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Activity of Analogs of Anticancer Drugs on the Serine Protease Enzymes Subtilisin and Chymotrypsin

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ACTIVITY OF ANALOGS OF ANTICANCER DRUGS ON THE SERINE PROTEASE ENZYMES SUBTILISIN AND CHYMOTRYPSIN

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ACTIVITY OF ANALOGS OF ANTICANCER DRUGS ON THE SERINE PROTEASE ENZYMES SUBTILISIN AND CHYMOTRYPSIN

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Dean, Graduate Studies and Research Date

I dedicate this thesis to my parents, who are great inspiration to me and also to my advisor Dr. Kevin Williams, who helped greatly in guiding me through out.

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I wish to express my hearty thanks and appreciation for the contribution and help of the many people who made this work possible: Dr. Kevin Williams for his everlasting support and patience, members of my research group especially Chaitanya Rapolu who helped me a lot in collecting my data. I owe special thanks to Dr. Kevin Williams who was involved in germination of this idea and also provided guidance and support till its completion.

I would like to acknowledge specially to Dr. Cathleen Webb and Dr. Bangbo Yan for giving right advice in order to accomplish my goals and supported me so much. I would also like to express my sincere gratitude and thanks to Shannon Marble for always helping me and making things easier and enjoyable.

Lastly I would like to thank my friends and family for their outstanding support to me when needed. The chemistry department and the entire faculty at WKU gave me the opportunity to enjoy my Western study life.

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Directed by: Dr. Kevin Williams, Dr. Bangbo Yan, and Dr. Eric Conte

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The anticancer activity of several platinum compounds is due to the formation of complexes with DNA. We hypothesize that the size and shape of the platinum compounds would impact interaction with proteins, and these interactions may be partly responsible for the anticancer activity. Chymotrypsin and subtilisin are serine proteases that have a histidine residue in the active site. We are investigating the inhibition of the digestive enzymes chymotrypsin and subtilisin by analogs of the anticancer drug cisplatin and trying to discern trends in the inhibition as the active site residues vary. In our research, we found that the enzyme subtilisin did not show any significant inhibition with different platinum compounds we used, while chymotrypsin showed inhibition only with the potassium tetrachloroplatinate and this inhibition is concentration dependent.

I. INTRODUCTION

A. HISTORY

Recent studies by the American Cancer Society reveal that nearly 25% of deaths in the United States are due to cancer; and more in men than in women. Statistics show that 1 in every 3 women are probable to be affected by some type of cancer.[1]

Cancer is as old as the human race. Since times immemorial, there have been various herbal treatments. The beginning of modern era of chemotherapy is with 20th century, when Paul Ehrilch compiled a book on Chemotherapy, in which he applied Paracelian idea of specific remedies for specific diseases to cancerous cells that can be damaged by chemicals without damage to healthy tissue. Later, profound Lymphoid and Mylenoid tissue suppression were noticed in the autopsy of bodies exposed to Mustard gas during World War I.[2] Based on this observation, Goodman and Gilman proposed that this gas can be used to treat Lymphoma. These findings led to the discovery of new chemical agents that can reach and destroy cancer cells in the body. Due to this early positive response to cancer treatment, the United States Congress created a National Cancer Chemotherapy Service Center (NCCSC) at the National Cancer Institute.

Transition metals play an important role in medicinal biochemistry due to their property of formation of coordinate complexes. [3, 4] They have the ability to exhibit different oxidation states and react with different negatively charged ions. The discovery of this property led to development of metal based drugs used in cancer therapy. Their use dates back to 16th century.

In 1845, Peyrone's chloride or cis-diammine dichloro platinum was first synthesized.[5] After 50 years, Alfred Warner deduced its structure [6]. Seventy years later,

scientists discovered that electrolytes produced from platinum compounds were responsible for bacterial elongation they observed. One of these products was discovered to be cis-diamminedichloro platinum.[7]

In December 1978, cisplatin was approved by USFDA for the treatment of testicular cancer. Since this has many side effects ,[8] second and third generation cisplatin were developed.[9,10] Carboplatin and oxaliplatin were less toxic and hence can be used in higher doses. They are used in the treatment of ovarian and colon cancers respectively.

B. CHEMISTRY OF PLATINUM COMPLEXES

Platinum was found to exhibit +1, +2, +3, +4 oxidation states but the most dominant forms are +2 and +4 [12]. The general structural formula of platinum complexes is cis $[PtX_2 (NHR_2)_2]$.[11] In which R = organic fragment, X = leaving group like chloride, carboxylate, nitrate or oxalate. The dominant valence states of platinum complexes +2, +4 form square planar and octahedral complexes respectively. Most of these platinum complexes follow certain rules for showing anticancer activity. One important rule to be followed by these anticancer complexes is that they need to be octahedral or square planar. The complexes should be electrically neutral and they should have NH group for formation of hydrogen bonding with the target species. Even though the complex is neutral electrically it becomes charged species after ligand exchange. The complexes exchange only some ligands in reaction with the biological molecules. The leaving group should be approximately 3.4 Å apart from the platinum on the molecule. There should be two cis monodentate or one bidentate leaving group should be required. The rates of exchange of these groups should fall into a restricted region; since too high a reactivity will mean that the chemical reacts immediately with blood constituents and never gets to

the tumor. Any deviations from these rules will result in absence of anticancer activity.[7, 12] But there are some exceptions where compound like trans form of cisplatin with bulkier ligands was found to show anticancer activity.

Ligand exchange kinetics of platinum complexes plays an important role in the anticancer activity.[8, 13] The platinum ligand bond, which has a thermodynamic strength of a typical coordination bond, is much weaker than C-C, C-N, or C-O single and double bonds. The ligand exchange behavior provides a reaction of minutes to days so that it can reach its target site despite of various interactions. In comparison to cisplatin, carboplatin and oxaliplatin have slower ligand exchange kinetics, which shows its decreased side effects and resistance.

C. MECHANISM OF ACTION ON DNA:

Cisplatin is a platinum containing anticancer drug, which is administered intravenously as a saline solution containing sodium chloride. Due to high concentration of chloride ions, cisplatin remains intact even after entering the blood stream. [8, 14] The neutral compound is then up taken by the cell either by active diffusion or passive diffusion. Cellular uptake of cisplatin and its analogs is influenced by copper transporters (Figure 1.1.)

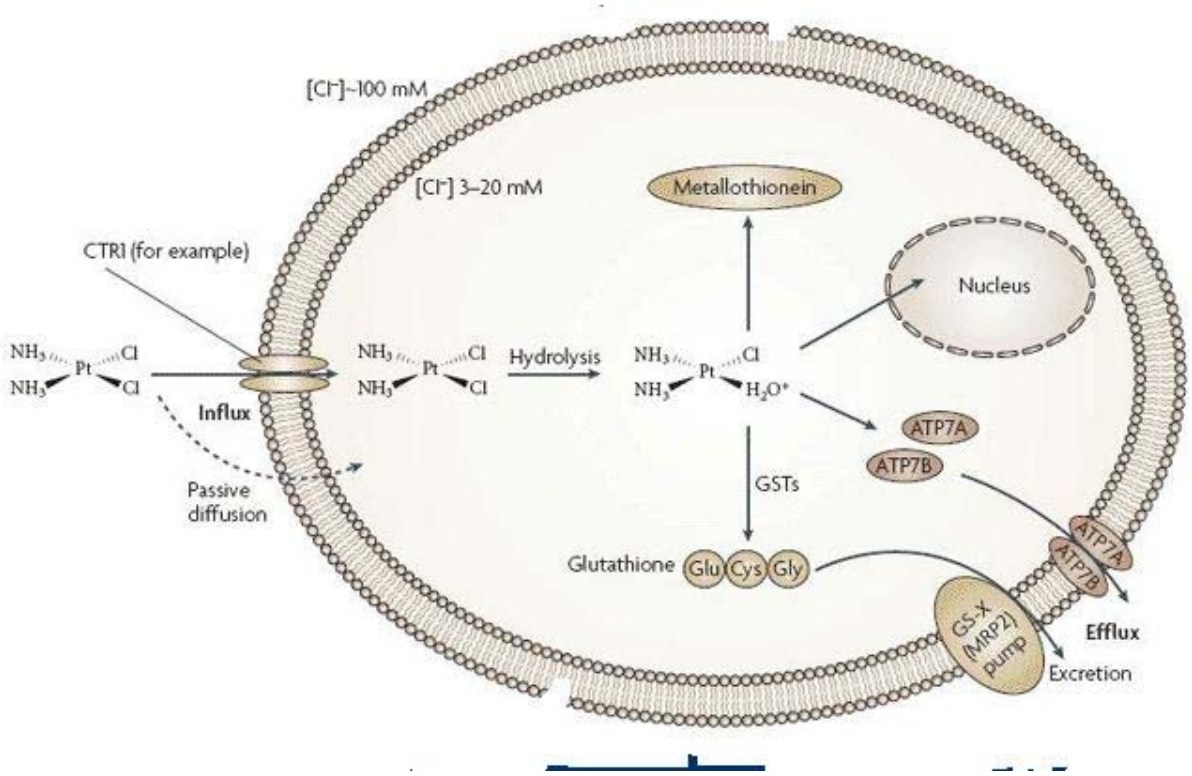

Figure 1.1. Mechanism of action of cisplatin [15]

Copper transporter (CRT1) is the major copper influx transporter that controls tumor cell accumulation and cytotoxic effects of cisplatin, carboplatin and oxaliplatin. Hence, if the CTR1 gene is mutated or deleted, this leads to cisplatin resistance and

reduced platinum levels. Degradation and disintegration of CTR1 gene can be triggered by simultaneous activity of copper and cisplatin, as both of them can reduce each other's uptake.[16-19] As the neutral Cisplatin molecule enters the cell, it undergoes hydrolysis (due to lower concentration of chloride ions); the water molecule replaces the chlorine ligand, generating a positively charged species. DNA is the primary target of hydrolyzed cisplatin; specifically the N7 guanine, leading to platinum cross links between adjacent guanines on same DNA strands (both interstrand and intrastrand cross links are formed).[14, 20]Major contribution to platinum drug resistance comes from two kinds of exporters, namely, ATP 7A and ATP 7B (copper exporters) and MRP2 or ABCC2 (Glutathione S-conjugate export GS-X pump).[21]

D. REACTION OF PLATINUM COMPOUNDS WITH PROTEINS AND AMINO ACIDS:

Since the discovery of cisplatin, much has been learned about how this drug affects the cell, and the DNA interactions have been studied. Despite the fact that DNA adducts are primarily responsible for the anticancer activity, up to 75-85% of the covalently bound cell associated cisplatin has been found to be bound to protein.[22] [Pt(dien)Cl]Cl reacts faster with methionine analogs than with guanosine 5'-monophosphate.[23] So, reaction with an amino acid can occur faster than reaction with guanine. It is very recently known that platinum complexes (cisplatin, carboplatin and oxaliplatin) react with Ctr1, copper transporter 1 protein.[18] Ctr1 transporters contain three transmembrane domains, an NH_2 terminal methionine rich motif consisting of three to five methionine in MxM and/or MxxxM arrangements, and a COOH-terminal cysteine or histidine motif. Platinum complexes interact with the Ctr1 at N terminal domain, rich in methionine and histidine residues, in 1:1 and 2:1 Pt:protein stoichiometries.[19,20]

The chemistry of the platinum is significant for its unique characteristics that can be utilized when it is combined with other compounds. Platinum is described as a "soft" acid. Elements can be considered soft when they have a larger radius, lower electronegativity and higher polarizability, which places then down in the periodic table among nonmetals. Hard or soft characteristic for an element is relative. By theory soft acids will prefer to react with soft bases, and hard acids will prefer to react with hard bases. Amino acids present metal ions with a variety of potential donor atoms. In amino acids, a sulfur donor residue is one of the primary targets owing to its relative softness for platinum.[23] The thioether functional group of methionine has an advantage on

binding with platinum over the thiol group of cysteine because thiol groups form disulfide bonds. Platinum can bind to the lone pair of nitrogen atoms of amino acid (histidine), only when S-donor groups are absent. Hence methionine, cysteine and histidine are amino acids that can interact with platinum complexes.[24] As selenium is softer than sulfur, it will react with the soft platinum (II). Researcher Steve Chmley in his experiments using an analog of cisplatin ([Pt (dien) Cl] Cl) and reacting it with both methionine and selenomethionine suggests that selenomethionine is kinetically favored in reaction with [Pt (dien) Cl] Cl to methionine.[25]

Cisplatin was reported to be associated with proteins initially; it is likely that many of these protein interactions occur prior to reaction with DNA. It is very difficult to determine the extent or specific site of reaction of a platinum (II) diamine complex with a particular protein. In our research we will utilize enzyme inhibition by a platinum complex as a means of probing for specific binding, most likely to the active site residues. Comparing the inhibition behavior of the platinum complexes with the kinetics of reaction as a function of bulk will enable us to determine whether the differences are due to coordination of the amino acids or to interactions with the surrounding protein surface.

In our research we are using the enzyme inhibition assays, which allows us to assess both the rate and extent of inhibition as a function of bulk and leaving ligand. The investigation of a variety of different enzymes will help us to discern trends in inhibition as the active site residues vary. In our research we are using subtilisin and chymotrypsin enzymes.

F. SERINE PROTEASES:

Enzymes are biological catalysts that accelerate the rate of reaction without being modified during process. Serine proteases are named after the reactive serine residue located in the active site that is essential for the function of the enzyme . Serine proteases are enzymes that cleave peptide bonds in proteins. The active site of serine proteases contains three critical amino acids: serine, histidine and aspartate. These residues are often referred to as the "catalytic triad." When the linear sequence of amino acids folds into its tertiary structure, these three residues are arranged in such a fashion that enables the side chain of the serine residue to become negatively charged through the loss of the hydrogen off the hydroxyl R group to histidine.[26-28] This nucleophile can then make an attack on the carbonyl group of the peptide bond that is to be cleaved.

G. SUBTILISIN:

Subtilisin is a non-specific protease obtained from *Bacillus subtilis,* it consists of 275 amino acid residue, which consists of several alpha –helices and a large β sheets (Figure 1.2).

The catalytic triad of subtilisin consists of Asp-32, His-64 and Ser-221.The carboxylate side-chain of Asp-32 hydrogen-bonds to a nitrogen-bonded proton on His-64's imidazole ring. The other nitrogen on His-64 hydrogen-bonds to the O-H proton of Ser-221, which results in charge-separation of O-H, with the oxygen atom being more nucleophilic. Therefore the oxygen atom of Ser-221 can attack incoming substrates.[30]

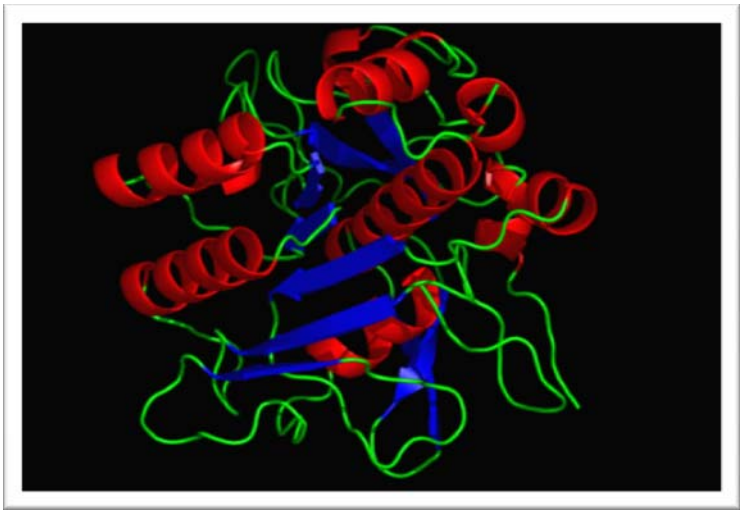

Figure 1.2 .Crystal structure of subtilisin [29]

Amino acid sequence of subtilisin

AQSVPYGISQIKAPALHSQGYTGSNVKVAVI'**D'**SGIDSSHPDLNVRGGASFVPSET

NPYQDGSS'**H'**GTHVAGTIAALNNSIGVLGVAPSASLYAVKVLDSTGSGQYSWIIN

GIEWAISNNMDVINMSLGGPTGSTALKTVVDKAVSSGIVVAAAAGNEGSSGSTS

TVGYPAKYPSTIAVGAVNSSNQRASFSSAGSELDVMAPGVSIQSTLPGGTYGAYN

GT'**S'**MATPHVAGAAALILSKHPTWTNAQVRDRLESTATYLGNSFYYGKGLINVQ

AAAQ.

H. CHYMOTRYPSIN

Chymotrypsin digests proteins in the intestine by hydrolyzing the peptide bond at the carboxy side of a hydrophobic amino acid. David Blow first determined the three-dimensional structure of chymotrypsin in 1967. Chymotrypsin is roughly spherical and consists of three polypeptide chains, which are linked by disulfide bonds. (Figure 1.3)

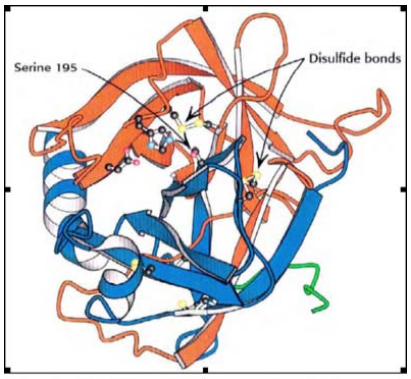

Figure 1.3. Three dimensional structure of chymotrypsin [31].

Chymotrypsin consists of 245 amino acids. In the catalytic triad of chymotrypsin, the side chain of serine 195 is hydrogen bonded to the imidazole ring of histidine 57. The –NH group of this imidazole ring is hydrogen bonded to the carboxylate group of aspartate 102.

Amino acid sequence of chymotrypsin:

CGVPAIQPVLSGLSRIVNGEEAVPGSWPWQVSLQDKTGFHFCGGSLINENWVVTAA'**H**'CGV

TTSDVVVAGEFDQGSSSEKIQKLKIAKVFKNSKYNSLTINN'**D**'ITLLKLSTAASFSQTVSA

VCLPSASDDFAAGTTCVTTGWGLTRYTNANTPDRLQQASLPLLSNTNCKKYWGTKIKDAM

ICAGA'**S**'GVSSCMGDSGGPLVCKKNGAWTLVGIVSWGSSTCSTSTPGVYARVTALVNWVQQ

II. EXPERIMENTAL

A. Materials used

The enzyme subtilisin (protease from *Bacillus licheniform*) was purchased from the Sigma Aldrich Company. The substrate used for subtilisin was N-Succinyl L-Phenylalanine p-nitroanilide purchased from Sigma Aldrich Company. We prepared the storage buffer (50 mM sodium acetate buffer, pH 5) and assay buffer (phosphate buffer, pH 7) in our laboratory.

The enzyme chymotrypsin from bovine pancreas was purchased from Sigma Aldrich Company; the substrate used for chymotrysin wass N-Benzoyl-L-Tyrosine Ethyl Ester Solution (BTEE) was purchased from the same company. The assay buffer we used for chymotrypsin is Tris HCl Buffer, pH 7.8 and we prepared both 1mM Hydrochloric Acid Solution and Calcium Chloride Solution 2M ($CaCl_2$) in our laboratory.

B. PREPARATION OF SOLUTIONS

Substrate Solution (BTEE)

Weigh about 37mg of BTEE into a 100 mL volumetric flask.

Calcium Chloride Solution 2M ($CaCl_2$)-

294mg/ml of Calcium chloride dihydrate is diluted 100 mL in water.

1mm Hydrochloric Acid Solution (HCl)-

Solution is made in purified water by diluting 0.10 ml of Hydrochloric Acid Solution to 100 ml in 100 ml volumetric flask

C. PLATINUM COMPOUNDS

Different types of platinum compounds were used.

1) Cis-diamine dichloroplatinum (II)-99% was purchased from Sigma Aldrich.

2) Ethylenediamine platinum (II) dinitrate was synthesized in our lab in previous research.

3) N, N, N', N'- tetrametylethylenediamine Platinum (II)-dinitrate was synthesized in the lab from previous research.

4) Potassium tetrachloroplatinate from Sigma Aldrich

5) Ethylenediamine platinum (II) oxalate was purchased from Sigma Aldrich.

6) N, N, N', N'- tetrametylethylenediamine platinum (II)-oxalate was synthesized in our lab in previous research.

The instrument we used is UV-visible spectrophotometer, models are uv-1201, and uv-1601 manufactured from SHIMADZU in year 2000.

A. ENZYME-ASSAY PROTOCOL USED FOR SUBTILISIN.

20 mg of N-Succinyl L-Phenylalanine P-Nitroanilide (substrate) is weighed and 230 microliters if DMSO is added to it.

Preparation of stock subtilisin:

10 mg of subtilisin was weighed and 900 µl of storage buffer was added and 100 µl of calcium chloride solution was added because there was no calcium in the subtilisin enzyme.

Preparation of control subtilisin

Add 400 µl of stock subtilisin, to a new vial and 400 µl of storage buffer.

Control assay:

In a clean cuvette, 900 µl of assay buffer and 20 µl of substrate, then 100 µl of control subtilisin sample, this solution was mixed briefly and gently and the activity was measured every 15 minutes.

Preparation of inhibited subtilisin

Add 400 µl of stock subtilisin, to a new vial and 400 µl of platinum compound in storage buffer. Some of the platinum compounds are not easily soluble in storage buffer, so we used a magnetic stirrer for the easy solubility of platinum compounds.

Inhibition assay:

In a clean cuvette, 900 µl of assay buffer and 20 µl of substrate were mixed, then 100 µl of inhibited subtilisin sample was added and the solution was mixed briefly and gently, and the activity was measured every 15 minutes.

The UV-spectrometer was used, the kinetics option was selected and the wavelength we were using here is 410 nm and the measuring time parameter was varied frequently. We used lag time of 120 sec for few experiments (Fig. 3.3), (Fig. 3.5), (Fig. 3.8) as the solution takes time to get settled and to show the enzymatic activity.

B. ENZYME-ASSAY PROTOCOL USED FOR CHYMOTRYPSIN:

Stock Chymotrypsin:

1mg of chymotrypsin was weighed and diluted with 10 ml of HCl solution.

Control Chymotrypsin

500 µl of stock chymotrypsin was added to a new vial and mixed with 400 µl of storage buffer.

Control Assay

In a clean cuvette, 710 µl of assay buffer and 700 µl of substrate, 40 µl of $CaCl_2$ and then 100 µl of control chymotrypsin sample and the solution was mixed briefly and gently, and the activity was measured.

Inhibited chymotrypsin:

500 µl of stock chymotrypsin and 500 µl of platinum compound in storage buffer were added to a new vial.

Inhibition assay:

In a clean cuvette, 710 µl of assay buffer and 700 µl of substrate, 40 µl of $CaCl_2$ and then 100 µl of inhibited chymotrypsin sample and the solution was mixed briefly and gently, and the activity was measured.

The UV-spectrometer was used, the kinetics option was selected and the wavelength we were using here was 256 nm and the measuring time parameter was varied.

For both the subtilisin and chymotrypsin enzymes, the enzymatic assays are done for about an hour. The activity (ΔA/min) of both the control sample and inhibited sample was measured for every 15 min, 30 min, 45 min, and 60 min and recorded. For some inhibition assays we even measured the activity for every 15 min interval for about 2 hours. Enzymes were incubated with different concentrations of specific platinum compounds at specific time and the enzymatic activity was measured. Enzymatic activity was determined relative to control that had no platinum compound. Each experiment was done three times to determine the precision.

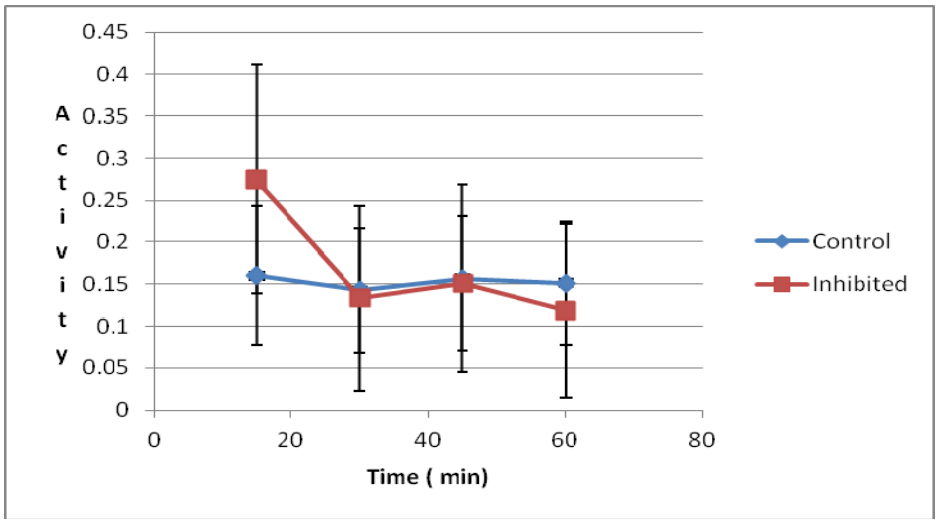

Figure 3.1. Activity of subtilisin with cisplatin (1mg in 3mL)

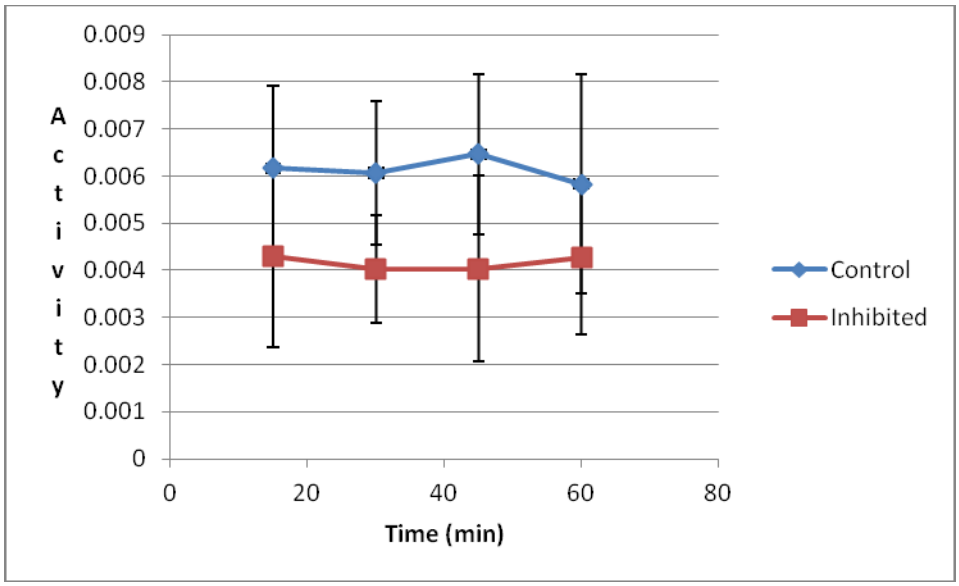

Figure 3.2. Activity of subtilisin with cisplatin (1mg in 1mL)

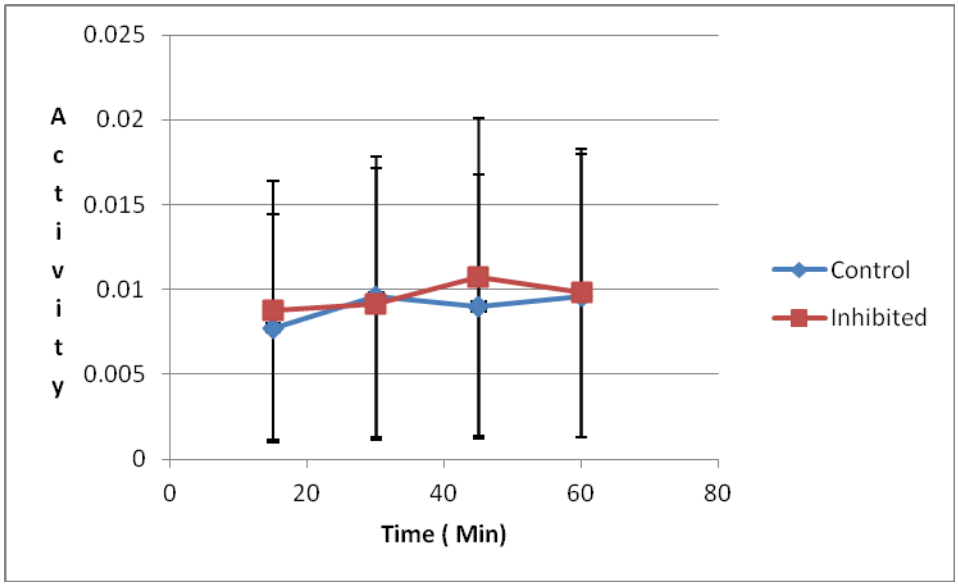

Figure 3.3. Activity of subtilisin with cisplatin (1mg in 1mL) with lag time 120 sec because solution will take time to settle and show enzymatic activity.

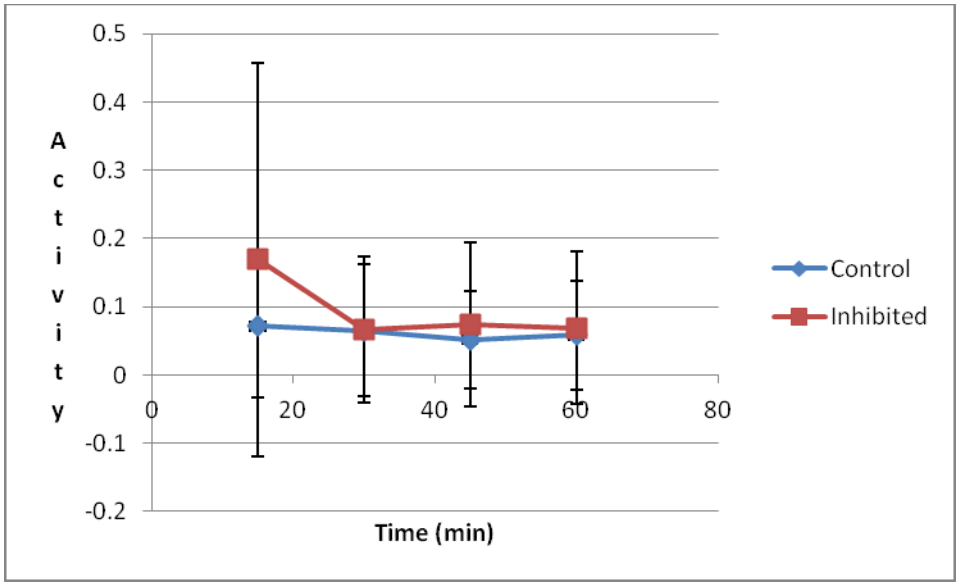

Figure 3.4. Activity of subtilisin with ethylenediamine platinum (II) oxalate (1mg in 3mL)

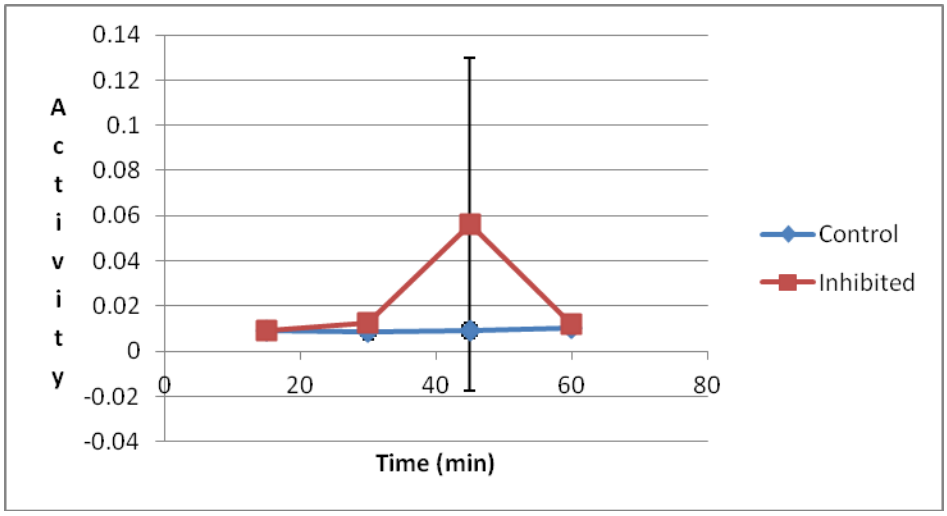

Figure 3.5.Activity of subtilisin with ethylenediamine platinum (II) dinitrate (1mg in 1mL) with 120 sec because solution will take time to settle and show enzymatic activity.

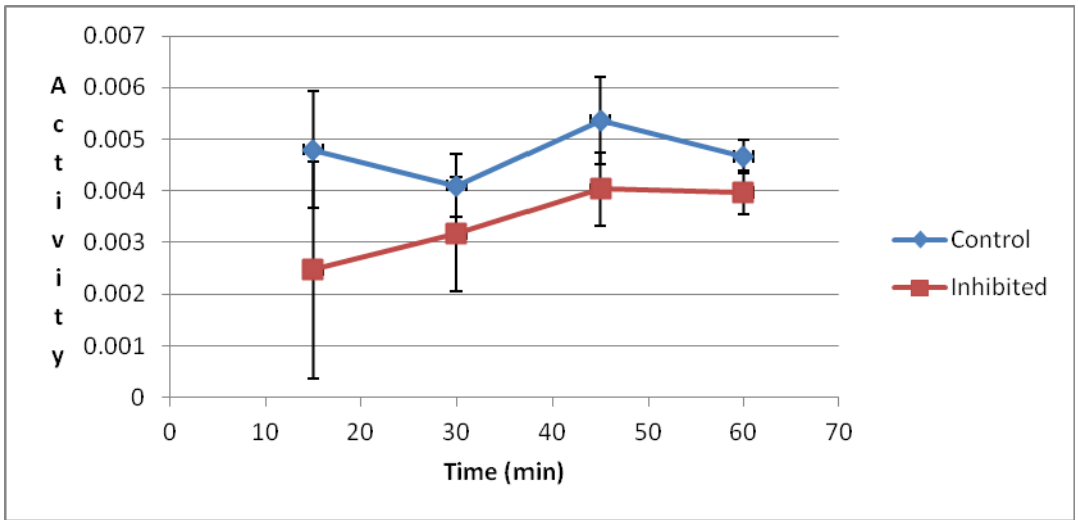

Figure 3.6.Activity of subtilisin with N,N,N',N'- tetramethylethylene diamine Pt(II)-dinitrate (1mg in 3mL)

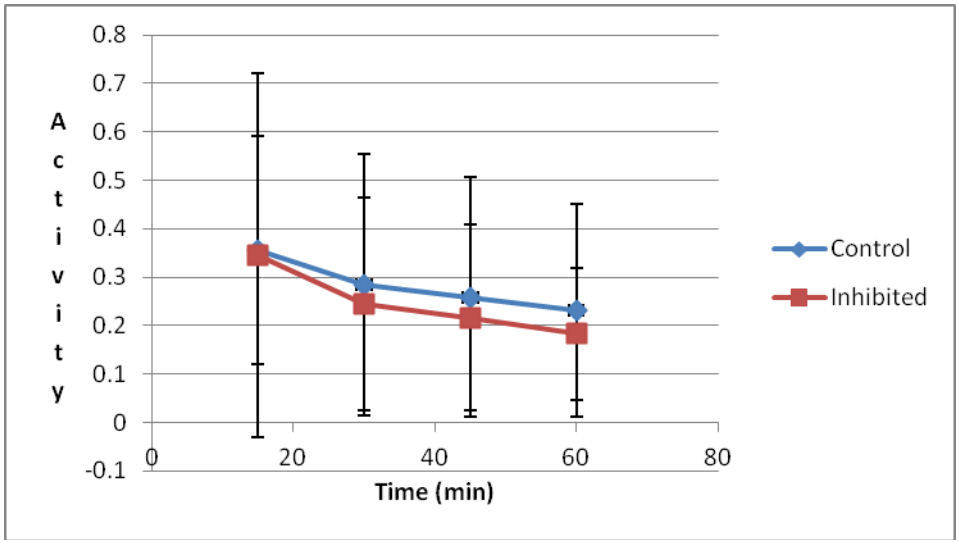

Figure 3.7. Activity of subtilisin with potassium tetrachloroplatinate (1mg in 1mL)

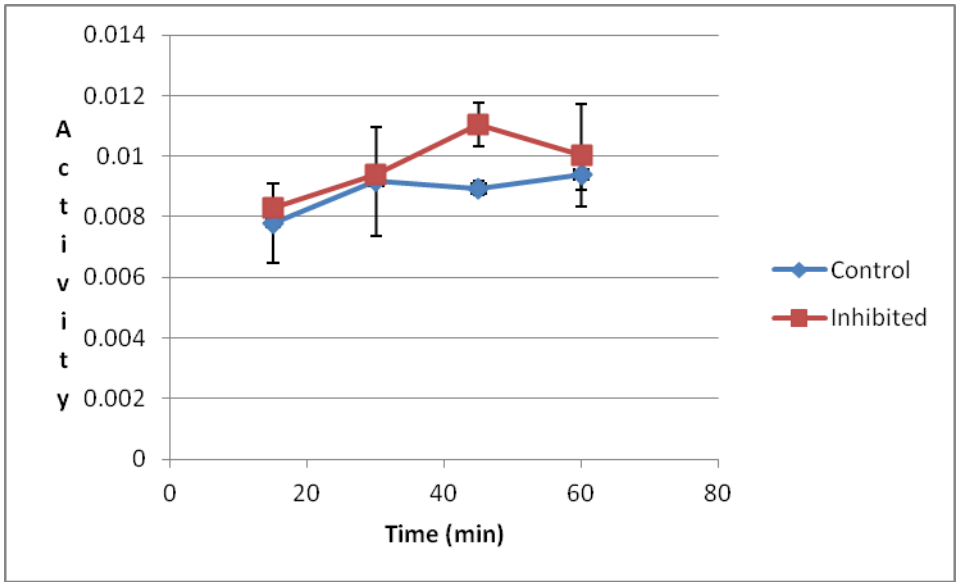

Figure 3.8. Activity of subtilisin with potassium tetrachloroplatinate (1mg in 1mL) with lag time 120 sec because solution will take time to settle and show enzymatic activity.

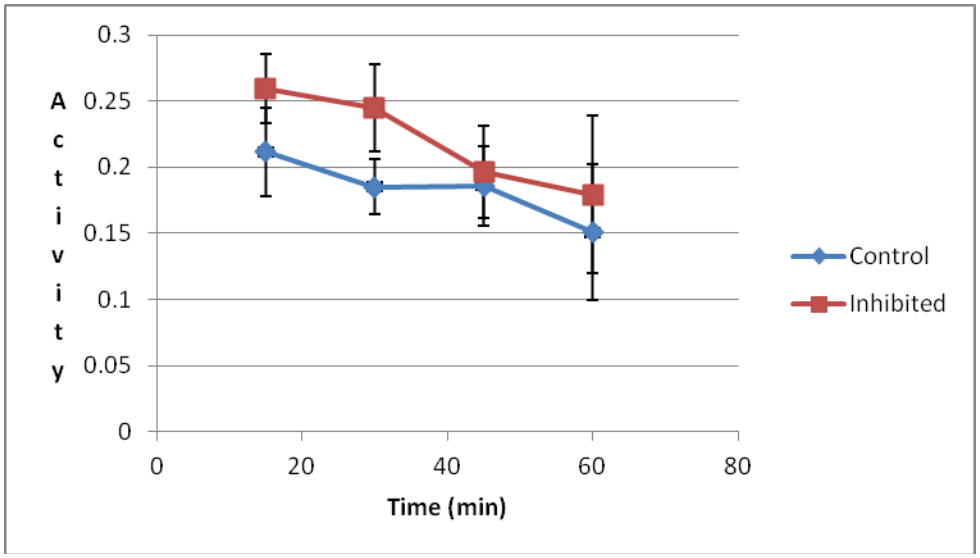

Figure 3.9. Activity of subtilisin with Potassium tetrachloroplatinate (5mg in 1mL)

We first tried the enzymatic assay for subtilisin with 1mg of cisplatin in 3 mL of storage buffer, when the enzymatic activity was measured for both the control and inhibition assays and the graph was plotted between activity and time. We observed that cisplatin at this concentration did not show any inhibition of the enzyme subtilisin, as the enzymatic activity of control and inhibitions assays were overlapping each other (Fig. 3.1). We then tried to increase the concentration of cisplatin 1mg in 1 mL of storage buffer, did the same procedure and when we plotted the graph (Fig. 3.2) , the increase in concentration showed some inhibition but this inhibition was inconclusive, as the error bars of control and inhibition assays activity of enzyme were overlapping each other. For the above two experiments the lag time was 0 sec. So, we changed the parameter of time with lag time 120 sec and measured the activity of enzyme with cisplatin 1mg in 1 mL of storage buffer; however the change measuring time parameter did not reveal any inhibition of the enzyme subtilisin (Fig. 3.3), as both the control and the inhibition enzymatic activity were overlapping each other.

As subtilisin did not show any significant inhibition with cisplatin, we then tried to see the activity of subtilisin with 1mg of ethylenediamine platinum (II) oxalate in 3 mL of storage buffer. We have done the same procedure and when the graph is plotted (Fig. 3.4), there was no inhibition of the enzyme subtilisin as both the activity of control and inhibitory assays were overlapping each other. We tried with 1mg of ethylene diamine dinitrate in 1 mL of storage buffer (Fig. 3.5) and 1mg of N,N,N'.N' tetramethylethyelnediamine platinum (ll) dinitrate in 3 mL of storage buffer, as this platinum compound had a bulkier ligand (nitrate) and this bulkier ligand may effect the enzymatic activity in 3 mL of storage buffer (Fig. 3.6) to see the activity of the enzyme

and when the graph was plotted, there was no inhibition of the enzyme with both the platinum compounds.

Later, we tried with 1mg of potassium tetrachloroplatinate in 1 mL of storage buffer to see the activity of the enzyme subtilisin, we have done the same procedure. When we plotted the graph it seen that there was some inhibition of enzyme subtilisin, but the inhibition was inconclusive as the error bars of both the control and inhibition enzyme activity were overlapping each other (Fig. 3.7). We tried with the same concentration of platinum compound, but we changed the measuring time parameter with lag time 120 sec and when the graph was plotted it is seemed that there was inhibition of the enzyme (Fig. 3.8). As there was no inhibition of enzyme with 1mg of potassium tetrachloroplatinate in 1 mL of storage buffer, we tried to increase the concentration to 5mg of potassium tetrachloroplatinate in 1 mL of storage buffer and measured the activity (Fig. 3.9). When the graph was plotted it was seen that there was no inhibition of the enzyme with this concentration of potassium tetrachloroplatinate.

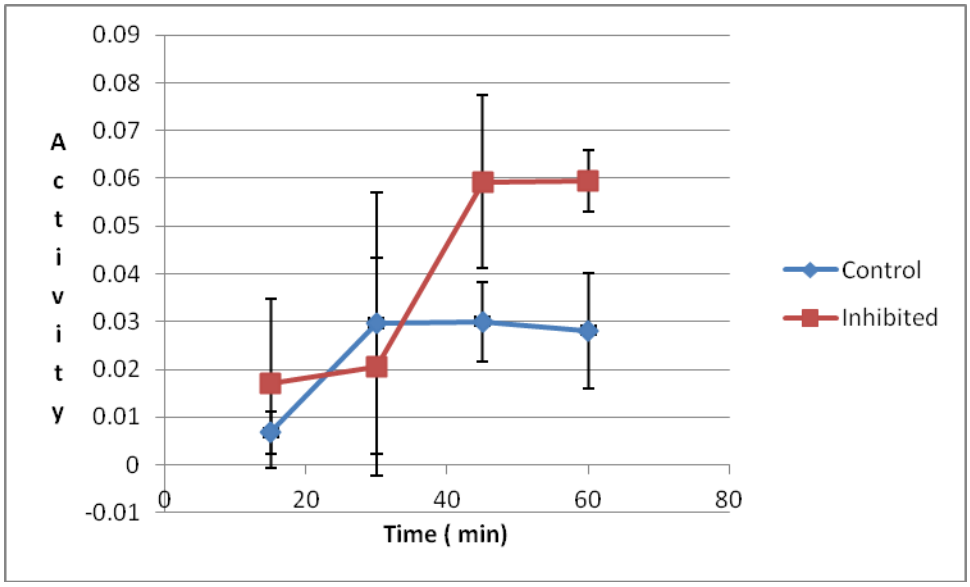

Figure 3.10. Activity of chymotrypsin with cisplatin (1mg in 1mL)

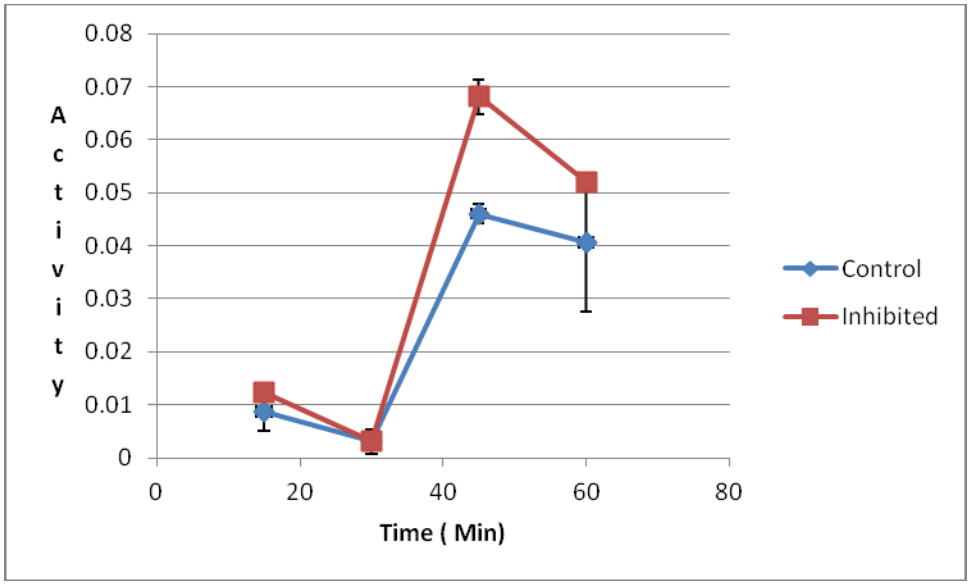

Figure 3.11. Activity of chymotrypsin with cisplatin (2mg in 1mL)

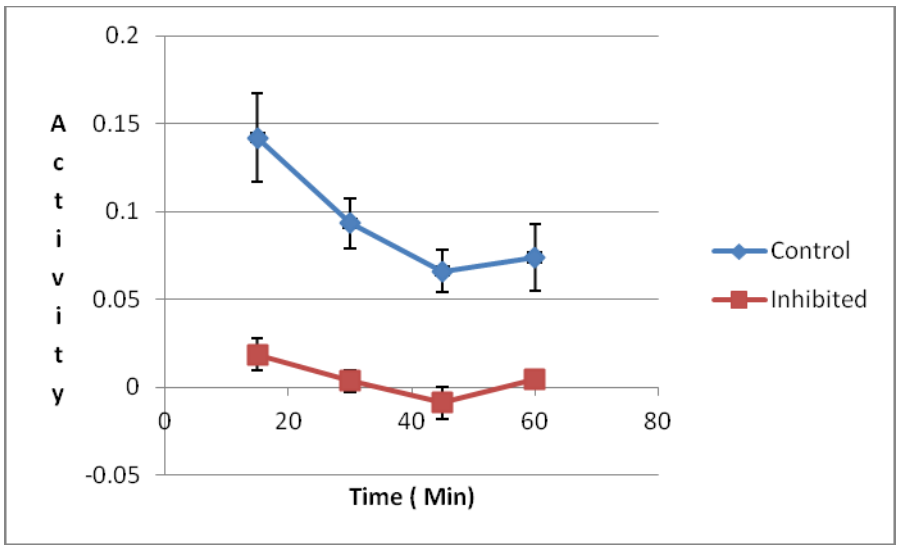

Figure 3.12. Activity of chymotrypsin with potassium tetrachloroplatinate (1mg in 1mL)

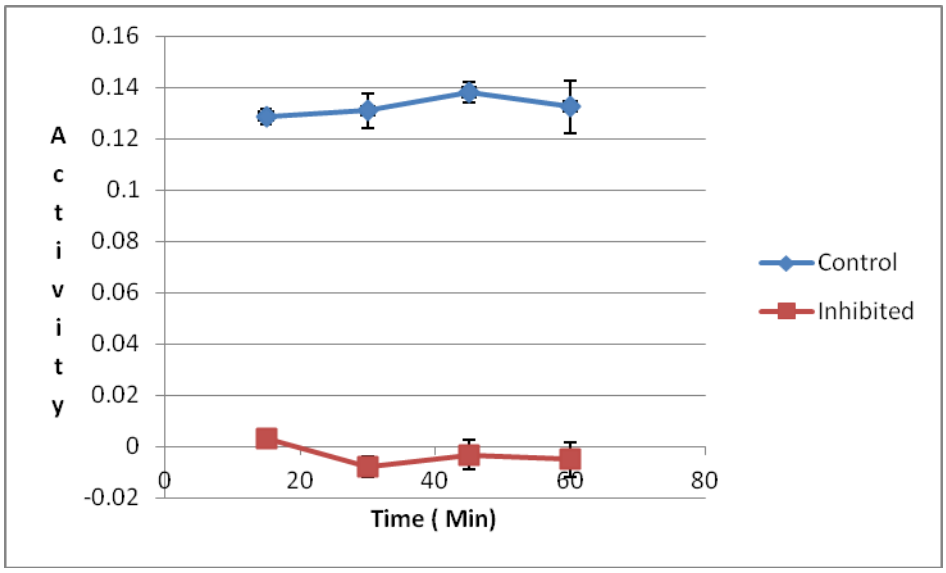

Figure 3.13. Activity of chymotrypsin with potassium tetrachloroplatinate (2mg in 1mL)

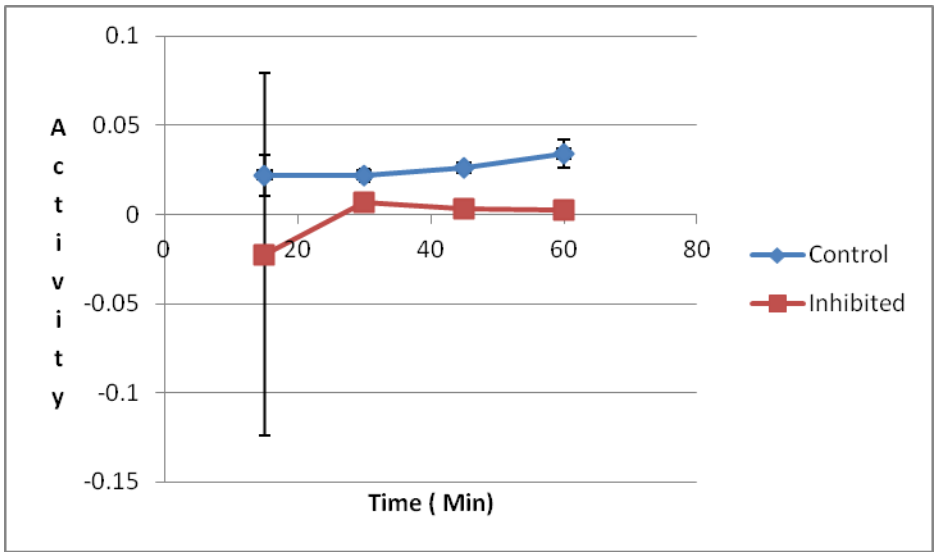

Figure 3.14. Activity of chymotrypsin with Potassium tetrachloroplatinate (0.2mg in 1mL)

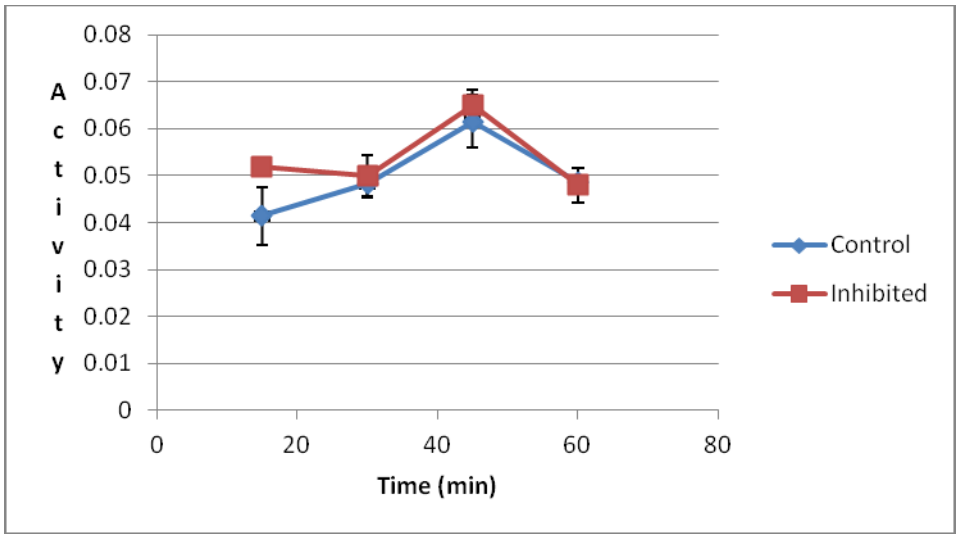

Figure 3.15 Activity of chymotrypsin with ethylenediamine platinum (II) dinitrate (1mg in 1mL)

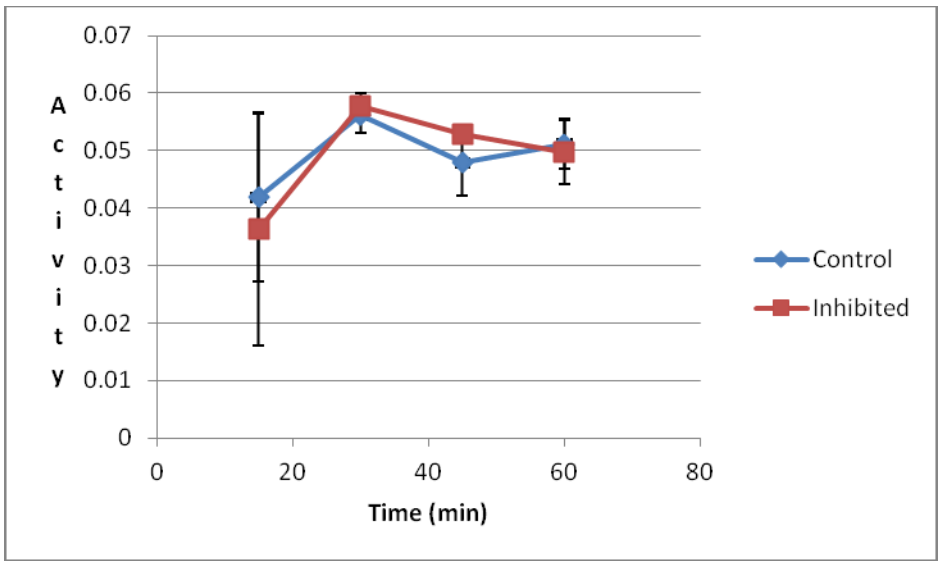

Figure 3.16. Activity of chymotrypsin with N,N,N',N'- tetrametylethylenediamine platinum(II) nitrate (1mg in 1mL)

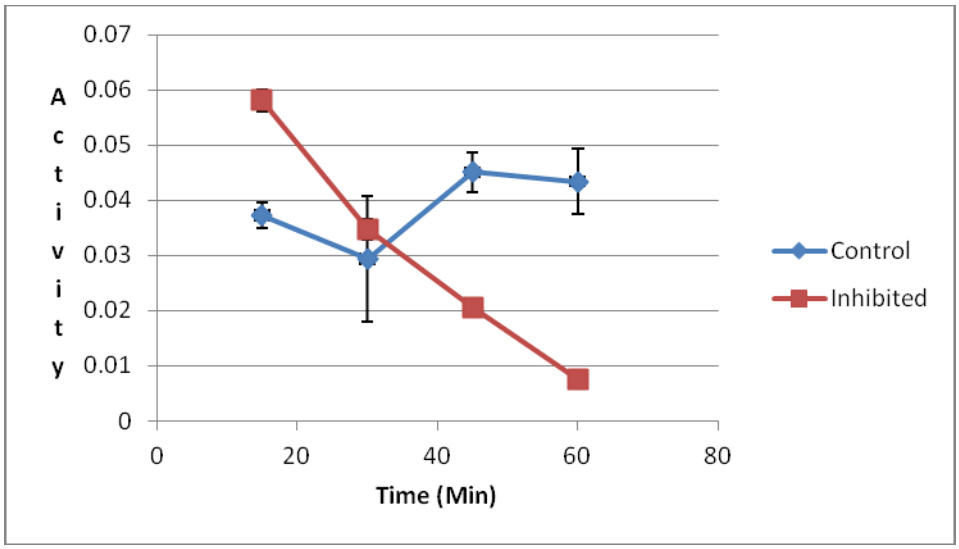

Figure 3.17. Activity of chymotrypsin with ethylenediamine platinum (II) dinitrate (1mg in 1mL)

We first tried the enzymatic assay for chymotrypsin with 1mg of cisplatin in 1 mL of storage buffer. When the enzymatic activity was measured for both the control and inhibition assays and the graph was plotted between activity and time (Fig. 3.10), as the enzyme activity was determined relative to control that had no platinum compound. We observed that cisplatin at this concentration did not show any inhibition of the enzyme chymotrypsin, as we measured the activity of the enzyme relative to control that had no platinum compound and the enzymatic activity of control and inhibitions assays were overlapping each other. We then tried to increase the concentration of cisplatin to 2mg in 1 mL of storage buffer, did the same procedure when we plotted the graph (Fig. 3.11). The increase in concentration showed some inhibition but this inhibition was inconclusive, as the error bars of control and inhibition assays activity of enzyme were overlapping each other.

As chymotrypsin did not show any significant inhibition with cisplatin, we then tried to see the activity of chymotrypsin with 1mg of potassium tetrachloroplatinate in 1 mL of storage buffer, when the enzymatic activity of the control and the inhibitory samples are measured for every 15 min interval for about an hour and the graph was plotted between activity and time. As we measured the enzyme activity relative to control that had no platinum compound, from the graph (Fig. 3.12) it was seen that chymotrypsin was inhibited by 1mg of potassium tetrachloroplatinate and the activity of the enzyme was found to be zero at 30 minutes. We then increased the concentration with 2mg of potassium tetrachloroplatinate in 1 mL of storage buffer and measured the activity. When the graph was plotted it was seen that (Fig. 3.13) the activity of the chymotrypsin was zero at 15 minutes. The results imply that an increase in the concentration of potassium

tetrachloroplatinate decreased the activity of the enzyme at lesser time. We even tried with very less concentration 0.2mg of potassium tetrachloroplatinate in 1 mL of storage buffer and measured the activity. From the graph (Fig. 3.14) it was seen that there was some amount of inhibition of the enzyme chymotrypsin but the inhibition was inconclusive, as the errors bars of the control and the inhibition enzymatic activity were overlapping each other.

Later, we tried to see the activity of the enzyme chymotrypsin with 0.001g of ethylenediamine platinum (II) dinitrate (Fig. 3.15), 1mg of N,N, N', N' tetramethylethylene diamine platinum (II) nitrate (Fig. 3.16) and 0.001g of ethylenediamine platinum (II) dinitrate (Fig. 3.17) in 1 mL of storage buffer. We did the same procedure, measured the activity of both the control and inhibition samples for every 15-minute interval for about an hour and plotted the graph. From the graph it was seen that there was no inhibition of the enzyme chymotrypsin with these platinum compounds at that particular concentration.

IV. DISCUSSION

The enzymatic assays we have done will allow us to assess both the rate and the extent of inhibition as a function of bulk and leaving ligand. The activity of enzyme was determined relative to the control that had no platinum (II) added to it. The UV/Vis spectrometer was used to measure the activity of enzyme.

Previously, the inhibition of thioredoxin reductases by cisplatin was done to see if the cisplatin targeted selenium residue of thioredoxin reductase. This enzyme showed dose and time dependent inhibition by cisplatin.[32] Cisplatin targets the selenium residue at the active site. The cysteine protease enzyme cathepsin-B did not show inhibition with platinum derivatives, even though the active site cathepsin-B was large enough to accommodate the Pt (II) moiety and the binding kinetics of Pt (II) was slow for efficient inhibition of cathepsin-B.[33] Acetyl cholinesterase, which occurred in neuronal as well as non-neural cells had the active site residues at Ser200, His440, Glu327 positions.[34] This enzyme showed inhibition with cisplatin in previous research done. Subtilisin and chymotrypsin both had histidine in their active sites similar to acetyl cholinesterase enzyme which also had histidine in its active site.

In our present research we are using serine proteases, subtilisin and chymotrypsin enzymes. The active site residues of subtilisin and chymotrypsin are as follows Asp 32, His 64, and Ser 221 and Asp 50, His 57, and Ser 195 respectively. As histidine is a known target for platinum, we expected the similar inhibition as in acetylcholinesterase.

Subtilisin was incubated with different concentration of specific platinum compounds at specific time and activity of enzyme was measured. Cisplatin, which

showed inhibition with acetyl cholinesterase, did not show any significant inhibition of subtilisin. There was some amount of inhibition with cisplatin but the inhibition is inconclusive because the error bars of control activity and inhibition activity were overlapping. The activity of subtilisin with other diamine platinum (II) complexes with different leaving ligands also did not show any inhibition. Even the potassium tetrachloroplatinate did not show any inhibition on subtilisin. There was no significant inhibition with any of the platinum compounds with different concentrations. Therefore these platinum compounds were not reacting with the histidine active site residue of subtilisin.

Chymotrypsin belongs to the same class of serine protease which has histidine in its active site. We tried the same platinum compounds that we tried for subtilisin; we did not see any significant inhibition with platinum diamine complexes with different leaving ligands but potassium tetrachloroplatinate showed inhibition. The inhibition of chymotrypsin with potassium tetrachloroplatinate was concentration dependent. The reason for only potassium tetrachloroplatinate showing inhibition was not completely understood. Potassium tetrachloroplatinate might be reacting outside the active site of the enzyme, the negative charge on the platinum counterion may affect the activity of the enzyme. Only chymotrypsin showed inhibition with potassium tetrachloroplatinate while subtilisin did not; the structure of the enzyme chymotrypsin may be the reason for the inhibition. Acetyl cholinesterase showed inhibition with cisplatin, while both subtilisin and chymotrypsin did not show any inhibition. If the cisplatin was reacting with the histidine active site residue in acetyl cholinesterase, even chymotrypsin and subtilisin

also should show inhibition. We think that the general structure of the enzyme acetlycholinesterase was responsible for inhibition.

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