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RESEARCH ARTICLE

INTERACTION OF BRAIN CYSTATIN WITH DOPAMINE: A NEUROTRANSMITTER

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ABSTRACT

The term Cystatin (Thiol Proteinaseinhibitor) refers to proteins that specifically inhibit the activity of papain like lysosomal cysteine proteinases. They serve a protective function and regulate the activities of endogenous proteinases, which if not regulated may cause uncontrolled proteolysis and damage to cells and tissues. They are non covalent tight binding proteins which are widely distributed in animals, plants and microorganisms. Dopamine abbreviated as "DA" is an amino acid neurotransmitter; it is a chemical substance which is able to transmit a nerve message across a synapse. It is obtained through the normal diet and also synthesized in the brain. The enzyme monoamine Oxidase helps in maintaining the level of dopamine, an increase or decrease in dopamine level leads to schizophrenia and Parkinson's disease respectively. Cystatin interacts with dopamine decreasing the level of dopamine in the brain and this might be a factor in Parkinson's disease. In the present study when (1μM) of cystatin was treated with increasing concentration of dopamine , it showed decrease in fluorescence intensity with 20 nM of blue shift which is indicative of binding and changes in native structure of protein upon complexation. Binding investigation in this work, gives significant information about the conformational changes in cystatin due to interaction with dopamine; such studies may be used as a tool for drug designing and it might shed some light on the mechanism of action of dopamine as well as its side effect.

INTRODUCTION

Neurotransmitters are endogenous chemicals that transmit signals from a neuron to a target cell across a synapse. (Dorland's Illustrated Medical Dictionary, 2007) Neurotransmitters are packaged into synaptic vesicles clustered beneath the membrane in the axon terminal, on the presynaptic side of a synapse. They are released into and diffuse across the synaptic cleft, where they bind to specific receptors in the membrane on the postsynaptic side of the synapse. (Elias and Saucier, 2005) Release of neurotransmitters usually follows arrival of an action potential at the synapse, There are many different ways to classify neurotransmitters. They are divided into amino acids, peptides, and monoamines. Dopamine and serotonin come under the section of monoamines. Several important diseases of the nervous system are associated with dysfunctions of the dopamine system. Parkinson's disease, a degenerative condition causing tremor and motor impairment, is caused by loss of dopamine-secreting neurons in the midbrain area called the *Substantia nigra*. Dopamine has a number of important functions in the brain which includes regulation of motor behavior, pleasures related to motivation and also emotional arousal. People with Parkinson's disease have been linked to low levels of dopamine and people with schizophrenia have been linked to high levels of dopamine (Sapolsky, 2005).

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The antipsychotic drugs that are frequently used to treat it have a primary effect of attenuating dopamine activity. Drugs targeting the neurotransmitter affect the whole system, this fact explains the complexity of action of some drugs. Cocaine blocks the reuptake of dopamine back into the presynaptic neuron, seizing the neurotransmitter molecules in the synaptic gap for long time. Since the dopamine remains in the synapse longer, the neurotransmitter continues to bind to the receptors on the postsynaptic neuron, eliciting a pleasurable emotional response. Physical addiction to cocaine may result from prolonged exposure to excess dopamine in the synapses, which leads to the down regulation of some postsynaptic receptors (Schacter *et al.,* 2011).

Neurotransmitters dopamine are able to diffuse away from their targeted synaptic junctions and are eliminated from the body via the kidneys, or destroyed in the liver. Each neurotransmitter has very specific degradation pathways at regulatory points, which may be the target of the body's own regulatory system. MAO concentration and too much or too little MAO activity is thought to be responsible for a number of neurological disorders like depression, schizophrenia and migraines. Monoamine oxidase A (MAOA) is an enzyme involved in the metabolism of monoamines, like serotonin and dopamine. It regulates both the free intraneuronal concentration and the releasable stores of serotonin. MAOA inhibitors (antidepressants) are used in the treatment of depression. People with depression have lower than normal levels of the

monoamines, the antidepressants restore the level and helps in the cure of depression (The American Heritage, 2005).

Cystatins are inhibitors of cysteine proteinases, most of which form equimolar complexes with their target enzymes. Cysteine proteinase inhibitors of cystatin super family are present in a variety of tissues and body fluids of human beings and animals to regulate the activities of cysteine proteinases. Cystatins are crucial for proper brain functioning. It has been reported that an imbalance of proteinases (cathepsins) and their endogenous inhibitor cystatins is closely associated with senile plaque, cerebrovascular amyloid deposits and neurofibirillary tangles in Alzheimer's disease. It has also been reported that Cystatin C is present in high concentration in CNS and is suggested to play an important role in diseases of the brain (Bernstein *et al.,* 1996). A proteinase inhibitor is of physiological importance because inhibition is achieved at physiological concentration of the inhibitor in a sufficiently short time with negligible dissociation of the complex. Endogenous thiol proteinase inhibitors the cystatins constitute a powerful regulatory system for overall cellular activity of cysteine proteinases (Sotiropoulou *et al.,* 1997). Cystatins are ubiquitously found in organisms, ranging from bacteria to mammals. They are classified into three distinct families based on their sequence homology, presence of disulphide bonds and molecular mass. Because of the various important physiological roles of cystatins they demand significant attention. Moreover they are associated with several neurodegenerative diseseas and pathological conditions including rheumatoid arthritis (Trabandt *et al.,* 1991), osteoporosis (Delaissé *et al.,* 1991), renal failure, cardiovascular and cancer diseases (Kabanda *et al.,* 1995; Servais *et al.,* 2008), resulting due to imbalance of endogenous cysteine proteinases and their inhibitors.

It has been shown that the distribution, free concentration and the metabolism of various drugs can be significantly altered as a result of their binding with proteins (Kragh-Hansen, 1981). In studying the interaction of drugs and proteins, fluorescence techniques are commonly used because of their high sensitivity, rapidity and ease of implementation. Several reports have been published studying the interaction of proteins with drugs by fluorescence technique (Tian *et al.,* 2003; Sereikaite and Bumelis 2006; Khan *et al.,* 2007). Moreover Fluorescence and UV–vis absorption spectroscopy are powerful tools for the study of the reactivities of chemical and biological systems since it allows non-intrusive measurements of substances in low concentration under physiological conditions. Studies showed that the emission of intrinsic fluorescence is mainly due to the presence of tryptophan residues excited at 280 nm. Another frequent amino acid in tyrosine shows high fluorescence intensity in pure solution however it presents weak emission when part of protein chains (Sun *et al.,* 2006).

Since there is a delicate balance between protease and antiprotease inhibitors, dopamine, a neurotransmitter binds to cystatin leads to decrease in the activity of dopamine causing several neurodegenerative diseases. Previous study shows that Mono amine oxidase inhibitor also binds to cystatin causing hindrance in the action of MAOI. Treatment of MAOI given to increase the concentration of dopamine to alleviate mood and memory etc). Which may therefore not be able to act on dopamine and dangerous effects continue causing several diseases.

MATERIALS AND METHODS

Experimental procedures

Materials

Papain (99% purity) and dopamine was obtained from Sigma Chemical Company (St. Louis, USA). The solutions were prepared in 50 mM phosphate buffer of pH 7.4. Salts were purchased from Merck (India). The protein concentration was determined spectro-photometrically. All other reagent were of analytical grade and double distilled water was used throughout.

Purification of Brain Cystatin

Fresh brain tissue (150 grams) was homogenized in 50 mM sodium phosphate buffer of pH 7.5 (30 ml) containing 1% NaCl, 3mM EDTA and 2% n-butanol. After centrifugation at 11000rpm for 15 minutes at 4^0C residue was discarded and the supernatant was further processed. The procedure involved a combination of alkaline treatment (pH 11.0), ammonium sulphate fractionation and gel filtration chromatography. Buffalo brain was homogenized and fractionated with ammonium sulfate between 40-60%, it was then dialyzed against 50 mM sodium phosphate buffer pH 7.4 containing 0.1 M NaCl. Elution profile showed two protein peak one major and one minor named as peak-I and peak-II. Peak-I corresponding to high molecular weight. Cystatin had significant inhibitory activity and protein content; however peak-II with insignificant protein concentration and low inhibitory activity was not taken into consideration for further studies. Peak-I renamed as BC was then purified with fold purification of 384.72 and yield of 64.13%. Papain inhibitory fractions of peak -I were pooled, concentrated and checked for purity. Five milliliter fractions were collected and assayed for protein by the method of lowry *et al.* (1951) and inhibitory activity against papain by the method of Kunitz (1947). Homogeneity of the preparation was investigated by 7.5% PAGE (Amin *et al.,* 2011).

Electrophoresis

To check the homogeneity and subunit structure of the purified preparation, native and SDS-PAGE was performed in the presence and absence of 2-mercaptoethanol by the method of Laemmli (Laemmli, 1970). Native gel was run at 7.5% and SDS PAGE was done with 12.5% agarose gel. The gels were stained with 0.1% coomassie brilliant blue (Fig. 4 and 5).

SPECTROCOPIC STUDIES

Fluorescence spectra of brain cystatin with dopamine

Brain cystatin (BC) (1μM) was incubated for 30 min with increasing concentrations of dopamine in 0.05 M sodium phosphate buffer pH 7.5 in a final reaction volume of 1ml at

room temperature. Drug solutions were prepared in the same buffer. Fluorescence measurements were carried out on a Shimadzu Spectrofluorimeter model RF-540 equipped with a data recorded DR-3 at 298K. The fluorescence was recorded in wavelength range of 300-400 nm after exciting the protein at 280 nm. The slits were set at 10 nm for excitation and emission. The path length of the sample was 1 cm. The data was analyzed by stern-Volmer equation

Stern- Volmer Constant

The fluorescence quenching was analyzed by the Stern-Volmer equation

$$\frac{F_o}{F} = 1 + Ksv\,[Q]$$

Where F_0 and F are the fluorescence intensities in the absence and presence of quencher respectively, Ksv the stern-volmer quenching constant and (Q) is the concentration of the quencher. Determination of binding constant (K) and number of binding sites (n)

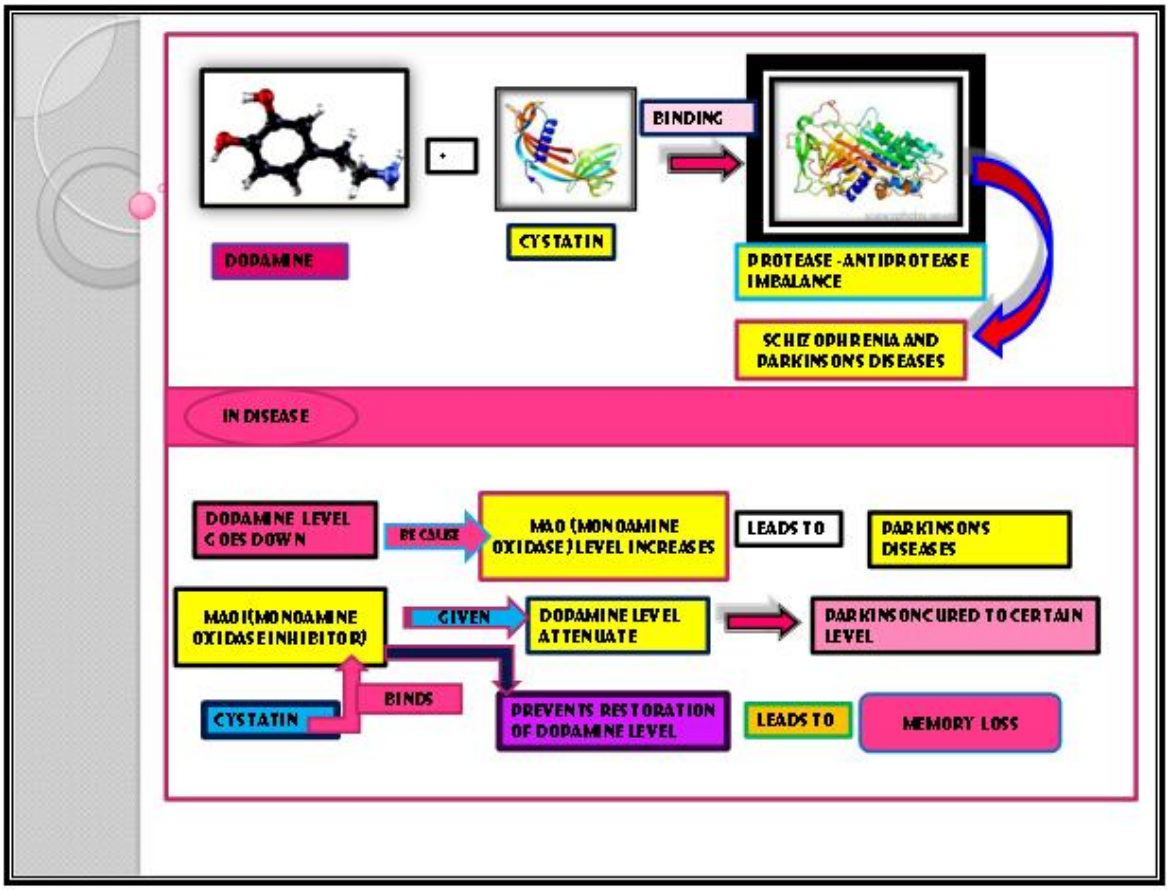
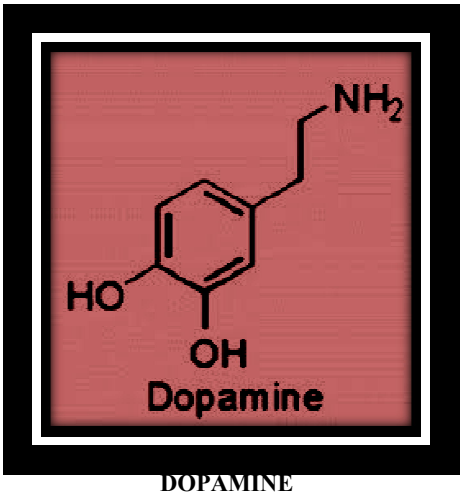


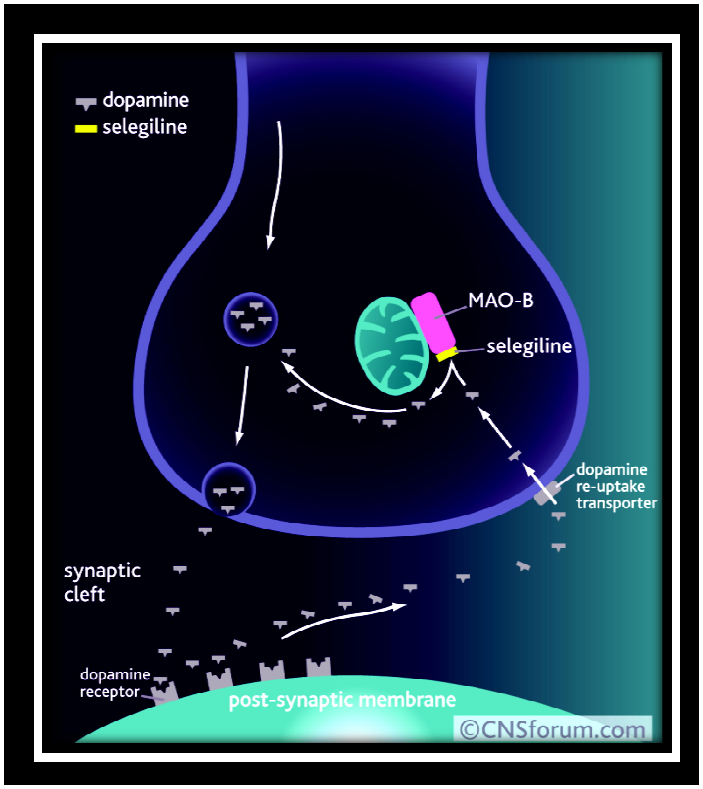
Fig.1. Proposed work



DOPAMINE

(The Placebo Effect 2011)The Placebo Effect; The chemical structure of dopamine, braintalks.wordpress.com March 14, 2011

Fig. 2. Chemical structure of neurotransmitters



(Dale *et al.*, 2001) Noradrenergic transmission. In: Pharmacology, 4th edition. Rang HP, Dale MM and Ritter JM. Edinburgh, UK: Harcourt Publishers Ltd, 2001:139–163

Fig. 3. Mechanism of dopamine receptors

When small molecules binds independtly to set of equivalent sites on a macromolecules, the equilibrium between free and bound molecules is given by the following equation. (Feng *et al.,* 1998; Gao *et al.,* 2004).

$$\mathbf{Log\frac{(F_0 - F)}{F} = LogK + n\,Log[Q]}$$

Where K and n are the binding constant and number of binding sites respectively thus a plot of Log ((Fo-F)/F versus (Q) can be used to determine K as well as n.

Calculation of the free enthalpy ΔG^0

The determination of the change of free enthalpy based on the van't Hoff equation:

$$\Delta G = -RT \ln K \left[\frac{J}{mol}\right],$$

$\mathbf{\Delta G^0}$ = -RTlnKJmol

Where *R* is the gas constant $\left(8.314 \frac{J}{mol \times K}\right)$8.314Jmol×K, *T* is the temperature (K), and *K* is the equilibrium constant.

UV spectra of cystatin in the presence of dopamine

The UV measurement of brain cystatin in the presence and absence of antidepressant was made in the range of 200-300 nm and the inhibitor (Cystatin) concentration was fixed at 1µM while the drug concentration was varied to different extent. Absorption spectra were recorded on a double beam Shimadzu UV-vis spectrophotometer UV-1700 using a cuvette of 1 cm path length. Activity measurement of brain cystatin in the presence of dopamine The inhibitory activity of the purified inhibitor (BC) under native conditions was assessed by its ability to inhibit caseinolytic activity of papain by the method of Kunitz (23). Cystatin (1 µM) was incubated with increasing concentrations of dopamine at 25^0C for 30 min before the activity was measured. Activity of untreated BC was taken as 100%.

RESULTS

Electrophoresis

Homogeneity of the pooled peak-I fractions obtained from gel filtration column was determined by polyacrylamide gel electrophoresis (PAGE) in the absence of SDS. The brain cystatin showed single band on 7.5% gel (Fig. 4).

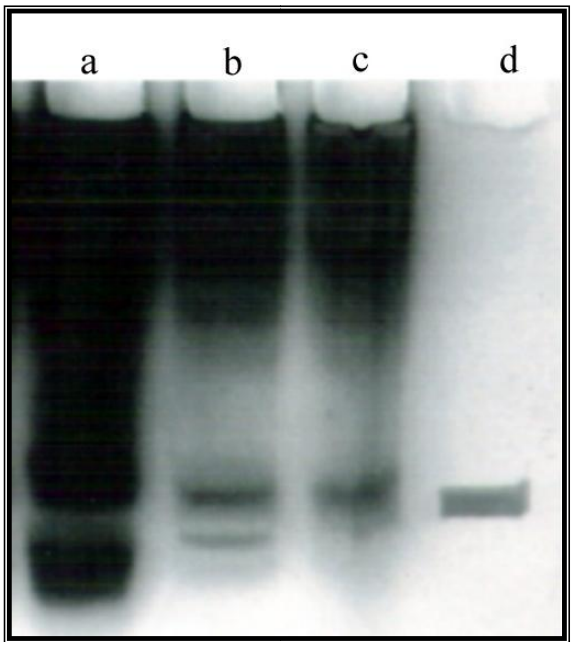


Fig. 4. Gel electrophoresis of Brain Cystatin during various stages of purification Electrophoresis was performed on 7.5% gel as described in the methods section at 25^0 C

Lane a: 60 µg of buffalo brain crude homogenate, Lane b: Supernatant after alkaline treatment, Lane c: Dialyzed fraction after 40-60% ammonium sulphate fractionation, Lane d: 60 µg of purified fraction after gel filteration chromatoraphy on sephadex G-75

REDUCING AND NON REDUCING SDS PAGE

Purified cystatin was also analyzed by SDS – PAGE under reducing conditions by the method of weber and Osborn (Weber and Osborn, 1969) (in the presence of β-mercaptoethonal) and non reducing conditions (in the absence

of β- mercaptoethonal) (Fig. 5). BC migrated as two band with different mobilities in both the conditions suggesting a two subunit structure which are held together by non-covalent forces

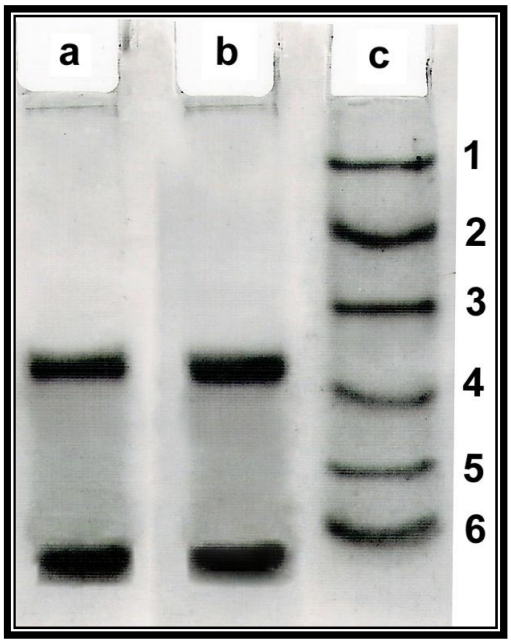


Fig. 5. SDS PAGE of the Brain Cystatin under reducing and non reducing conditions

Electrophoresis of BC was performed 12.5 % polyacrylamide gel Lane a, Contain BC treated with SDS alone (non reducing conditions) Dye. Lane b, Contain BC Treated with SDS + βME (reducing conditions). Both giving two subunits, Lane C contained molecular weight marker's of standard proteins. 1- Phosphorylase (97.4 kDa), 2-BSA (Bovine serum albumin) (68 kDa), 3-Oval albumin (45 KDa), 4-Carbonic anhydrase (29.1 kDa), 5-Soya bean trypsin inhibitor (20 kDa), 6-Lysozyme (14.3 kDa).

Interaction of dopamine with brain cystatin spectral studies

Fluorescence analysis of brain cystatin in the presence of Dopamine

The fluorescence spectra of BC (1μM) was recorded in the presence of different concentrations (2-10 μM) of Dopamine after 30 min of incubation intensities were recorded in the range of 300-400 nm upon excitation at 280 nm. Dopamine caused quenching of the intrinsic fluorescence of BC accompanied by a blue shift (20 nm) (Fig. 6) at 2μM concentration. These results indicated that there was interactions between dopamine and brain cystatin and the binding reactions resulted in non-fluorescent complex.

Fluorescence quenching data was analyzed by the Stern-Volmer equation

Stern-Volmer equation gave BC-Dopamine complex the K_{sv} value at 298K was 0.5 x10 ^{6} mol $^{-1}$ which is shown in Table 1.

Determination of binding constant (K) and number of binding sites (n)

When small molecules bind independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecules is given by the equation given in the methods section (Feng *et al.,* 1998; Gao *et al.,* 2004) the value of binding constant K was found to be 2.59 x10 ^{6} mol $^{-1}$ and the number of binding sites calculated as 1 (Table-1)

ΔG^0 of interaction between Dopamine and Cystatin

The forces of interaction between drugs and biomolecules are generally noncovalent. In order to identify the interacting forces between dopamine with Cystatin the thermodynamic parameter, that is. Free energy change (ΔG^0), of the interactions were calculated from the stern volmer equations, it was found to be -36.58 KJ/mol (Table-1) Cystatin (1μM) was incubated with various concentrations of Dopamine varying from 2μM to 10μM for 30min. The fluorescence was recorded in wavelength region 300-400 nm after exciting the protein solution at 280 nm for total protein fluorescence. The slits were set at 10 nm for excitation and emission. The path length of the sample was 1 cm.

UV-vis absorption Spectra of Dopamine - Cystatin complex

The interaction between Dopamine-Cystatin was studied from UV-vis absorption spectral data. Absorption spectra of native BC in the presence and absence of Dopamine were recorded in the range of 200-300 nm. Cystatin concentration was fixed at 1μM while the Dopamine concentrations were varied from 2-10μM. The UV absorption intensity of Cystatin increased with the increase in dopamine concentration (Fig. 7). This evidences clearly indicated the interaction and some complex formation between Dopamine and Cystatin as reported for other proteins (Cui *et al.,* 2004; Hu *et al.,* 2004). Cystatin concentration was fixed at 1μM while the Dopamine concentrations were varied from 2μM-10μM. Absorption spectra of native Cystatin in the presence and absence of Dopamine were recorded in the wave length range of 200-300 nm for 30min in the final reaction volume of 1 ml in 0.05 M sodium phosphate buffer pH 7.5.

Table 1. Different parameter of the drugs obtained by stern volmer equation for interaction with cystatin

DRUG PARAMETER	K_{SV} (Stern-volmer Constant) Mol-1	K (Binding constant) Mol-1	N (number of binding sites)	ΔG^0 (Free energy change) KJ/mol
DOPAMINE	0.5x106±0.97	2.592 x 106±0.71	0.798±0.02	-36.589±0.11

Inhibitory activity of Cystatin in the presence of Dopamine

Changes in the inhibitory activity of Cystatin after incubation for 30 min with increasing concentration of Dopamine is shown in Table-2.

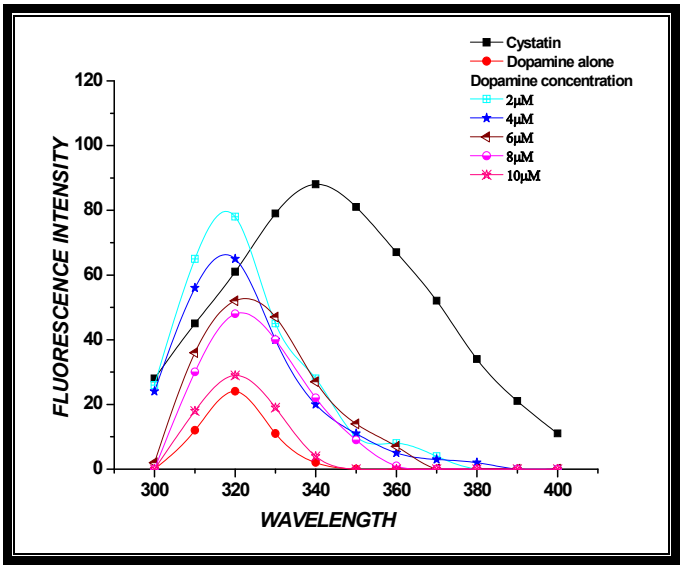


Fig. 6. Fluorescence Spectra of Cystatin in the presence and absence of Dopamine

The result showed that cystatin lost significant amount of inhibitory activity (34%) at 2μM of dopamine concentration, this loss in inhibitory activity increased with increasing concentration of Dopamine, cystatin lost half of its inhibitory activity at 6 μM concentration indicating changes on complex formation.

Changes in the inhibitory activity of Brain cystatin after incubation with increasing concentration of Dopamine are shown in the table-2 Cystatin (1μM) was treated with varying concentration of Dopamine (2 -10μM) for 30min in the final reaction volume of 1 ml in 0.05M sodium phosphate buffer pH 7.5.

DISCUSSION

Dopamine is a member of the catecholamine family, it is a precursor of nor epinephrine and epinephrine and is the neurotransmitter that helps transmit messages to the striatum it initiates as well as controls the movement and balance of the body. These dopamine messages make sure that muscles work

Percent decrease in tryptophan fluorescence in the presence of Dopamine

Cystatin alone fluorescence	Dopamine alone	2 μM	4 μM	6 μM	8 μM	10 μM
100	98±0.71	68±0.65	77±0.92	69±0.11	75±0.62	95±0.92

Table 2. Inhibitory activity of cystatin in the presence of dopamine

S.NO	Dopamine Concentration	% Remaining inhibitory activity
1	Cystatin alone	100
2	Cystatin + 2 μM Dopamine	66 ± 0.985
3	Cystatin + 4 μM Dopamine	56 ± 0.1.22
4	Cystatin + 6 μM Dopamine	44 ± 0.605
5	Cystatin + 8 μM Dopamine	38 ± 0.772
6	Cystatin + 10 μM Dopamine	28 ± 0.788

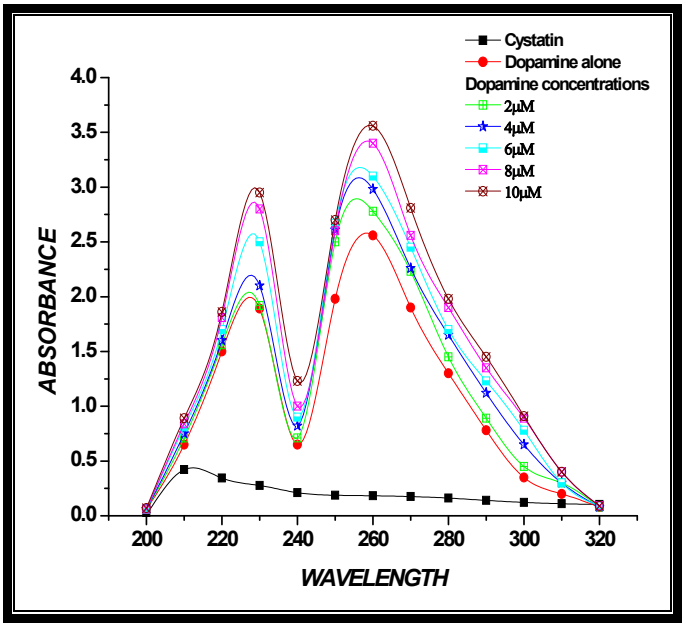


Fig-7. Uv-vis spectra of brain cystatin in the presence and absence of dopamine

smoothly, under precise control, and no unwanted movement occur. Many endogenous compounds exist in human body and drugs can bind to them to form stable complexes, which effect the function of regulating proteins directly or indirectly (Silva *et al.,* 2004). In addition, the effectiveness of drugs depends on their binding ability to the target proteins. It has been shown that the metabolism of various drugs may be strongly affected by drug protein interaction in the blood stream (Kamat and Seetharamappa, 2004; Seedher, 2000; Cui *et al.,* 2004). Therefore, study of the interactions between proteins and drug molecules help to provide basic information on the pharmacological actions and bio-distribution of drugs. Such studies also provide the information about the side effects of drugs that is if the drug has any effect on the structure and function of protein. In the present work purification of buffalo brain cystatin was achieved using a three step procedure including alkaline treatment at pH 11.0, ammonium sulphate fractionation and gel filteration chromatography (Amin *et al.,* 2011). (Our simple procedure has given better yield and fold purification as compared to the values reported in literature for some other species (Bige *et al.,* 1985; Anastasi *et al.,* 1983; Evans *et al.,* 1987; Baba *et al.,* 2005). The inhibitor protein migrated as single band on native PAGE 7.5 % gel, however in reducing and non reducing SDS PAGE (12.5% gel) it showed

two bands, indicating that the two subunits are joined by non covalent forces (Fig-5).

The molecular weight obtained by SDS PAGE for the two subunits were 31.62 kDa and 12.58 kDa which is equivalent to 44.2 kDa. When cystatin was interacted with dopamine it showed decrease in fluorescence. The quenching of fluorescence is accompanied by the blue-shift in spectra (Fig-6). This indicates the increase of polarity of the fluorophore environment in the cystatin, probably due to the hydrogen bonding between dopamine and NH_2, OH and SH groups in the inhibitor which stabilizes the complex (Bures *et al.,* 1990) Negative value of ΔG^0 -36.589KJ/mol (Table- 1) showed that complex formation between dopamine and Cystatin occur spontaneously, Our finding is similar to the binding of Methotrexate and Berberine Hydrochloride drug with human serum albumin (Khan *et al.,* 2007). The number of binding sites (n) for dopamine – BC complex showed the value approximately equal to 0.798 indicating that there is one independent binding site for interaction.

Inhibitory activity assay and fluorescence result of cystatin in the presence of dopamine clearly indicates that dopamine causes functional and structural modification in cystatin. Dopamine was found to be a modest inactivator of BC. The treated inhibitor lost only 44% of its antiproteolytic activity in the presence of 4µM dopamine (Table-2). In case of dopamine marked blue shift of 20nm was observed indicating a change in microenvironment of tryptophan residues towards polar environment resulting in the loss of native folded state of cystatin. The obtained result is in accordance with the binding of sodium diethyl dithiocarbamate (SDD) with phytocystatin (Sharma *et al.,* 2005) and is further supported by the similar other results in literature (Khan *et al.,* 2008).

Interaction between dopamine- cystatin complexe was also studied from UV-vis absorption spectroscopy. Equimolar complex of cystatin and dopamine showed only a broad absorption band in the region of 200-300 nm, suggesting that the native structure of the proteins is altered on complexation. (Fig. 7). These evidences clearly indicated that there is interaction and complex formation between dopamine and cystatin causing imbalance of proteases antiprotease effecting the structure and function of proteins the results are supported from other studies too (Cui *et al.,* 2004; Hu *et al.,* 2004). The information may be use full in treatment of patients with dopamine as it may be considered as one of the side effect of dopamine

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