsin and IMP-trypsin system.

CPF K_D (M) 10^{-3} M	DZN K_D (M) 10^{-3} M	TCPy K_D (M) 10^{-4} M	IMP K_D (M) 10^{-3} M
1.31 ±0.34	1.22 ±0.52	1.85 ±0.24	2.77 ±0.14

5.4.4 Molecular Docking Study

Molecular docking is used to analyze the binding sites on the trypsin. The interacting residues, bond angle, and binding forces for CPF-trypsin, DZN-trypsin, TCPy-trypsin, IMP-trypsin are shown in table 5.5. Figure 5.10 depicts the mode of interactions through hydrogen bonding as well as hydrophobic interactions of test ligands with trypsin. The hydrogen bonding and hydrophobic interactions could change the conformation of trypsin and also influence the enzyme activity. The amino acid residues ALA132, ILE162, PHE181, LYS 230 take part in binding CPF-trypsin through hydrophobic interaction, whereas LEU163, LYS230, LA129, LYS230 form hydrogen bonding with oxygen and nitrogen atom of the ligand. Trypsin shows the catalytic triad HIS 57, ASP 102, SER 195. In the case of DZN-trypsin, hydrophobic interaction occurs for amino acid residues ALA132, ILE162, PHE181, LYS23, and hydrogen bonding exists between ALA132, ALA129, LYS230 amino acid residues of trypsin and oxygen and the nitrogen atom of DZN. ARG117 shows hydrogen bonding for TCPy, while ALA24 shows hydrophobic interaction. In the case of IMP, hydrogen bonding is involved with amino acid residues ASN25, SER116, ARG 117. The docking results for test OP shows that the S_1 the binding pocket is involved in binding of OP-trypsin. Table 5.5 summarizes the docking results for test OP and metabolites with trypsin [Liu et al, 2015; Ren et al, 2019; Wu et al, 2018].

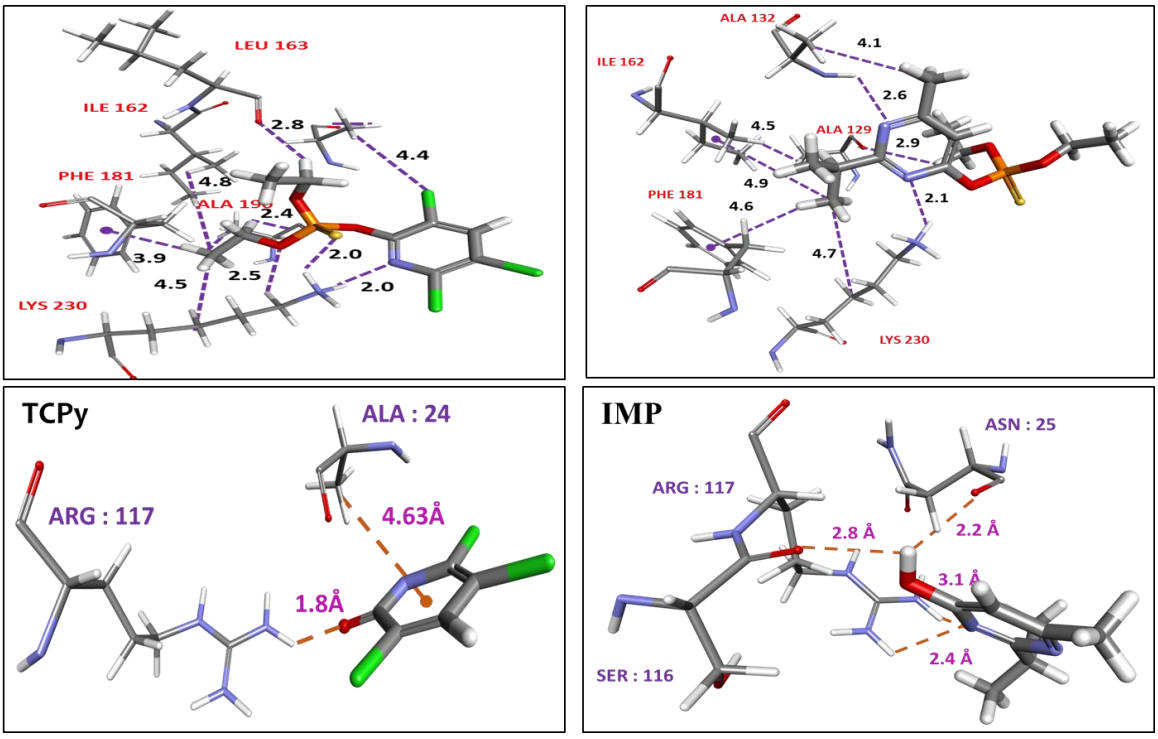

Figure 5.10 : The molecular docking results for CPF, DZN, TCPy, and IMP

Table 5.5 : Summary of interactions between OP/OP metabolite-trypsin (CPF, DZN, TCPy, and IMP).

Interacting Residues	Bond Angle (Å)	Interaction type
CPF		
ALA132 :Cl1	4.4	Hydrophobic
LEU163:H19	2.8	Hydrogen
ILE162:C13	4.8	Hydrophobic
ILE162 :C14	4.8	Hydrophobic
PHE181 :C14	3.9	Hydrophobic
LYS230 :C14	4.5	Hydrophobic
LYS230:O7	2.5	Hydrogen
ALA129 :H22	2.4	Hydrogen
LYS230:S4	2.0	Hydrogen
LYS230:N9	2.0	Hydrogen
DZN		
ALA132: C15	4.1	Hydrophobic
ALA132:N7	2.6	Hydrogen
ALA129: H31	2.9	Hydrogen
ILE162 : C11	4.5	Hydrophobic
ILE162 : C10	4.9	Hydrophobic
PHE181 :C10	4.6	Hydrophobic
LYS23 :C10	4.7	Hydrophobic
LYS230:N6	2.1	Hydrogen
TCPy		
ARG117:O4	1.8	Hydrogen bond
ALA24 : 23017	4.6	Hydrophobic
IMP		
ASN25:H23	2.24	Hydrogen Bond
SER116:H23	2.84	Hydrogen Bond
ARG117:N2	2.73	Hydrogen Bond
ARG117 :N2	3.10	Hydrogen Bond

5.4.5 Fluorescence Spectroscopy

Fluorescence quenching analysis is carried out to quantify and complement the NMR and molecular docking studies of OP-trypsin interaction. Trypsin contains four tryptophan (TRP 51, Trp 141, Trp 215, and Trp 237) residues as intrinsic fluorophores. The representative fluorescence quenching spectra of trypsin in the absence and presence of OP and their metabolites are shown

in Figure 5.11. The trypsin fluorescence intensity quenches gradually with increasing concentration of above-mentioned OP, which is known as fluorescence quenching effect. The Stern-Volmer equation is used to describe the possible quenching mechanism as given in Chapter 2 and shown in Figure 5.12 [Dahiya et al, 2019; Dahiya, et al, 2017; Dahiya and Pal, 2018].

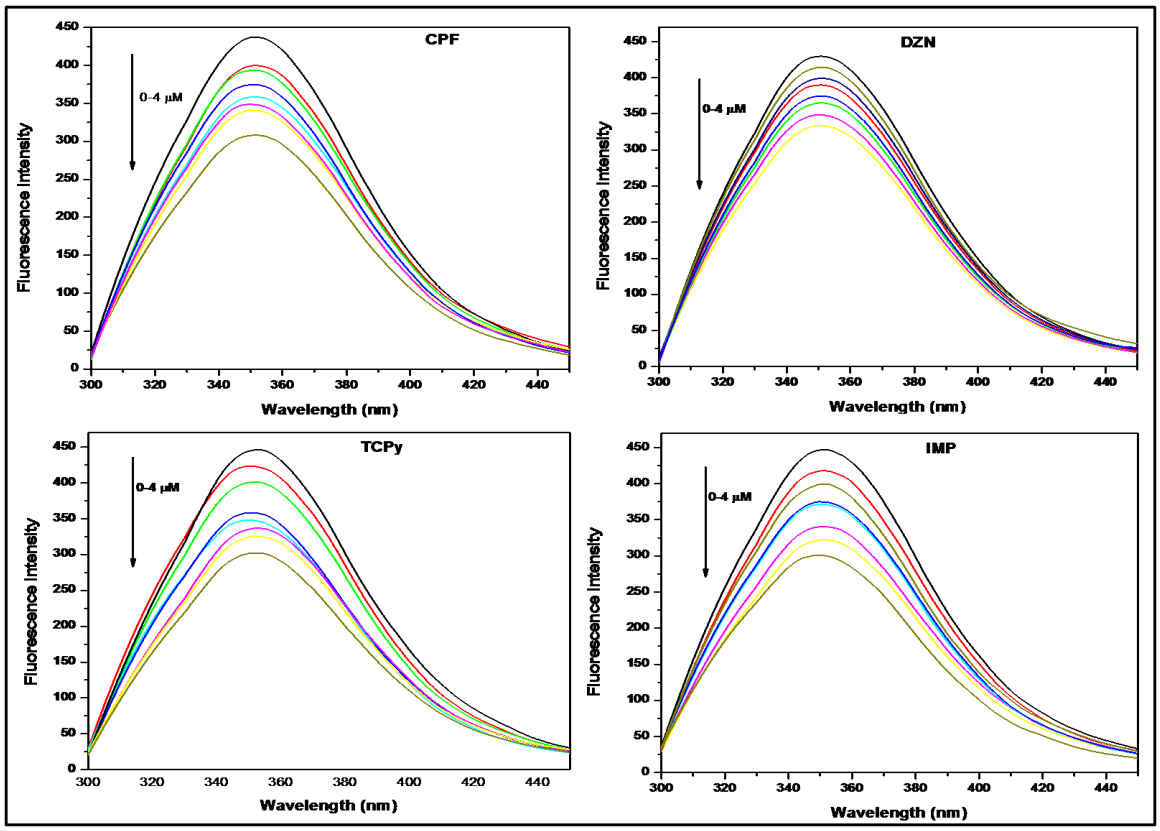

Figure 5.11 : Effect of OP on the fluorescence spectra of trypsin (a) CPF-trypsin (b) DZN-trypsin (c) TCPy-trypsin (d) IMP-trypsin. λ_{ex} = 280 nm; λ_{em} = 350 nm; pH = 7.40; T = 298 K.

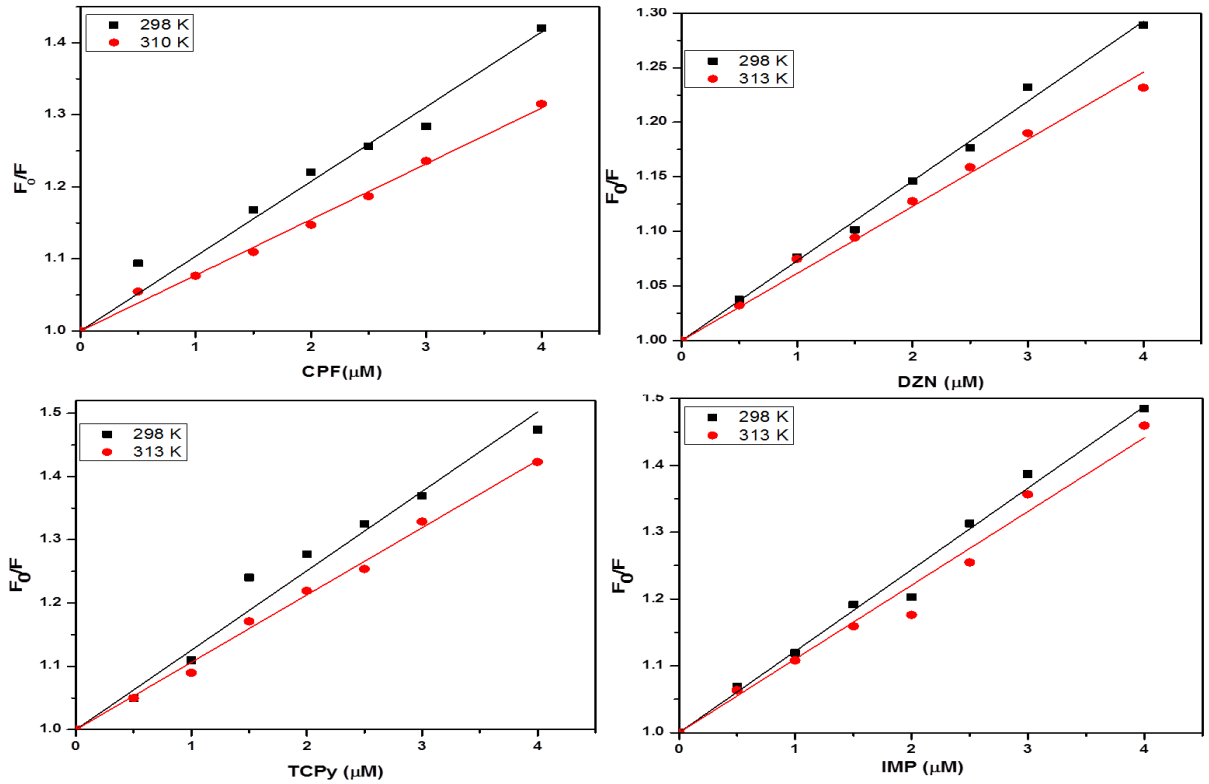

Figure 5.12 : Stern–Volmer plots for fluorescence quenching of the CPF-trypsin, DZN-trypsin, TCPy-trypsin and IMP-tryspin system at different temperatures. c(trypsin) = 1 μM; c(ligand) = 0, 0.5, 1, 1.5, 2.0, 2.5, 3.0 , 4.0 μM; pH= 7.40; T = 298, and 313 K.

The Stern-Volmer results summarized in Table 5.6 clearly indicated that the test OP and their metabolites could bind to trypsin and form the OP-trypsin and OP metabolite-trypsin adducts. The quenching rate constant is more than 2×10^{10} $M^{-1}s^{-1}$. Also, the K_{SV} is inversely related to temperature, which indicated static quenching between test molecule-OP. The stability of complex decreases with an increase in temperature, confirming the static quenching model for all the test OP [Dewey, 1991b,Eftink, 1991,Joseph R. Lakowicz, 2006].

Table 5.6 : The Stern-Volmer constant and the quenching-constant value for mentioned ligands.

	CPF		DZN		TCPy		IMP		
T (K)	$K_{SV}\times10^5$ $Lmol^{-1}$	$K_q\times10^{12}$ $Lmol^{-1}s^{-1}$	$K_{SV}\times10^5$ $Lmol^{-1}$	$K_q\times10^{12}$ $Lmol^{-1}$	$K_{SV}\times10^5$ $Lmol^{-1}$	$K_q\times10^{12}$ $Lmol^{-1}s^{-1}$	$K_{SV}\times10^5$ $Lmol^{-1}$	$K_q\times10^{12}$ $Lmol^{-1}s^{-1}$	R^2
298	1.037 0.035	10.37	0.731 ±0.013	7.31	1.255 ±0.042	12.55	1.220 ±0.029	12.20	0.999
313	0.774 ±0.013	7.74	0.614 ±0.013	6.14	1.064 ±0.016	10.64	1.104 ±0.036	11.04	

Further, to analyze the binding constant (K_A) and the number of binding sites (n), Eq.(2.26) is used. The binding strength of test ligands to trypsin is the main factor in determining OP and their metabolite metabolism in the body. The values of K_A and n at 298 K and 313 K, are listed in Table 5.7. The value of n shows that there is only one binding site in trypsin for these test OP and

their metabolites. On the other hand, the binding affinities of metabolites are much stronger than parents. The results are in line with STD NMR data. Also, it is clear from the Table that the temperature has a significant effect on the stability of the OP-trypsin complexes. The binding constant for DZN, TCPy, and IMP decreases with an increase in temperature and indicate that the binding process is exothermic, and the complex is not stable at high temperature. Whereas in the case of CPF-trypsin, the binding constant increases and shows that the reaction is endothermic and different from other test ligands. This can be the reason for different trends shown by CPF in case of alteration of trypsin enzyme activity. Further, the molecular interaction forces involved in the ligand-protein interaction include hydrogen bonds, van der Waals forces, electrostatic interactions, and hydrophobic interactions. In order to obtain more comprehensive knowledge on the contributions of the interaction forces, the corresponding thermodynamic parameters, including ΔH (enthalpy change) and ΔS (entropy change), are calculated and shown in Table 5.7. As given in Table 5.7, the negative ΔG values indicate that the test ligand interaction with trypsin is a spontaneous process. In the case of DZN and TCPy, the negative ΔH and ΔS show the van der Walls and hydrogen bonds playing a major role in the binding process. In case of IMP, the negative ΔH and positive ΔS show that electrostatic interaction with hydrogen bonding are effective molecular forces during the complexation. In the case of CPF, the positive ΔH and ΔS show the role of hydrophobic interaction [Ross & Subramanian, 1981].

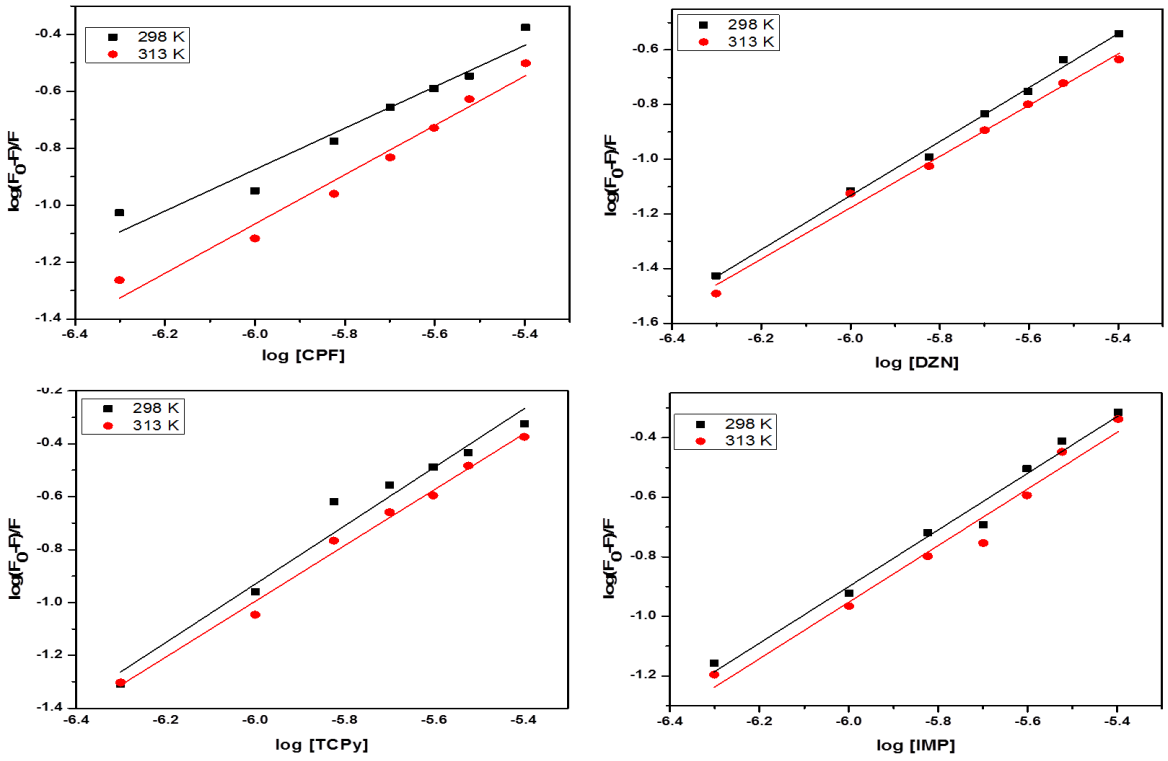

Figure 5.13 : The double log plot of fluorescence of trypsin by CPF, DZN, TCPy, and IMP.

Table 5.7 : Binding constant and thermodynamic parameters for CPF-trypsin, DZN-trypsin, TCPy-trypsin, and IMP-trypsin.

Compounds	Temp.	$K_a \times 10^4$	n	ΔG (KJ mol^{-1})	ΔH (KJ mol^{-1})	ΔS ($JK^{-1}mol^{-1}$)
CPF-trypsin	298	0.3083	0.72	-19.9087	75.9789	321.771
	313	1.343	0.86	-24.735	75.9789	321.771
DZN-trypsin	298	6.234	0.98	-27.353	-160.42	-446.534
	313	2.80	0.93	-20.655	-160.42	-446.534
TCPy-trypsin	298	48.5	1.10	-32.436	-42.783	-34.723
	313	21.2	1.05	-31.915	-42.783	-34.723
IMP-trypsin	298	6.4	0.95	-27.4183	-6.906	68.83
	313	5.6	0.95	-28.45	-6.906	68.83

5.5 CONCLUSION

The experimental results obtained from STD NMR and fluorescence confirms that the OP-protein and OP metabolite-protein show moderate interaction with trypsin and form ground state complex. The docking results show that both hydrogen bonding and hydrophobic forces are involved in CPF-trypsin, DZN-trypsin, TCPy-trypsin, whereas IMP-trypsin interaction occurs through hydrogen bonding. The enzyme activity assay shows that OP and metabolites concentrations altered the trypsin activity significantly. This study is pivotal in terms of knowledge about the effects of these OP on digestive enzymes. It demonstrates the toxic effect of OP and their metabolite on humans and animals. OP have tremendous benefits in agriculture, medicine, and veterinary. But the knowledge about the indirect side effects of these OP is not well known and need to be explored continuously. The knowledge of side effects of these OP can be used as a secondary factor to help to select suitable OP and their doses. Although the current study is not showing complete inhibition of target enzymes by these OP, but a partial decrease in trypsin activity dictates consideration for limited use of these OP for insect control.

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