STUDIES ON ANTIDEPRESSANT DRUG AND OXIDATIVE STRESS INDUCED STRUCTURAL AND FUNCTIONAL MODIFICATION OF BUFFALO BRAIN PURIFIED CYSTATIN

ABSTRACT

OF THE

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

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BY

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ABSTRACT

The term Cystatin refers to proteins that specifically inhibit the activity of papain like lysosomal cysteine proteinase. 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 . These endogenous thiol proteinase inhibitors are non covalent tight binding proteins which are widely distributed in animals, plants and microorganisms. Cystatin have been found to be evolutionary, structurally and functionally related proteins forming the cystatin superfamily .Members of this family have been divided into three groups of proteins known as family 1,2 and 3 . This classification is based on differences in amino acid sequences, molecular weight, sub cellular localization, disulphide bonds and number of amino acid residues. Family I cystatins called as Stefins are intracellular inhibitors which include members containing about 100 amino acid residues (MW 11,000), it lacks disulphide bridges and carbohydrate content. Family II cystatins known as type 2 cystatins they include inhibitors of about 115 amino acid residues (MW 13,000) and contain two disulphide loops towards the carboxyl terminal with no carbohydrates, They are found both in the cells and body fluids. Kininogens or Family III cystatins are large precursor molecules of the vasoactive kinins. They are single chain glycoproteins, which serve a variety of biological functions such as kinin delivery, induction of endogenous blood coagulation cascade and mediation of the acute phase response. They are found only in blood plasma.

Cystatins have been purified from several mammalian sources like muscle, heart, kidney, spleen, liver, skin and placenta. In the present study a cystatin of molecular mass 44,000 has been purified from buffalo brain ***(Bubalus bubalis).*** It has been characterized in terms of its biochemical and biophysical properties. The purification has been achieved by a simple three step procedure involving alkaline treatment at pH 11, ammonium sulphate fractionation (40-60%) and gel filtration chromatography on Sephadex G-75. gel filteration profile showed two protein peaks (peak-I and peak - II) with significant inhibitory activity in peak -I it was named as BC (Brain Cystatin) the

pooled fractions of the peak-I showed homogeneity on the basis of charge on 7.5% polyacrylamide gel electrophoresis, was obtained with 64.13% yield and 384.72 fold purification .Peak – II was not taken into consideration because of its insignificant inhibitory activity and low protein content . Studies on the isolated cystatin include molecular mass determination, kinetics pH and temperature stability studies, carbohydrate content , sulphydryl group determination, fluorescence and UV spectral analysis. BC consist of two polypeptide chains as determined by SDS-PAGE in the presence and absence of β-mercaptoethanol. Indicating that the two chains are joined by noncovalent forces The molecular weight of the two subunits were 31.62 KDa and 12.58 KDa as determined by SDS PAGE, (44.2 KDa) this value was 43.6 KDa by gel filtration chromatography . The stoke's radius of BC was found to be 27 A^0 indicating its compact globular structure. Cystatin was lacking in both carbohydrate content and disulphide bonds. The purified protien was found to be maximally active in the temperature range of 25 - 60 ^{0}C and the pH stability was in between pH 6.0 – 10.0. The inhibitor was found to be stable upto 40 minutes at 90^{0}C showing 60% inhibitory activity.

Purified BC strongly inhibited activities of proteases like papain, bromelain and ficin, but it did not inhibit serine proteases namely trypsin and chymotrypsin. The inhibition kinetics revealed that the purified cystatin is a tight binding inhibitor. The respective Ki values of BC obtained for papain, ficin and bromelain were 1.0 nm 1.85 nm and 2.25nm respectively. The IC_{50} values for BC were 0.09μM, 0.12μM and 0.15μM for papain, ficin and bromelain, respectively. kinetic parameters taken together imply that the cystatin binds more effectively to papain, then bromelain and least with ficin.

Ultraviolet spectra of the complex of BC with papain indicated that the environment of several aromatic amino acids residues in proteins have been perturbed by the interactions. It showed Changes in the fluorescence emission intensity by a 10 nm red shift in spectra, upon formation of inhibitor - papain complex indicating that there is some conformational change or local interactions affecting chromophoric groups of the two proteins involved in the complex formation.

Because of the absence of carbohydrate content, lack of disulphide linkages, high molecular weight and analysis of all other results obtained for BC, it can be considered as a variant of class I cystatin, that is the Stefins class of cystatin superfamily..

A review of the existing literature showed that cystatins from mammalian sources have species as well as organ differences, they differ not only in size but in several other physicochemical properties owing to the specific physiological functions they perform in the mammalian body Therefore, in view of the reported differences in molecular and functional properties of cystatin from various species and different tissues it was thought worthwhile to isolate and purify cystatin from a hitherto uninvestigated source and to characterize it on physicochemical basis.

To investigate the effect of reactive nitrogen species on cystatin .The effect of nitric oxide on structural and functional modifications of BC was investigated. It was found that nitric oxide quenched the tryptophan fluorescence and lead to 66% loss of the inhibitory activity in the presence of NO. Effect of Curcumin and querecitin (widely used as scavengers of NO) were analyzed for their protective effect in the presence of NO. Both the scavengers were found to have very significant preventive effect against NO induced damage of BC. This protection was maximally effective by 50μM of curcumin (loss was only 8%) however at 250μM querecitin loss in tryptophan fluorescence was 13% , both the scavengers were able to protect 90% of antiproteolytic activity of cystatin in the presence of NO .

In an attempt to explore the effect of reactive oxygen species (ROS) on cystatin HOCl and H_2O_2 were used as oxidants. The results reveal that BC, in the presence of HOCl lost its antiproteolytic activity rapidly (within 30 min, 82% loss occurred at 5μM HOCl). Another oxidant hydrogen peroxide at 250mM concentration showed significant functional and structural damage of cystatin , 60% loss of antiproteolytic activity occurred in 30 min of incubation, moreover at the same H_2O_2 concentration , cystatin showed 82% decrease in tryptophan fluorescence. The use of scavengers and antioxidants showed that hydroxyl radicals were involved in the damage of the purified inhibitor curcumin and quercetin both of them provided 80% protection to damage.

Riboflavin is a common reactive oxidants produced in the mammalian system under various metabolic conditions. Interaction of riboflavin (50μM) with BC showed that oxidant inactivated the inhibitor to the extent of 76% for 30 min of incubation with complete loss of inhibitory activity at the same concentration for longer time of incubation. Oxidation by riboflavin was also observed in the form of changes in fluorescence spectra .It showed quenching of tryptophan fluorescence. Free radical scavengers were used for detection of the type of ROS involved in the damage .the scavengers used were Glucose,KI ,Sodium azide , Thiourea ,Mannitol and Sodium benzoate . Out of these sodium benzoate (89%) thiourea(86%) and mannitol (83%) provide significant protection . Results suggested that singlet oxygen and flavin triplet state were predominantly responsible for BC damage by riboflavin .

Drug induced structural changes in BC were monitored by intrinsic fluorescence and UV vis spectrophotometric analysis. Four drugs (amytriptyline, fluoxetine , haloperidol , donepezil) and two neurotransmitter (dopamine and serotonin) were analyzed for their effect on BC .

Amytriptyline is a tricyclic antidepressant drug. It caused quenching of the intrinsic fluorescence of Cystatin, increasing concentration of amytriptyline caused no shift in wavelength, but fluorescence intensity decreased with increasing concentration of drug.A decrease in antiproteolytic activity was also noticed.

Fluoxetine is approved for the treatment of major depression .The Drug caused quenching of the intrinsic fluorescence of Cystatin with 10 nm of blue shift in wavelength (λ_{max} was shifted from 340nm to 330nm) at 2 μM fluoxetine concentration. On interaction of cystatin with 0.5 μM fluoxetine, 48% loss of cystatin inhibitory activity was observed .

Haloperidol is a typical antipsychotic drug. Due to its strong central antidopaminergic action, it is also classified as a highly potent neuroleptic drug. Haloperidol at 0.02μM did not induce any change in emission λ_{max} , however 27% decline in fluorescence intensity was observed. A decline in antiproteolytic activity was also noticed.

The thermodynamic parameters stern volmer constant (K_{sv}) for all the three drugs calculated indicate that both hydrogen bonds and hydrophobic interaction played a major role in the binding of antidepressants with BC .

Donepezil, marketed under the trade name Aricept,is a reversible acetylcholinesterase inhibitor. Donepezil caused unfolding of the Cystatin as indicated by enhancement in fluorescence intensity accompanied by the red shift of 40nm while the drug (native) shows λ_{max} at 370 nm , however when it forms complex with Cystatin there was a shift in λ_{max} of 10nm with significant enhancement in fluorescence intensity at 1.6 µM of the drug concentration .

The inhibitor was analyzed for its interaction with well known neurotransmitters dopamine and serotonin. Dopamine is a neurotransmitter essential for the normal functioning of the central nervous system. It initiates as well as controls the movement and balance of the body . It causes quenching of the intrinsic fluorescence of BC accompanied by a blue shift (20 nm) in wavelength at 10µM concentration. These results indicated that there were interactions between dopamine and brain cystatin, this binding reactions resulted in non-fluorescent complex.

Serotonin a monoamine neurotransmitter helps to relay signals from one area of the brain to another and has various functions in biological system including depression alcoholism, pain , anxiety control of appetite, mood and anger . On interaction with BC it caused unfolding of the cystatin as indicated by enhancement in fluorescence intensity. The study showed that cystatin lost significant amount of inhibitory activity at 10 nM concentration of serotonin.

Results of fluorescence for interaction of cystatin with the drugs in which binding was observed were analyzed by stern volmer equation , for calculation of the number of binding sites, free energy change and binding affinity of the drugs with cystatin, results revealed that there was one binding site for each drug the values were 0.89 , 0.79 , 1.1 and 0.798 for amtriptyline Fluoxetine haloperidol dopamine respectively . The binding affinity values for the four drugs were as follows amtriptyline (3.018×10^6) Fluoxetine (5.0317×10^6) haloperidol (7.988×10^6) and dopamine (2.592×10^6). Indicating highest binding affinity for haloperidol.The free energy change (ΔG^0) values were in the order of -39.377 > -38.232 > -36.966 >-36.589 for haloperidol ,

Fluoxetine , amtriptyline and dopamine respectively showing the reactions to be spontaneous. The stern volmer constant values were in the range for amtriptyline (0.115×10^6) , Fluoxetine (0.5×10^6) , haloperidol (15×10^6) and dopamine (0.5×10^6) respectively .indicating the type of quenching to be static

Since cystatins have important roles to play in normal body processes owing to their cysteine proteinase inhibitory activity, it is of utmost importance that their conformation should be stable for maximum functional activity. The present study on cystatin not only gives a detailed characterization of the inhibitor isolated from buffalo brain on physiochemical basis but the investigations in this work also provide useful information on the drug-protein interaction. Understanding the conformational changes that result in a protein by various drug treatments may provide a powerful tool for drug design.

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Dedicated to my Loving Parents and Tayyab uncle

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Certificate

This is to certify that the Ph.D thesis entitled "***STUDIES ON ANTIDEPRESSANT DRUG AND OXIDATIVE STRESS INDUCED STRUCTURAL AND FUNCTIONAL MODIFICATION OF BUFFALO BRAIN PURIFIED CYSTATIN***" is an original work of ***FAKHRA AMIN.*** The thesis incorporates the results of the independent study carried out by the candidate herself under my supervision. It has not formed the basis for any degree or diploma prior to this. Therefore, she is allowed to submit this thesis for the award of Ph.D degree in ***ZOOLOGY*** of Aligarh Muslim University, Aligarh India.

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This is to certify that the work in this thesis entitled "*STUDIES ON ANTIDEPRESSANT DRUG AND OXIDATIVE STRESS INDUCED STRUCTURAL AND FUNCTIONAL MODIFICATION OF BUFFALO BRAIN PURIFIED CYSTATIN* "is an original work done by *FAKHRA AMIN* under my co-supervision at *Interdisciplinary Brain Research Center (IBRC) , J.N. Medical College, A.M.U* and is suitable for the award of Ph.D degree in *ZOOLOGY.*

13/4/2010

Co-supervisor
(Prof. Shameem J. Rizvi)

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ACCOLADE

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(Fakhra Amin)

LIST OF ABBREVIATIONS

A	Angstrom
AD	Alzheimer disease
BC	Brain cystatin
CP	Cysteine proteinase
CPI	Cysteine proteinase inhibitor
CSF	Cerebrospinal fluid
DTNB	Dithionitrobenzoic acid
EDTA	Ethylenediaminetetraacetic acid
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
kDa	Kilo Dalton
µg	Microgram
µl	Microlitre
µM	Micromolar
M	Molar
MHC	Major histocompatibility complex
min	Minutes
Mr	Molecular weight
nm	Nanometer
NO	Nitric oxide
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulphate
STI	Soya bean trypsin inhibitor
TCA	Trichloroacetic acid
TEMED	Tetraethylmethyl ethylene diamine
v/v	Volume by volume
w/v	Weight by volume

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Introduction

1. INTRODUCTION

Proteolytic enzymes also termed as proteases, hydrolyze susceptible peptide bonds in proteins and are of universal occurrence in mammalian system. The term proteinase is a general term applied for both exopeptidases and endopeptidases. A protease is classified as a proteinase if it exhibits a significant endopeptidases activity regardless of whether it exhibits exopeptidases activity. However proteases and proteinases are synonymously used in literature. *Proteinases are further divided into fours classes* – serine proteinases, acid proteinases, metalloproteinases and thiol proteinases. Serine proteinases require the presence of serine residue at their active sites, acid proteinases require low pH conditions for their activity, metalloproteinases need metal ions for their full activity and thiol proteinases have cysteine residue at their sites. Serine proteinases and their endogenous inhibitors are much more explored as compared to thiol proteinases and their inhibitors.

Thiol proteinases synonymous with cysteine proteinases comprise a group of proteolytic enzymes which are widely distributed among living organisms *[Barrett et al. (1986a)].* They are proteins of MW of about 25-35 KDs. These proteinases are synthesized in a precursor form and are later subjected to co-translational and post translational modifications that convert them to catalytically active mature enzymes *[Cygler and Mort (1997), Turk et al. (2000)].* They contain two principal catalytic amino acid residues, cysteine and histidine at their active sites which are involved in the cleavage of peptide bonds *[Stennicke (1999)].* Cysteine proteinases play an important role in the catabolism of proteins and peptides, processing of prohormones, maturation and cleavage of a number of precursor proteins *[Siewinski (1993)].*

About 40 different families of cysteine proteinases have so far been characterized *[Barrett et al. (1998)].* The first clearly recognized and extensively studied cysteine proteinase was papain, isolated from the latex of the plant, *Carica papaya.* Nearly half of the known families of cysteine proteinases are represented only in viruses some occur in bacteria, whereas others exist in eukaryotic organisms *[Rawlings and Barrett (1994)].* Current evidence suggests that eleven lysosomal cathepsins *[B, H, L, S, C, K, O, F, V,*

X and W] among the known to date proteinases are members of papain family *[Kotyza and Krepela (2002)].*

Mammalian thiol proteinases have been classified into four different classes

1. Cathepsins 2. Legumain 3. Calpains 4. Caspases

Cathepsins They are relatively small proteins with the exception of cathepsin C which is an oligomeric enzyme of molecular weight of about 200 KDa. They are present in the lysosomes of cells and are involved in intracellular protein turnover, proteolytic degradation and cleavage of a number of precursor proteins and hormones. These are present in high concentrations in spleen *[McDonald and ELIS (1975)],* kidney, liver *[Knight (1980), Quinn and Judah (1978)]* and brain *[Suhar and Marks (1979)]*

Legumain It is an asparaginyl endopeptidase present in lysosomes. These are involved in MHC class II – restricted antigen presentation *[Manoury et al. (1998)]* and local negative regulation of osteoclasts formation and activity *[Choi et al. (1994)].*

Calpains and **Caspases** they are cytoplasmic thiol proteinases. **Calpains** participate in many intracellular processes such as turnover of cytoskeletal proteins, cell differentiations and regulation of signal peptides. Mammalian calpains have molecular mass of about 110 KDa *[Yajima and Kawashima (2001)].*

Caspases are the cysteine aspartate specific proteinases. The central component of cell apoptosis consists of proteolytic systems involving caspases *[Nicholson (1999), Stennicke and Salvensen (2000)].*

The lysosomal cysteine proteinases have been associated with a number of pathologies including cancer *[Koppel et al (1994)]*, inflammation, rheumatoid arthritis *[Trabandt et al. (1991)]* and osteoarthritis, Alzheimer disease *[Nakamura et al. (1991)]*, multiple sclerosis *[Bever et al. (1995)]*, muscular dystrophy *[Sohar et al. (1998)]*, pancreatitis, liver disorders, lung disorders, lysosomal disorders *[Sloane and Honn (1984)]*, diabetes and

myocardial disorders *[Hopsu-Havu et al. (1983b)]* In many of these diseased lysosomal enzymes have been found in the extracellular and extra lysosomal environments in their proforms, which are substantially more stable than their mature enzymes *[Barrett et al. (1998) Turk et al. (2001)].*

1.1 REGULATION OF LYSOSOMAL THIOL PROTEINASE ACTIVITY

Proteolysis is a key process in all living organisms and must be carefully controlled in order not to be hazardous to the organism itself. It is, therefore, not surprising that a large number of proteinase inhibitors have been found in animals, plants and micro organisms *[Ryan (1990)]* Naturally occurring proteinaceous PIs (protein inhibitors) are primarily classified into different classes, based on the type(s) of enzyme they inhibit *[Bode and Huber (2000)]* such as serine proteinase inhibitors. Which inhibit serine proteinases. Next to this criterion, classification of Proteinase inhibitor is also performed based on sequence homology, such as is done for Kunitz-type inhibitors. A further classification of these inhibitors has been done on the basis of their molecular mass, their protein architecture (monomeric or multimeric), the number of disulfide bridges present and their isoelectric points as in the case of thiol proteinase inhibitors. These criteria determine in which family a proteinase inhibitor can be classified in animals and plants,

1. Serine proteinase inhibitors

The serine proteinase inhibitors are by far the largest class of proteinase inhibitors when looking at the number of families. Serine proteinase inhibitors can be classified into 13 structurally distinct families, based on their origin.

2. Aspartate proteinase inhibitors

In contrast to the widespread distribution of the aspartate proteinases, the aspartate proteinase inhibitors are relatively uncommon. The aspartate proteinase inhibitors from Solanaceae are the most well known and well characterised in plant *[Mares et al. (1989)].* Until now, only one family has been described. This family comprises proteinase inhibitors with a molecular mass of 20-22 kDa, and that contain two S-S bridges. They show sequence homology with the Kunitz-type inhibitors and are therefore, classified as

members of the Kunitz-type family. They all inhibit cathepsin D and in some cases they inhibit trypsin also.

3 Metallo proteinase inhibitors

Only one family of the class of metallo proteinase inhibitors has so far been discovered. The potato carboxypeptidase inhibitor (PCI) from *Solanum tuberosum [Molina et al. (1994)].* PCI is a small of 38-39 amino acid residues, containing 3 S-S bridges and is known to be highly thermostable *[Huang et al. (1981)].*

4 Cysteine proteinase inhibitors

Cysteine proteinase in animals plants and microorganism such as e.g. papain, bromelain, cathepsin B, H, and L and also several other *lysosomal cysteine proteinases*, which are responsible for protein degradation. *[Barrett (1987)].* The class of cysteine proteinase inhibitors which regulate the activity of these proteins is also known as the "Cystatin superfamily".Generally members of Cystatin superfamily are monomeric proteins and contain a highly conserved region of 5 amino acids (Gln-X-Val-X Gly) always located in position starting from 57.

The process of proteolysis in cells is under biological control acting at the level of protein biosynthesis or mediated through protein inhibitors.The biological activity of cysteine proteinases is regulated not only by specific and non specific inhibitors but also by pH and zymogen activation *[Turk et al (1997)].* The Non - specific inhibitors of these peptidases in general are α-macroglobulin , which bind and inhibit irreversibly most proteinases, peptide aldehydes like leupeptin and antipain, (acting as reversible transition-state analogues) and peptide chloromethyl ketones which inactivate proteinases by binding the inhibitor and alkylating the active site *[Salvesen and Nagase (1989)].* Peptide diazomethanes and peptide epoxides alkylate active-site cysteine inhibiting particularly cysteine proteinases but they cannot discriminate between different cysteine proteinases *[Salvesen and Nagase (1989)].* All papain-like cysteine proteinases are synthesized as inactive proenzymes (zymogens). The proregion is needed for proper folding and transport of newly synthesized enzymes *[Cygler and Mort (1997)].* Cathepsins

are transported from the Golgi apparatus to the lysosomes where the pH is acidic *[Cygler and Mort (1997)]*.

This acidic environment has two functions it triggers the proteolytic cleavage of propeptide yielding mature cathepsin *[Jerala et al. (1998)]* and provides the optimum pH for these enzymes to function *[Kirschke et al. (1995)]*. A neutral pH as well too acidic pH rapidly inactivates cathepsins *[Turk et al. (1999)]*. Further, one mechanism of regulation is that cathepsins are stable only in a certain pH range which differs for every cathepsin. Throughout the maturation the pH declines in lysosomes. Therefore different cathepsins are activated at different maturation stages of lysosomes *[Turk and Bode (1991)]*. In mature lysosomes pH can reach values as low as 3.8 which is sufficiently low to cause irreversible denaturation of cathepsins B, S and L *[Turk et al. (2000)]*.

Several processes lead to increase of proteolysis in the cells e.g, starvation of animals increases the rate of protein degradation in liver *[Dice et al. (1978)]* and muscle *[Li and Goldberg (1976)]* and causes about two fold increase of Cathepsin D and A levels in the liver *[Katunuma et al. (1982)]*.

Similarly, several hormones are known to regulate average rates of protein degradation. Various pathological conditions have been associated with increased thiol proteinase activities like metastasizing cancer *[Koppel et al. 1994)]*, muscular dystrophy *[Noda et al. (1981)]*, renal failure *[Kabanda et al. (1995)]* and rheumatoid arthritis *[Gabrijeleic et al. (1990)]*. Hence the wide implications of the thiol proteinases in various biological functions need to be effectively regulated by opposing effects of activators and inhibitors.

Specific Proteinase inhibitors are ubiquitously present in the living system and they constitute a powerful regulatory system for the endogenous thiol proteinases. The members of cystatin superfamily, include **stefins, cystatins** and **kininogens**. These cystatins play a key role in regulatory process for endogenous cysteine proteinases whose activities if not controlled will lead to unwanted tissue destruction.

1.2 DISCOVERY OF CYSTATIN SUPERFAMILY

Protein inhibitors of cysteine proteinases in mammalian tissues were first reported by *Finkelstadt (1957)*, who reported a heat stable inhibitor of

cathepsin B and dipeptidyl peptidase-I in Rat liver cytosol. *Sen and Whitaker* discovered and partially characterized a protein from chicken egg white that inhibits papain and ficin *[Sen and Whitaker (1973)]*. This protein also inhibits cathepsin B and dipeptidyl peptidase-I as reported by *Keilova and Tomasek (1975)*.

The term cystatin was originally coined by *Barrett (1981)* for these inhibitors because of their unique property of arresting the activity of cysteine proteinases (CPs). Later, the name was given to a superfamily of evolutionary, structurally and functionally related proteins involved in the inhibition of CP activities.

Later on, CPIs (cysteine proteinase inhibitors) were isolated and characterized from various mammalian tissues. These inhibitors resembled chicken cystatin in relative molecular mass and physicochemical properties. Subsequently, *Machleidt et al. (1983)* reported that the sequences of *stefin and chicken cystatin are homologous [Green et al. (1984), Barrett (1985)]*. Stefins however, differed in lacking cysteine residues and disulphide bonds and hence, were placed in a different family within "cystatin superfamily".

All those cystatins falling into a single family that is a group within which all the members can be shown to have a statistically significant relation with each other but do no resemble the members of the other super families comprise one family *[Dayhoff et al. (1979a)]*. Cystatin super family include three distinct families, the members of which are expected to have atleast half of their amino acid residues in common *[Dayhoff et al. (1979b)]*.

CLASSIFICATION OF CYSTATIN SUPERFAMILY

The first international symposium on cysteine proteinases and their inhibitors in 1985 decided that these inhibitors should be divided into three families 1, 2 and 3 *[Barrett et al. (1986b)]*, Hence the proteins of cystatin superfamily are divided on the basis of sequence homology, molecular structure and the number of amino acids in the following three families *[Rawlings and Barrett (1990), Turk et al. (1997) , Brzin et al. (1984)]* ***[Fig - 1]***

FAMILY I (TYPE I CYSTATIN): STEFINS

The term stefin has been assigned to this family to emphasize the structural difference between these proteins and other members of the super family. The proteins of this family are the smallest among the members of the cystatin superfamily lacking in disulphide bonds and carbohydrate moieties *[Brzin et al. (1983)]*. Stefins are single chain proteins, synthesized without signal peptide and consist of about 100 amino acid residues *(MW 11000)*. All type I cystatins have blocked N-terminal and contain methionine representing the points of initiation of translation *[Turk et al. (1995)]*, There are three important members of this family, cystatin A and B isolated from human *[Green et al. (1984) Lenney et al. (1979)]* and stefin C purified from bovine tissues. Cystatins α and β are considered to be simply the rat species variants of cystatins A and B*) [Lenney (1979)]*. The amino acid sequence of these proteins closely resembles each other *[Barrett et al. (1986)]*. ***[Fig 2 a & b]***

Cystatin A

It is an inhibitor of cathepsin B in human skin discovered by *Fraki (1976)*. Later on *Jarvinen (1978)* studied it as 'acid cysteine proteinase inhibitor' ***(ACPI)***. *Brzin et al. (1983)* purified an inhibitor from blood leucocytes and named it as 'stefin'. The amino acid sequence was determined by *Machleidt et al. (1983). Green et al. (1984)* characterized same type of CPI from human liver and later renamed it as cystatin A. Cystatin A occurs in multiple isoelectric forms with predominantly acidic pi values in the range 4.5-5.0 *[Jarvinen (1978), Hopsu-Havu et al. (1983a)].Rinnie et al. (1978)* detected cystatin A in extracts of squamous epithelia from oesophagus. It was also found in dendritic reticulum cells of the lymph nodes *[Rinnie et al. (1983)]*, seminal plasma *[Minakata and Asano (1985)]*, saliva, bovine skin *[Turk et al. (1995)*, human nails *[Tsushima (1993)]* and in a number of epidermoid carcinomas *[Rinnie et al. (1980, 1984a)]*.

Physiological concentrations of human cystatin A was reported by *Hopsu-Havu et al. (1983b)* in serum as (10-15μg/ml). In tissues the concentrations were: epidermis of tongue and mouth, 65-200μg/g; bone marrow, 8μ g/g, liver 5μg/g, spleen 3μg/g and lung 0.3μg/g.The N-terminal sequence of bovine stefin A was identified as a truncated form of bovine

Fig-1 Diagrammatic representation of the chain structure of proteins in the cystatin super family

The **stefins** are single chain proteins without disulphide linkages. The **cystatin** ar also single chain but possess two disulhide bonds, The structure indicated for **kininogens** is that of L- kininogens, H-kininogens have a longer carboxyl terminal extension. The symbol ♦ marks potential sites for the attachment of the carbohydrate chains

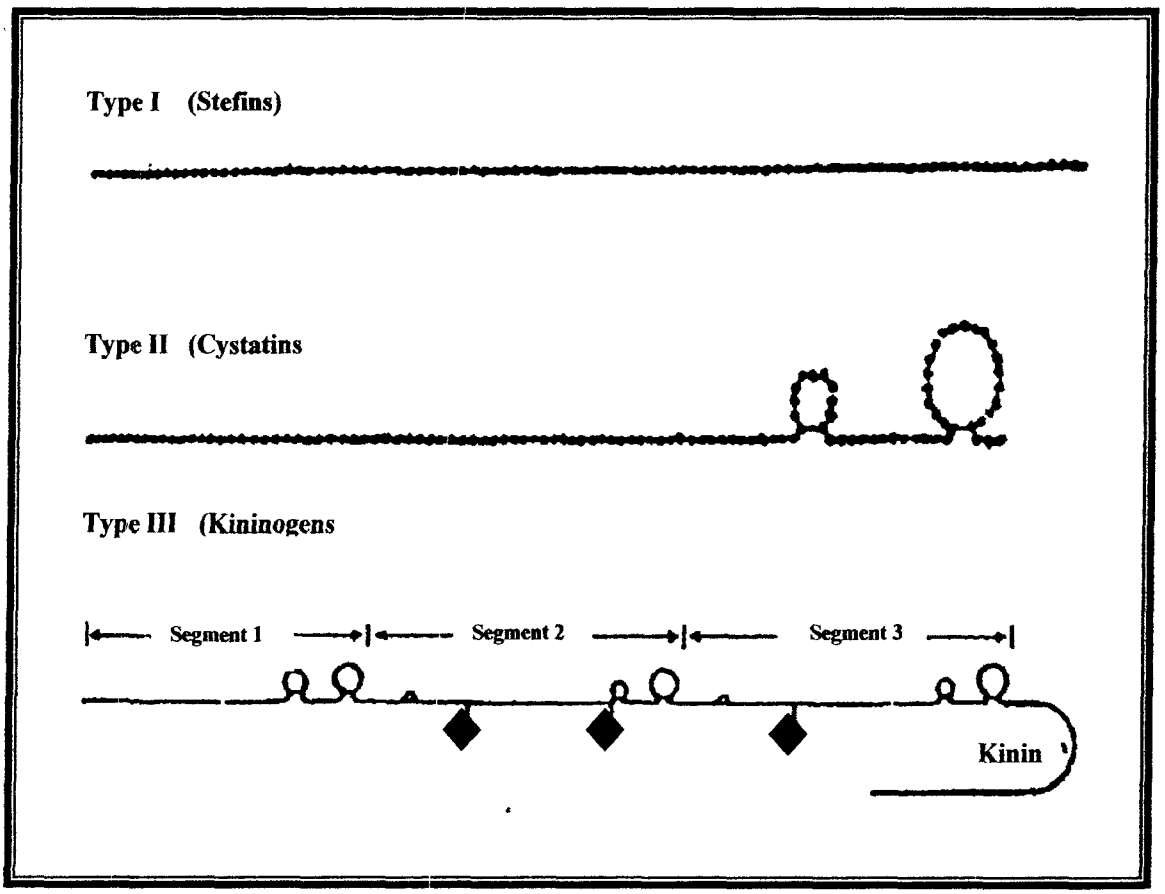

Type I (Stefins)
Type II (Cystatins
Type III (Kininogens
Segment 1
Segment 2
Segment 3
Kinin

Fig- 2(a) 3D structure of stefin A

(Member of type–I of cystatin superfamily)

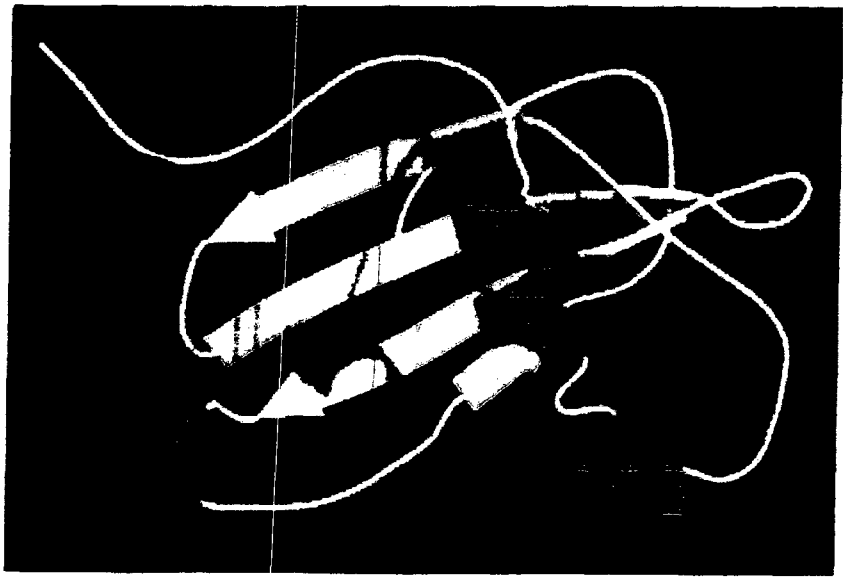
(b) Three dimensional structure of stefin B

(Member of type–I of cystatin superfamily) antiparallel β sheet are in green colour and α- helix is in red colour

a

STEFIN A

b



STEFIN B

cystatin C *[Hirado et al. (1985) Turk et at. (1995)].* The sequence identity of this stefin A is 86% and 88% respectively with -N and C terminal parts of *human cystatin A.*

Cystatin α

Cystatin α is assumed to be a species variant of cystatin A found in rats. This protein was characterized by *Jarvinen (1976)* as specific inhibitor of CP from rat skin having MW of 13000. Cystatin α is generally found on the epidermal layer *[Jarvinen et al. (1978)]* and various other squamous epithelia *[Rinnie et al. (1978)]. Kominami et al. (1984)* and *Katunuma and Kominami (1985)* quantified the concentration in skin and small intestine as 2800 µg/ml protein and 820 µg/ml protein respectively. The serum level of cystatin α in rats was 2.5µg/ml.*Green et al. (1984)* purified it from human spleen and liver with separation of multiple forms, Cystatin B is a relatively basic protein with pI values of 6.25 and 6.35 for the two forms of cystatin B *[Green et al. (1984)].*

Cystatin B

Cystatin B was detected as an inhibitor of cathepsin B and H in human tissues by *Lenney el al. (1979). Jarvinen and Rinnie (1982)* purified cystatin B from human spleen *(1982)* and reported its pI values of 6.0 and 6.5 *Rinnie et al. (1984)* reported the value as 6.5 and 7.5. Human cystatin B forms dimers but dimeric form does not show inhibitory activity *[Jarvinen and Rinnie (1982) , Green et al. (1984)].* Cystatin B is ubiquitously distributed in various cells and tissues like epithelial cells, lymphocytes *[Davies and Barrett (1984)],* monocytes *[Hopsu-Havu et al. (1984, 1985b)]* and to much lesser extent in seminal plasma *[Brzin (1982)]. Jarvinen et al. (1983)* have shown cystatin B in cells of the upper layers of the epithelium of the oral mucosa. Confocal microscopic analysis showed the localization of cystatin mainly in the proliferating cells and both in the nucleus and cytoplasm of differentiated cells *[Riccio (2001)].* This cystatin is involved in the protection against uncontrolled activity of lysosomal CP *[Henskens (1996)].* Crystal structure of recombinant human cystatin B has been elucidated by X-ray crystallographic analysis *[Stubbs et al. (1990)].* Cystalin B is more abundant in all cells than cystatin A

with the exception of PMNs. Stefin B from bovine was reported by *Krizaj et al. (1992).*

Cystatin β

Finkelstadt (1957) and Lenney (1979) reported the existence of a thermostable and dialyzable inhibitor of cathepsin B, H and dipeptidyl peptidase I in the supernatant of rat liver homogenate. Subsequently *Hirado et al. (1981)* isolated cystatin β from rat liver cytosol. The p1 values for cystatin β ranges from 5.04 to 5.6 *[Kominami et al. (1981), Hirado et al. (1981)]* Cystatin β has an almost even distribution in the tissues and is more abundant than cystatin α in all tissues except skin. This characteristic of cystatin β resembles cystatin B of human variant.

Stefin C

Several low MW CPIs have been found in bovine thymus, although kinetically similar they differ in the primary structure and pi values, Stefin C is unique among the inhibitors from stefin family which was found in multiple forms resulting from the cleavage of Asn 5-Leu 6 bond of the inhibitor, *[Kopitar et al .(1989)].* The protein consists of 101 amino acid residues and its MW is calculated lo be 11,546. The inhibitor was found to be acidic with pI values from 4.5 and 5.6 Stefin C exhibits considerable sequence homology with other inhibitors from stefin family with maximum homology of 87.4% with bovine stefin B, 72.4% with human cystatin B and 47.9% with human stefin A, It was identified as the first tryptophan containing stefin (at position 2) and it had prolonged N-terminal *[Turk et al. (1993)].*

FAMILY 2 (TYPE-II): CYSTATINS

Family 2 cystatins like slefins are low MW Cystatins. These inhibitors are composed of 115-120 amino acid residues having MW of about 13000. Cystatins contain two disulphide bonds and lack carbohydrate contents. They are synthesized with the singel peptide and found at a relatively high concentration in secretions like chicken egg white, seminal plasma and saliva. They originate in cell and function intracellularly. The known members of this family are chicken cystatin, human cystatin C, D, S, SN, SA, E, F and M.

Measurements of the concentration of the cystatin in human biological fluids reveal that all such fluids contain inhibitors, but the total molar ratios of different inhibitors vary markedly. Cystatins are found both in cells, tissues and secretions *[Lofberg et al. (1983)]*. The general pattern seems that secretions contain the highest concentration of family 2 cystatins *[Abrahamson (1994)]*. Family 2 cystatin genes are clustered in multigene locus on chromosome 20 together with two pseudogenes *[Abrahamson et al. (1990) Thiesse et al. (1994)]*. The best known representatives of this class of cystatins are human cystatin C, chicken cystatin and cystatin S. ***[Fig -3]*** .The pI value ranges from 4.4 to 9.3.

Cystatin C

Originally cystatin C was termed as γ-trace or post γ-globulin from CSF because of its basic natures and γ-electrophoretic mobility *[Barrett et al. (1984a) Brzin (1984)]*. It was also found in urine from patients with renal failure *[Butler and Flynn (1961)]* and ascetic and pleural fluids *[Hochwald and Thombecke (1962)]*. Later, cystatin C was also detected in saliva, normal serum *[Cejka and Fleischmann (1973)]* and in seminal plasma *[Collic et al. (1976)]. Lofberg and Grubb (1979)* determined cystatin C concentration of 5.8µg/ml in CSF [cerebrospinal fluid], 1.8mg/ml in saliva, 0.095ug/ml in normal urine, 21µg/ml in urine from patients with renal tubular disorders and 1.1µg/ml in normal plasma. Seminal plasma contains 50µg/ml that is. 36 fold higher concentration of inhibitor than in normal blood plasma *[Grubb et al. (1983), Abrahamson (1990)]* found cystatin C concentration of 3,4ug/ml in saliva from submandibular glands and 0.9µg/ml from the parotid gland. The high level of cystatin C in the CSF compared with blood plasma suggests the production of cystatin in the central nervous system *[Lofberg and Grubb (1979)]*. The protein is able to cross blood brain barrier to the vascular space and thereby rapidly filtered in the glomeruli and catabolised in the renal tubular cells. Despite widespread extracellular distribution, cystatin C has also been detected intracellularly in brain cortical nerves *[Lofberg et al. (1981a)]*, normal and neoplastic neuroendocrine cells in the adrenal medulla *[Lofberg et al. (1982)]*. A cells of pancreatic islets *[Lofberg et al. (1981b)]*, thyroid and pituitary glands *[Lofberg et al. (1983), Moller et al. (1985)]*. Homologues of

human cystatin C have been found in several mammals including mouse, rat, cow and dog *[Solan et al. (1990), Esnard et al. (1990), Hirado et al. (1985), Poulik et al. (1981)].* It is also found to be linked with epileptogenesis and epilepsy along with other neurodegenerative diseases *[McGeer (1995)].Cejka and Fleischmann (1973)* determined the isoelectric point of cystatin C to be 9.0 although, values ranging from 8.0 to 9.5 have been reported. *[Brzin et al. (1984)]* Degradation of N-terminal region takes place during storage which results in a lower pI value, N-terminal truncation of cystatin C by cathepsin L has been reported by *Popovic et al. (1999)* leading to loss in its activity and lowering of pI to 8,1 and 8.25 due to loss of one or more basic residues. It was suggested that cleavage could be of physiological significance to control the extent of inhibition by cathepsin B at the site of inflammation *[Abrahamson el al. (1991)].* The truncation results in the exposure of N-terminal and loss of ordered structure of cystatin in solution *[Ekiel et al. (1997)].*

Chicken cystatin

Chicken cystatin was first isolated from chicken egg white by *Fossum and Whitaker in (1968) and Sen and Whitaker in (1973)* reported it as low MW light binding CPI of ficin and papain and of cathepsin B and C by *Keilova and Tomasek (1974, 1975).* Chicken cystatin ***[Fig-4]*** has also been detected in the serum of both male and female chickens at a concentration of 80µg/ml in egg white and 1µg/ml in the serum *[Anastasi et al. (1983)].*it was found to be heat stable and could be boiled for 30 min without much loss of activity, but was unstable to freezing or freeze drying. It has also been found in chicken muscle cells *[Wood et al. (1985)]* and showed resemblance to cystatin C Mw of about 13,000 has been determined for chiken cystatin from its amino acid composition *[Lindahl el at. (1988), Nicklin and Barren (1984)]* resolved this inhibitor as two isoelectric forms I and II having pI values of 6.5 and 5.6, respectively.

Cystatin D

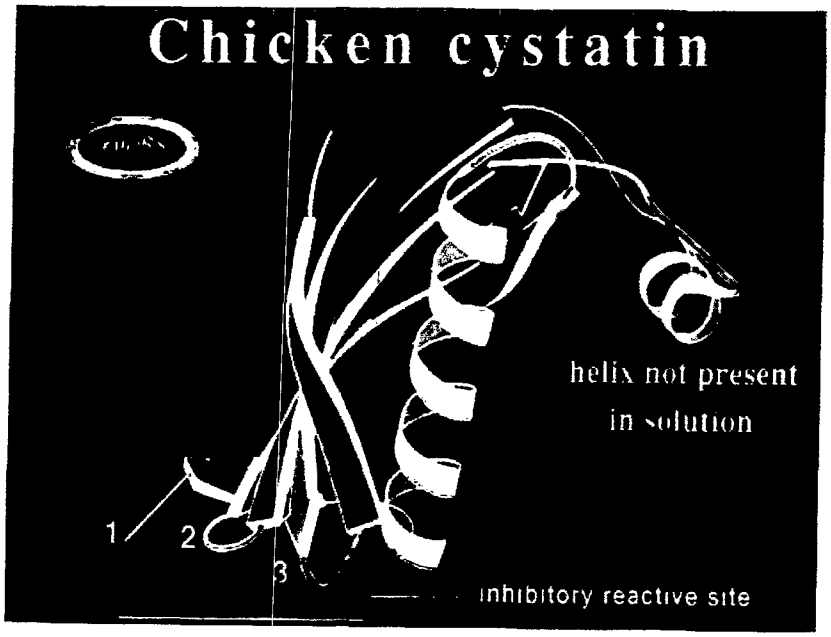
Frieje et al. (1991) cloned a new member of human cystatin multigene family from a genomic library using cystatin C cDNA probe.This inhibitor consists of 122 amino acid residues having a Mw of 13,885. The deduced

Fig-3 Three dimensional structure of Human cystatin

(Member of the type-I cystatin superfamily)

Fig-4 Three dimensional structure of Human cystatin along with its inhibitory reactive sites

HUMAN CYSTATIN



amino acid composition includes a putative signal and has 51-55% homology with either cystatin C or secretory gland cystatins S, SA and SN. Cystatin D is relatively neutral protein with PI in the range 6.8-7.0 *[Frieje et al. (1991)]*. It is expressed in parotid glands, saliva and tears *[Balbin et al. (1994)]*. This tissue restricted expression is in marked contrast with a wider distribution of all other family 2 cystatins.

Cystatin S

Human saliva contains several low MW acidic proteins which include CPI also *[Minakata and Asano (1985)]*. The first salivary inhibitor purified and sequenced was SAP-I (salivary acidic proteinase-I) *[Isemura et al. (1984a)]*, which was renamed as 'cystatin S'. It was found to have 54 and 41% sequence homology with cystatin C and chicken cystatin, respectively *[Isemura et al. (1984a and b)]*. This inhibitor has also been isolated from human submaxillary, submandibular and sublingual glands and found to be present in the serous cells of parotid and submaxillary glands *[Isemura et al. (1984b)]*. It is assumed that these cells are responsible for its synthesis. The protein has also been found in tears, serum, urine, bile, pancreas and bronchus *[Isemura et al. (1986)]* Cystatin S contains no phosphate, in contrast to other salivary proteins.

Variants of cystatin S

Several molecular variants of cystatin S have been studied by *Isemura et al. (1986)*. They differ in their N-terminal sequences and pI values differences in PI values resulted from phosphorylation of residues Ser 3 and Ser 1 in salivary cystatin *[Isemura et al .(1991)]*.

Cystatin SN

It was originally known as cystatin SV or SA-I *[Abrahamson et al. (1986)]*. The protein consists of 121 amino acid residues and MW value of 14,316. The pI value is in the range of 6.6-6.8.

Cytatin SA

The protein consists of 1221 amino acid residues and Mw value is slightly higher than SN, which is 14,351. It has acidic pI value of 4.6 *[Isemura*

et al. (1991)]. Cystatin SA isolated from saliva had N-terminal residue Glutamic acid *[Isemura et al.(1986)].*

Cystatin E

Ni et al. (1997) found this type of cystatin by expression of amniotic fluid and fetal skin epithelial cell cDNA libraries. The mature protein is a polypeptide of 121 amino acid residues and has 28 residue signal peptide having a Mw of 15,000. Cystatin E resembles family 2 cystatins structurally in containing two protective disulphide bridges and by being a secreted protein. The inhibitor has unusual characteristic of being a glycoprotein carrying an N-linked oligosaccharide at Asn 108 *[Stenmann et al. (1997)].* Cystatin E is a functional tight binding CPI it has amino acid sequence similarities with human family 2 cystatins [C,D,S, SN and SA] in the range of 26-34% , 18 and 23% homology with family 1 (cystatin A and B) and less than 30% with domains 2 and 3 of the family 3 cystatins, that is kininogens *[Ni et al. (1997)].* The protein has Pro-Trp pair towards the C-terminal like that of family 2 and 3 cystatins. Cystatin E gene is localized on chromosome 11 *[Stenmann et al. (1997)].*Cystatin E has been detected in a variety of specialized tissues and organs *[Ni et al .(1997)].* Its high concentration is found in uterus and liver, but significant amounts in placenta, pancreas, heart, spleen, small intestine, and peripheral blood leucocytes, in brain, testis and kidney of mammals. The inhibitor serves a protective role during fetal development.

Cystatin F

Another described human member of the cystatin superfamily was identified as cystatin F by *Ni et al. in (1998*). Its sequence contained signal peptide and the mature protein of 126 amino acid residues has two disulphide bonds which resemble family 2 cystatins. Unlike other members, cystatin F has two additional Cys residues indicating the presence of an extra disulphide linkage which stabilizes N-terminal region of the molecule. The mature protein has an extended N-terminal segment, being 6-10 residues longer than the other single domain cystatins. Cystatin F has a Mw of 14,543 as determined by sequence analysis *[Ni et al. (1998)].*Cystatin F has two N-linked carbohydrate chains at positions 36 and 88. It has 30-34% homologous

sequence to human family 2 cystatins, only 29% homology to cystatin E, even lower 22-20% identities to cystatin A and B and 24% with domain 3 of kininogens *[Ni et al. (1998)].*The distribution pattern of cystatin F is very different from that of cystatin C or E. Blood contains low levels of this inhibitor (0.9 µg/ml). The highest concentration is present in the spleen and peripheral blood leucocytes, moderate levels in thymus and small intestine. Cystatin F is found mainly in the immune cells. However, it could not be detected in B cell lines but appreciable amounts were detected from primary dentritic cells, bone marrow, activated T-cells, multiple sclerosis lesions, osteoarthritic, lymph nodes, apoptotic T-cells, osteosarcoma cells, pancreatic islet tumour cells, thymus, helper T-cells and peripheral blood mononuclear cells. The function of cystatin F is important for specialized cells of the myeloid or T-cell lineage. Taken alltogether the expression pattern, inhibitory profile and intracellular localization predispose cystatin F as possible regulator of antigen processsing and presentation *[Ni et al. (1998)].*

Cystatin M

Cystatin M was discovered in the primary tumour cell lines. It is synthesized and secreted as a precursor protein *[Von Heinjie (1985)].* The predicted molecular mass is approximately 14.3 KDa and pI of 7.8 for the mature protein. It containes a single disulphide bond *[Sotiropoulou et al. (1997)]* An N-glycosylated form of cystatin M of 20-22KDa was immunoprecipitated and accounted for about 30-40% of total cystatin M protein. Both forms of native cystatin M occurred intracellularly.Cystatin M resembles other members of the family in having the three conserved domains. It has 40% homology to human type 2 cystatins the overall homology between cystatin M and other cystatins range from 30-40% for conserved amino acid residues and 25 to 33% for identical amino acids. Cystatin M shows the closest homology to cystatin C, they share 33% identical and 38% conserved amino acid residues *[Sotiropoulou et al. (1997)].* Cystatin M is expressed by normal mammary epithelial cells and a variety of human tissues but it is not expressed in many metastatic mammary tumour cell lines. High levels of cystatin M are present in placenta, lung, skeletal

muscle, kindly and pancreas its lower level was observed in heart tissue *[Sotiropoulou et al. (1997)].*

FAMILY 3 (TYPE III): KININOGENS

Kininogens are the high molecular weight thiol proteinase inhibitors found only in plasma .In mammals, three types of kininogens have been found differing in size, structure and function. The largest kinin precursor, commonly referred to as high molecular weight (HMW) kininogen, has a Mw of 88000-114000, depending on its species and tissue origin *[Kato et al. (1981)].* Among the smaller kininogens, a low molecular weight (LMw) kininiogen with a Mw of 50000-70000 is found in throughout the mammalian lineage *[Muller-Esterl et al. (1986)].* The third type, T-kininogen of MW 68000, seems to be unique of rats *[Okamoto and Greenbaum (1983)].* The various types of kininogen have the same basic structure, a heavy chain of 50-60KD at the amino-terminus, the kinin segment in the core part of the molecules and a light chain of variable length at the carboxy-terminus *[Kitamure et al .(1985), Lottspeich et al. (1985)].* The primary structure of the heavy chain, kinin portion and a segment of 12 amino acid residues are identical in HMW and LMW kininogens, but the structure of their light chain diverges considerably. HMW kininogen has a large light chain of 45-58Da which harbours unique histidine-rich region *[Sugo et al. (1980)].* The HMw kininogen light chain contains the binding site or prekallikrein and factor XI. The structure of T-kininogen closely resembles that of LMw kininogen in its heavy and light chain portions but differs at the amino-terminal flanking region of the kinin segment *[Cole et al. (1985)].*The primary site of kininogen biosynthesis is the liver with only minor expression in other tissues. The kininogens are secretory proteins synthesized with a signal peptide and undergoes glycosylation. Important functions of kininogens are the release of vasoactive peptide bradykinin, by acting as substrate for kallikrein, intrinsic blood coagulation, acute phase reactions and inhibition of cysteine proteinases *[Muller-Esterl et al. (1986)].* pI values reported for the multiple isoelectric forms of L and H kininogens fall in the range 4.0-4.9 *[Ryley (1979) , Muller-Esterl et al. (1982 , 1983)].* The proteins are stable to temperature in the range of 50-90^{0}C *[Taniguchi et al. (1981)].*

Plasma concentrations of kininogens have been determined by radio immunoassay to be 109-271 µg/ml (L-kininogens) and 69-116 µg/ml (H-kininogen) *[Adam et al. (1985), Salvesen et al. (1985)]* found that three cystatin like segments are present in kininogens, although the first the first lacks preserved sequence Gln-Val-Val-Ala-Gly. It is known that the LMW-kininogen contain no free thiol groups *[Salvesen et al. (1985)]*. Kininogens inhibit papain, ficin, cathepsin B, H and L *[Jarvinen (1979), Gounaris et al. (1984)]*. ***Table 1*** summarizes the various mammalian and non mammalian sources from where cystatins have been isolated.

1.3 OTHER TYPES OF CYSTEINE PROTEINASE INHIBITORS

There are a number of other cystatin or cystatin related proteins, which are structurally related to cystatins with no inhibitory activity against papain like enzymes *[Turk and Turk (2008)]*. CRES (Cystatin Related Epididymal Spermatogenic protein.*[Sutton et al. (1999)]*, testatin *(expression restricted to mouse pre-Sartoli cells) [Tohonen et al. (1999)]*, cystatin SC and cystatin TE-1 (expressed in testis and epididymis, respectively) *[Li et al. (2002)]*, and several other genes of cystatins with no inhibitory activity were found expressed specifically in the male reproductive tract *[Hamil et al. (2002) , Xiang et al. (2005) , Shoemaker et al. (2000)]* indicating the existence of a new subgroup in the type two cystatins *[Cornwall et al. (2003) , Sutton-Walsh et al. (2006)]*. These CRE Statins show homology to cystatins, with the exception of the two hairpin loops responsible for the cysteine proteinase inhibition Their role could be regulation of proteolysis in the reproductive tract as well as protection against invading pathogens, as shown by cystatin 11 *[Hamil et al. (2002)]*.

The CRES protein tend to form oligomers similar to cystatin C *[Janowski et al. (2001), Wahlbom et al. (2007)]* and stefin B *[Jenko-Kokalj et al. (2007)]*. Another type 2 cystatin, cystatin 10, expressed in cartilage, localized in prehypertrophic and hypertophic chondrocytes is known to be an inducer of chondrocyte maturation followed by apoptosis *[Koshizuka et al. (2003)]*. A novel cystatin type 2 protein namely CLM expressed widely in normal tissues playing role in hematopoietic differentiation or inflammation, different from CRES was characterized by *Sun and coworkers [2003]*.

TABLE - 1 CP1 FROM DIFFERENT SOURCES

Source	Tissue	Mr	pI	*Reference*
African puff	Adder	13,000	6,5	*Evans and Barrett, 1987*
Beef	Spleen	13,000	4.8-7.0	*Brzin et al, 1982*
Bovine	Hoof	11,406	-	*Tsushima et al., 1996*
	Muscle	14,000	6.2	*Bige et al., 1985*
	Colostrum	12,787	10.0-10,3	*Hiradot et al., 1984*
Carp	Ovary	12,000	-	*Tsai et al., 1996*
Horse shoe Crab	Hemocytes	12,600	-	*Agarwala et al., 1996*
Human	Liver	12,400	-	*Green et al,. 1984*
	Placenta	12,300	-	*Warwas & Samicki, 1985*
	Spleen	11,400	6.0-6.5	*Rinnie et al., 1980*
Rabbit	Liver	5000-10,000	-	*Pontremoli et al., 1983*
	Skin	12,500	6.6	*Udaka & Hayashi, 1965*
Sheep	Plasma	280,000	-	*Baba et al., 2005*
Goat	Kidney	67,000	-	*Zehra et al., 2005*
	Brain	70,300 12,720	-	*Sumbul & Bano, 2006*
Trypanasoma cruzi	-	11,000	-	*Monteiro et al., 2001*

1.4 NEW MEMBERS OF THE CYSTATIN SUPERFAMILY

The feutins and histidine-rich glycoproteins (HRG) comprise fourth family of cystatins. The feutin family consists of two tendem cystatin domains. Bovine feutin was first characterized by *Pedersen in (1944)* and its relation to cystatin superfamily was described in 1988 *[Elzanowski et al. (1988)]*. Human feutin (α_2-HS glycoprotein) was confirmed in 1987 *[Dziegielewska et al. (1987, 1990) Dziegielewska and Brown (1995)]*. Since then, protein and/or cDNA sequences of this protein have been reported for human, cow, mouse and Habu snake. Almost all the feutin sequences contain 12 cysteine residues, showing homology to the cystatins and cystatin domains in kiniogens *[Dziegielewska and Brown (1995)]*.

HRG has been characterized in the plasma of man, mouse, rabbit and cow *[Leung (1993)]*, sharing good sequence homology with human and bovine HMW kininogen *[Koide et al. (1986)]*. A large number of proteins have been discovered recently, which posses cystatin domains e.g. latexin *[Agaard et al. (2005)]*. However, feutin, HRG and latexin all seem to lack CPI activity.

Thyropins

Constitute a new family of papain-like CP inhibitors *[Lenarcic and Bevec (1998)]*, classified as family I31 *[Rawlings et al. (2004)]*. The p41 invariant chain (Ii)-fragment of the MHC class II-Ii complex 104, 105 and equistatin from the sea anemone *[Lenarcic et al. (1997)]* are best characterized members of this family Thyropins show inhibitory activity against CPs and also towards aspartic and metalloproteinases *[Mihelic and Turk (2007) , Lenarcic and Turk (1999)]*.

Tick cystatins

Syalostatin L and *syalostatin L2 [Kotsyfakis et al. (2007)]* have been characterized from salivary glands of the tick *Ixodes seapularis*. Both show 75% sequence identity and inhibit cathepsin L with a Ki of 4.7 nM and cathepsin V with Ki of 57 nM.

Statphostatins

These are specific inhibitors of staphylococcal CPs. Three members of this family have been described-staphostatins A and B from *Staphylococcus*

aureus and staphostatin A from *Staphylococcus epidermidis [Filipek et al. (2003)]*.

Clitocybin

It is a new type of CPI from a mushroom appearing to be related to fungal lectins and hence a new family of CPIs is suggested for them called mycocypins *[Brzin et al. (2000)]*. Chagasin is a cysteine proteinase inhibitor from *Trypanozoma cruzi* inhibiting both cruzipain and papain, but has no homology with cystains *[Monteiro et al. (2001)]*.

Phytocytatins

In plants, inhibitors of CPs are known as phytocystatins. They contain the QXVXG region of type 2 cystatins, but also resemble stefins in the absence of disulphide bonds *[Arai et al. (2002)]*, providing a transitional link between type 1 and type 2 cystatins.

There are numerous phytocystatins expressed and characterized on the basis of protein level from corn *[Abe et al. (1992)]*, rice *[Chen et al. (1992)]*, soyabean and sugarcane *[Oliva et al. (2004)]*. C-terminal extended phytocystatins were found as bifunctional inhibitors of papain and legumain *[Martinez et al. (2007)]*. In addition, a "multicystatin" containing two cystatin like domains were isolated from cowpea leaves *[Diop et al. (2004)]*.

Also there are certain plant proteins like monellin which lack the CPI activity but have a cystatin like three dimensional structure *[Grzonka et al.(2001)]*. Phytocystatins and other inhibitors important for plant defence response to insect predation may act to resist infection by some nematodes *[Koiwa et al. (1997)]*. They play a crucial role in response to various conditions *[Diop et al. (2004), Brzin and Kidric (1995)]* and show great potential tools for genetically engineered resistance of crop plants against pests *[Aguiar et al. (2006)]*.

VARIANT CYSTATINS

Divergent cystatins showing significant homology to stefins, cystatins and kiniogens have been expressed/purified and characterized from venom of African puff adder *(Bitis arietans) [Evans and Barrett (1987)]* from perilymph of

flesh fly larvae *[Suzuki and Natori (1985)]* from Drosophila melanogaster *[Delbridge and Kelly (1990)]*.

1.5 EVOLUTIONARY RELATIONSHIP

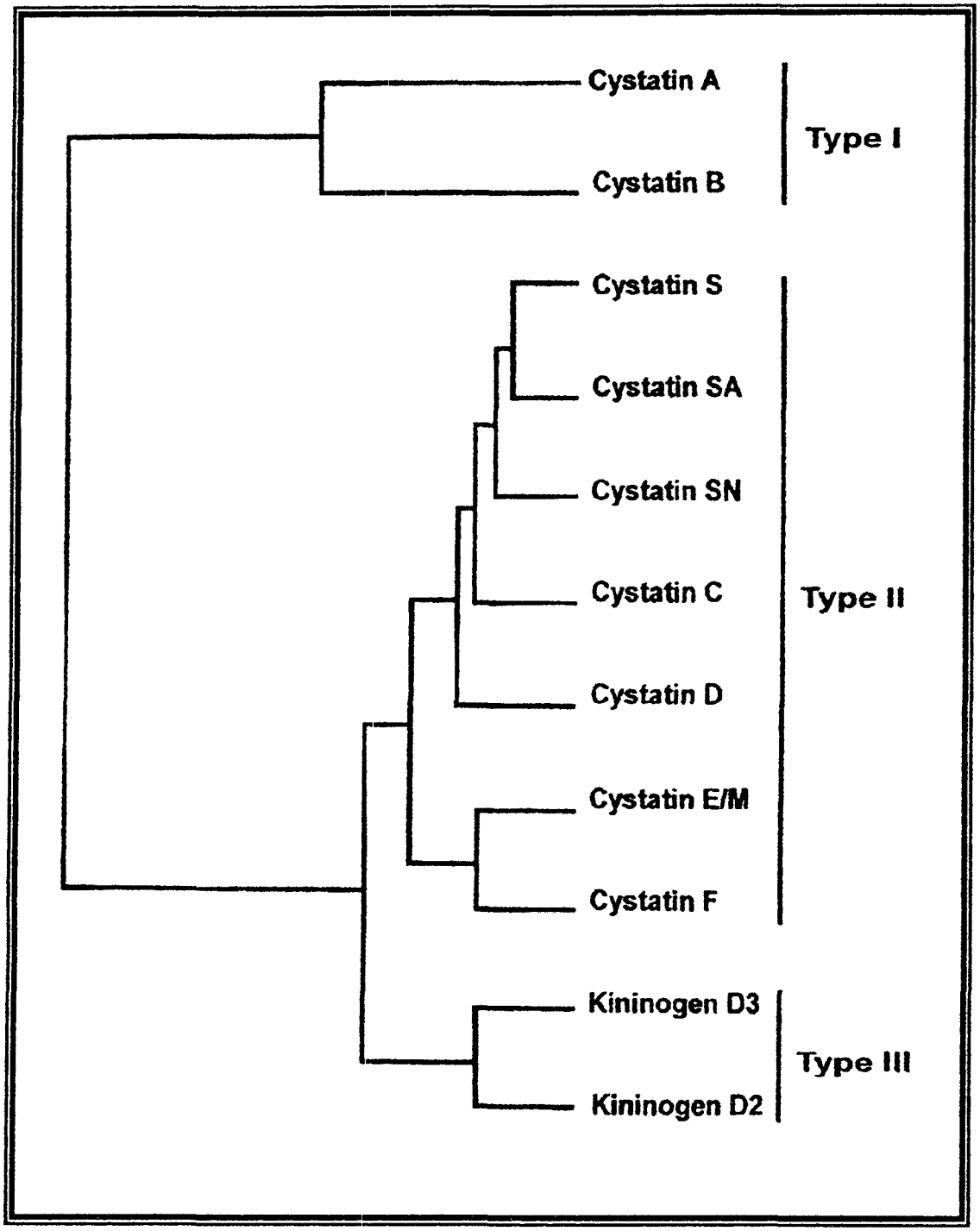
Statistical analysis of the similarities between the aligned sequences in Cystatins is suggestive that they have evolved from a single primitive sequence *[Barker and Dayhoff (1972)]*. Difference matrix of the sequences of the members of the superfamily revealed that the type-I forms one family and type II forms a second family whereas kininogens also form a single family. However, members of the cystatin superfamily are closely related to each other. ***[Fig.5]*** *demonstrates the relationship between all known inhibitory* active human cystatin domains according to their sequence homology and physicochemical properties.

The structure of the cystatin superfamily indicates that the members of the family involed atleast six duplications of genetic material from a single primordial sequence to produce the cystatins A, B, C, S and 3 segments of kininogens. Gene structures of kininogens show that each of the three cystatin like segments of kininogens are coded by three exons for which, the cystatin gene was the precursor *[Kitamura et al. (1985)]*. Some workers reported that only the first two of the three exons of the primitive family 2 gene was represented in family I cystatin gene, the segment is acquired during the evolution of family 2 cystatins *[Keil-Dlouha (1986)]*.Analysis of amino acid sequence data led to the conclusion that the differences in the length of the polypeptides are accounted by the insertion and deletion of the genetic material in small blocks. Since disulphide bonds are often acquired in the evolution of protein but are rarely lost, it is assumed that the primitive precursor of the cystatin was not unlike a modern type I cystatin. The evolutionary relationship among the various CPIs remained largely obscure until *Muller-Esterl et al. (1985)* suggested a common evolutionary origin of stefins, cystatins and kininogens.

The superfamily of mammalian CPI is constituted by atleast three distinct families with stefin, cystatin and kininogens as their prototype.The phylogenetic dendogram depicts that the divergence between chicken cystatin and human cystatin C and S may have occurred 300 million years ago, the

Fig-5 Schematic illustration of evolutionary relationship of cystatins of type I, II, III

The Figure shows evolutionary relation ship between all known inhibitory active human cystatin families


Cystatin A
Cystatin B
Type I
Cystatin S
Cystatin SA
Cystatin SN
Cystatin C
Cystatin D
Cystatin E/M
Cystatin F
Type II
Kininogen D3
Kininogen D2
Type III

time of appearance of first reptile. The divergence of cystatin A and B appears to be about 230 million years ago, before the appearance of true mammals *[Muller-Esterl (1985)]*.

1.6 PRIMARY STRUCTURE

Most of the member of cystatin super family are polypeptides of 98-126 amino acids residues with Mr values falling in the range of 11000-14000 it can be seen that cystatin A and α are devoid of cysteine residues where as cystatin B and β contain one and two residues respectively.

Cystatin F is reported to have two or more cysteine residues apart from the usual four cysteine resides although tryptophan is absent from type I cystatin it is reported to be present in stefin C. An interesting feature of the stefins B is the conserved QVVAG region in the stefins of mammalian origin.Val 54 is replaced with Leu 54.*[Grubb et al. (1984)]* The disulphide linked peptides have been identified from human cystatin C and chiken cystatin between cys 71- cys81 and cys 95 –cys 115. *Anastasi et al. (1983)* found that cystatin type I contains no free thiol groups .cystatin E apart from other usual conserved sequences characteristic of family 2 cystatins has a five residues insertion between amino acid 76 and 77 and a deletion of residues 91 (cystatin C numbering).*Ni et al. (1998)* deciphered the presence of a second trp residues , in addition to the conserved trp-106 in cystatin F.

1.7 SECONDARY STRUCTURE

The secondary structure of chicken cystatin and human cystatin C has been studied extensively. One line of evidence from analysis of amino acid sequence for the distribution of hydrophilic and hydrophobic residues, has predicted that hydrophilic residues are on the surface whereas hydrophobic regions are in the interior of the protein inhibitors like other compact globular proteins.Each sequence posses a large N-terminal hydrophilic region (15-42 residues) and a prominent central hydrophobic region (55-70 residues) followed by another large hydrophilic region at C-terminus. The secondary structure of human cystatin C in solution is very similar to that reported for chicken cystatin *[Dieckmann et al. (1992)]*. The crystalline form of chicken cystatin has been reported by *[Bode et al (1988)]*.Both human cystatin C and

chicken cystatin as well as the family 1 cystatins (A and B) consist of five stranded antiparallel β-pleated sheets *[Martin et al. (1995)]* which is wrapped around a straight five turn α-helix. X-ray diffraction analysis showed an appending segment of partial α – helical in chicken cystatin *[Saxena and Tayyab (1997)]* which is not found in human cystatin C The confirmation of this region seems to be closer to that found in chicken cystatin *[Dieckmann et al. (1993)]*.

The N-terminal segment upto Val 10 seems to be flexible similar to that of other cystatins *[Martin et al. (1995)]*. Trp was found only in the second hairpin loop of cystatins *[Bode et al (1988)]*.Circular dichroism and computer prediction of secondary structure from the sequence indicate that the chicken cystatin has about 20% α-helix, 42% β-structure, 24% β-turn and 12% random coil structure. *Janowski et al. (2001)* proposed that the human cystatin C undergoes dimerization under mild denaturing conditions. NMR studies revealed that the majority of the proteins are not affected by dimerization however, the CP binding site is significantly affected which accounts for the activity loss *[Ekiel et al. (1997)]*.

Hence, it is concluded that hydrophobic CP binding site is involved in the monomer-monomer contact. The dimerization may be required for regulation of cystatin C inhibitory activity in vivo *[Abrahamson (2001)]*. Three dimensional swapping studies indicated the dimerization of human cystatin C to be a step towards protein oligomerization *[Janowski et al. (2001)]*.

1.8 INHIBITION OF PROTEASES

Specificity

All the enzymes known to be inhibited by the cystatins are proteases with cysteine at the active sites. No example of serine, aspartic or metalloproteinase or non proteolytic enzymes being inhibited by cystatins is available. Results that have been reported include those on cystatin A and B with trypsin, chymotraypsin, pancreatic elastase, cathepsin G and thermolysin *[Fraki (1976), Green et al. (1984)]*. The available data indicates that all cystatins inhibit CP of papain superfamily including papain, chymopapain,

papaya, proteinase III, ficin, actinidin and the lysosomal CP and cathepsisn B, H and L.

Of the type II cystatins, chicken cystatin has been shown to inhibit papain, ficin, chymopapain, papaya proteinase III, actinidin and cathepsin B,H, L and N *[Fossum and Whitaker (1968), Keilova and Tomasek (1974 , 1975) Barrett (1985), Etherigton (1976)]* cystatin S inhibits papain and ficin *[Isemura et al.(1984b)]*, beef colostrums cystatin inhibits papain and cathepsin B and H *[Hirado et al. (1984)]*.

Cystatin M inhibits papain *[Sotiropoulou et al. (1997)]*. Cathepsin B and papain are inhibited by cystatin E *[Ni et al. (1997)]*. Cystatin F inhibits papain and cathepsin L but not cathepsin B *[Ni et al. (1998)]*. Cystatin D inhibits cathepsin L, H and S but not cathepsin B *[Balbin et al. (1994)]*.Ironically, papain homologue bromelain from pineapple stem or fruit is not inhibited by cystatin. The low Mw cystatin B do not inhibit calpains, chicken cystatin *[Johnson et al. (1984)]* and human amniotic fluid *[Rohrlich et al (1985)]*.

Human cystatin A and chicken cystatin partially inhibit clostripain and show no inhibition of streptococcal cysteine proteinase *[Green et al. (1984)]*. Clostripain was also not inhibited by cystatin S *[Isemura et al. (1984b)] Keilova and Tomasek (1975)* showed that chicken cystatin inhibits dipeptidyl peptidase I, this proteinase is also known to be inhibited by human cystatin A, B, C, and S *[Green et al (1984), Isemura et al. (1986)]*, cystatin α *[Jarvinen and Hopsu-Havu (1975)]* and cystatin β. He also reported Inhibition of lysosomal carboxypeptidase B by cystatin β

Kinetic Behaviour

The cystatins are the first group of protein inhibitors of CP for which the mechanism of inhibition has been investigated. cystatins are a group of potent, non covalent and reversible inhibitors of CP of papain superfamily *[Barrett (1987), Ekiel et al. (1997)]*. Some of the reported values of Ki are presented in ***Table-2.*** It can be seen that the inhibition constants extend down to a value of 5×10^{-12}. Inhibition of cathepsin B tends to be weaker than that of papain and cathepsin H and L, except with cystatin C which is a strong inhibitor of cathepsin B.

TABLE-2 Ki VALUES FOR INHIBITION OF CYSTEINE PROTEASE BY LOW MW CYSTATINS

Enzyme	A[a]	B[a]	C[a]	D[b]	F[c]	E[d]	Chicken cystatin	Bovine stefin	Puff adder
Papain	0.019	0.12	0.005	0.9	1.1	0.39	0.005	0.18	0.1
Cath. B	8.2	73	0.25	1000	-	32	1.7	>100	2.7
Cath. H	0.31	0.58	0.28	8.5	-	nd	0.064	nd	nd
Cath. L	1.3	0.23	0.005	25	0.31	nd	0.019	0.0065	nd
Dipeptidyl	33	0.23	3.5	nd	nd	nd	nd	nd	0.23
Cath. S	nd	nd	nd	0.24	nd	0.35	nd	nd	nd

Values are given in nanomolar concentration.

nd – not determined

a *Barrett et al. (1984) Gounaris et al. (1984) and Machleidt et al. (1986).*

b *Balbin et al. (1994)*

c *Ni et al. (1998) and d Ni et al. (1997).*

Reactive site

The reactive site of the folded, native cystatin molecule could obviously involve parts of the molecule well separated in primary sequence. The elucidation of the crystallographic structure of cystatin / enzyme complexes classified the precise location of the reactive site ***[Fig 6 a & b].*** The central region of the sequence contains the highly conserved block, usually Gln-Val-Ala-Gly at 53-57, Gln 57 are common to all cystatins and inhibitory kininogen segments .The neighbouring residues also show strong tendency to conserve this type of sequence. In chicken cystatin the sequence is QLVSG instead of QVVAG *[Barrett et al. (1986), Barrett (1987)].* Residues 60-62 are all uncharged generally with large hydrophobic side chains in small cystatins.

The C-terminal part of the sequence tends to be very varied, although Cys 71 and Tyr 100 are conserved in the large majority of sequences. There also appear to be deletions and insertions in the C-terminal segments that suggest variability in structure but the disulphide bonds presumably stabilize the folding of these segments in type I cystatins and kininogens. Chicken ystatin has Trp 104 as the reactive group during the interaction with Cp. All other type II cystatins also contain the conserved second hairpin loop around Trp 104. *Estrada et al. (1998)* emphasized the role Gly4 on *human stefin A (cystatin A)* in the binding of target proteinases. Even the smallest replacement in this protein by site directed mutagenesis by Ala resulted approximately 1000, 10 and 6000-fold decreased affinities for papain, cathepsin L and cathepsin B, respectively.

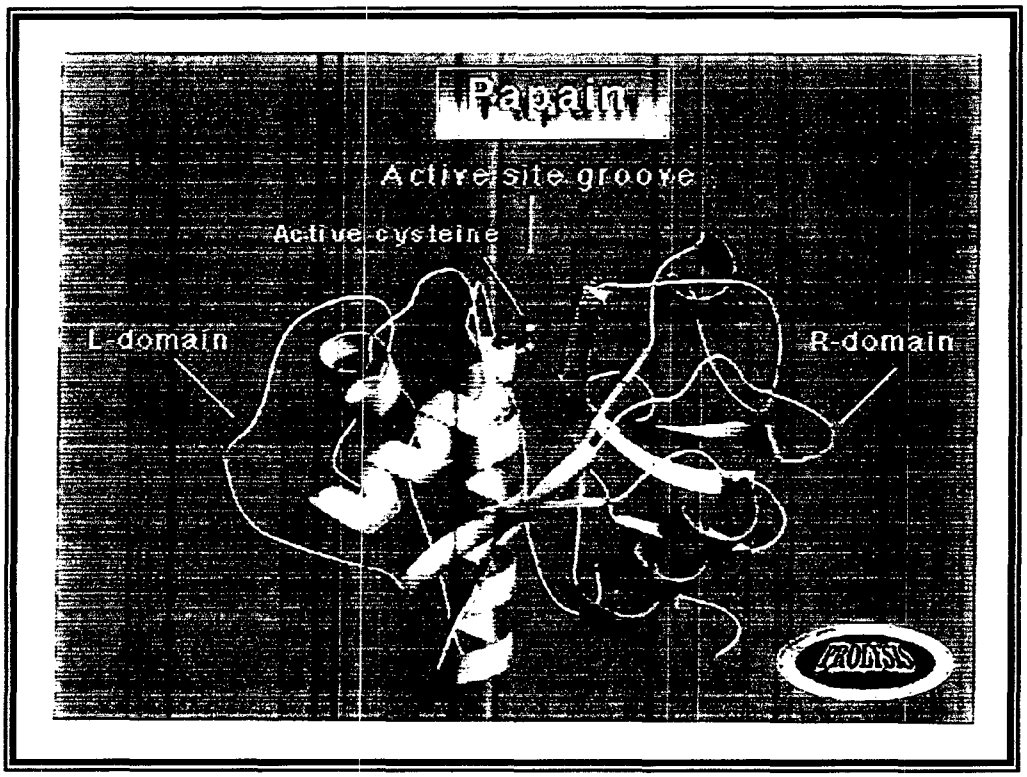
As the side chain of the substituted amino acid residue became more bulky, the inhibitory activity of the variant decreased markedly *[Shibuya et al. (1995)]*, the single cysteine residue, Cys 3 of human and bovine cystatin B has appreciable importance for inhibition of CP, particular for cathepsin B *[Pol and Bjork (2001)].*The contact residues in the hairpin loop, Leu 73 and His 75 when replaced by Gly it resulted in appreciably reduced affinities for papain, cathepsins H and B. These two residues contribute 20-30% of the free energy of binding of cystatin B.

Replacement of the contact residue in the C-terminal end, Tyr-97, with Ala resulted in the loss in affinities for papain, cathepsins H and L. All affinity

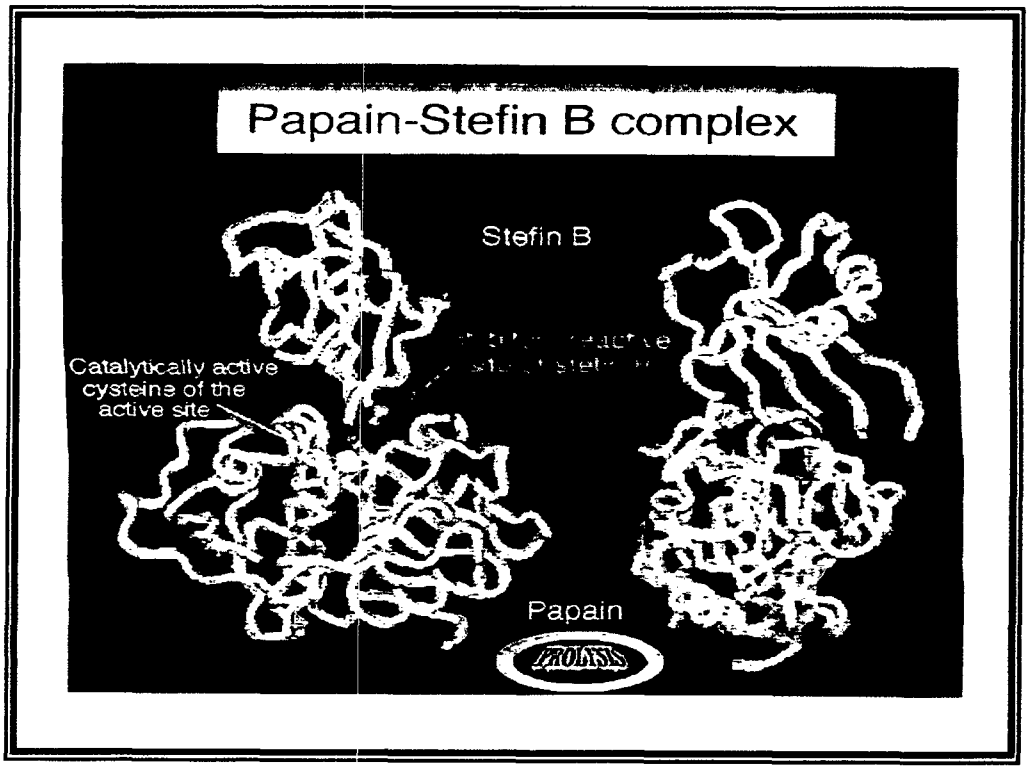
Fig-6 Proposed model for the structure of papain and papain inhibitor complex

Part a and b of the figures give a diagrammatic representation of the 3D structure of papain and its interaction with proteases inhibitor

a


Papain
Active site groove
Active cysteine
L-domain
R-domain

b


Papain-Stefin B complex
Stefin B
Catalytically active cysteine of the active site
Papain

decreases were due to increased dissociation rate constants *[Pol and Bjork (2001)]*. Cystatins form tight equimolar complexes with CP *Nicklin and Barrett (1984)]*. The binding ratio between cystatin β and rat cathepsin H is found to be 1:1 on the basis of titration of the active site of proteinase. *Abrahamson et al. (1987)* also reported the rapid formation of 1:1 complex between cystatin C and papain. Previous studies of chicken cystatin revealed that about two molecules of the inhibitor are required for the inhibition of each molecule of papain. *Nicklin and Barrett (1984)* Found that dipeptidyl peptidase and papain competed for binding site of cystatin *[Bjork et al. (1989)]* contrary to previous report in a separate experiment cathepsin B was shown to compete for binding to the same site too *[Nicklin and Barrett (1984)]*.

1.9 MECHANISM OF INTERACTION OF CYSTATINS WITH PROTEASES

It has been established that no disulphide bond is formed between the active site cysteine and the inhibitor because complexes dissociate when denatured without reduction as was found in chicken cystatin and kininogens *[Gounaris et al. (1984)]*. Carboxymethylation of enzyme and inhibitor does not prevent complex formation *[Anastasi et al. (1983)]*. It became clear that complex formation is reversible competitive and non-covalent with enzyme.

The nature of interaction resembles the standard mechanism of inhibition described by *Laskowski and Kato (1980)*. The active site of papain in complex with chicken cystatin is unreactive with 5,5'-dithio-bis-(2-nitrobenzoic acid) at pH 8, 2,2 '-dipyridyl disulphite and [^{14}C] iodo acetate at pH 4 *[Nicklin and Barrett (1984)]*. It has also been reported that the complex formation is accompanied by pronounced spectroscopic changes, most likely reflecting local perturbation of the environment of aromatic residues in both enzyme and inhibitor *[Bjork et al. (1989)]*.

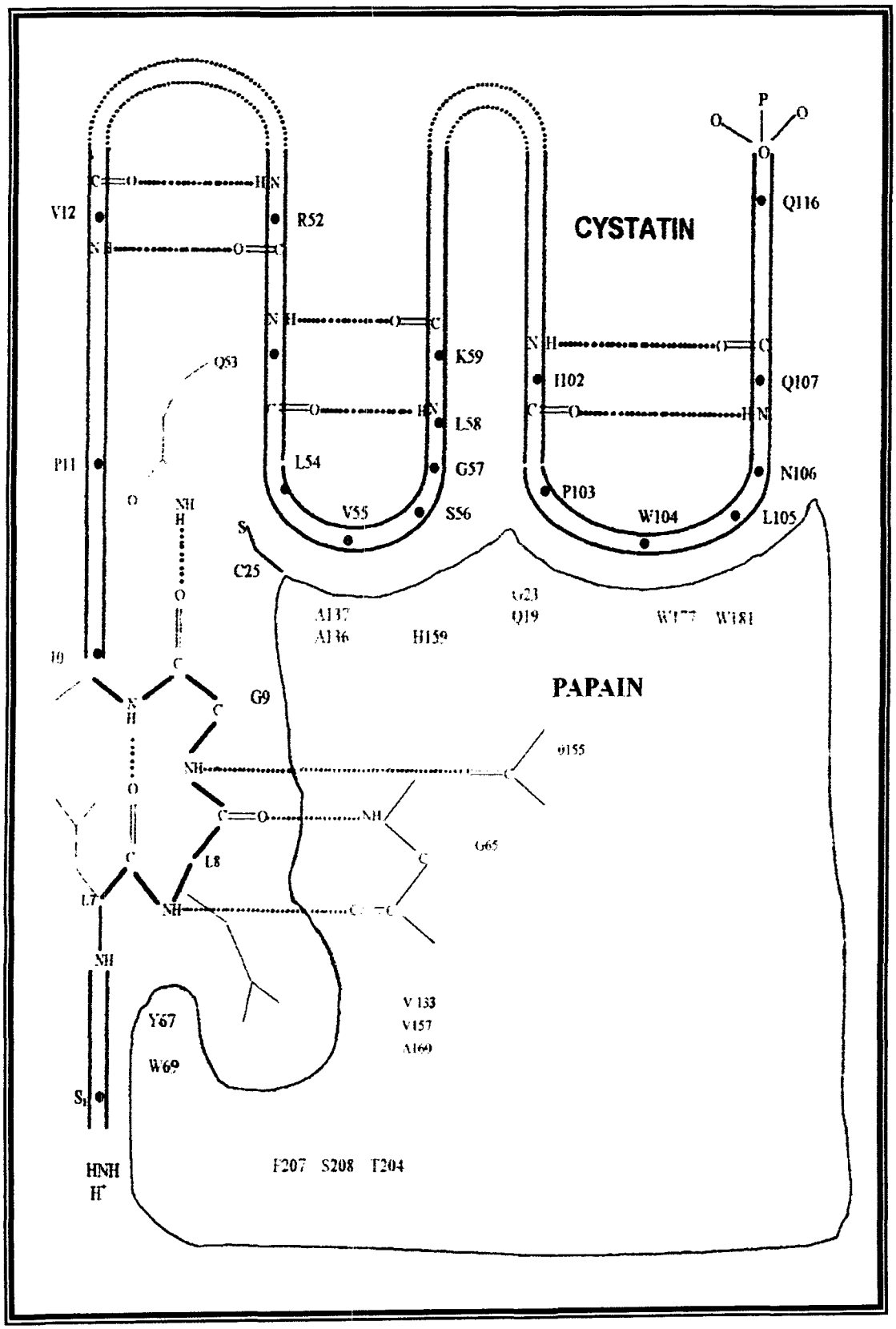
The mechanism of inhibition of papain by chicken egg white cystatin has been extensively studied. *Bode et al. (1988)* proposed a model for the interaction of cystatin with CP based on X-ray crystal structure of chicken cystatin and subsequent studies based on docking experiments indicate that three parts of the inhibitor are in close contact with the active site cleft of papain, the amino acid terminus with conserved Gly-9, a first hairpin loop from

residues 53-57, containing the prototype sequence, QVVAG *[Barrett et al. (1986) , Barrett (1987)]* and a second loop (residues 102-107) containing conserved Trp 104. *Bode et al. (1988)* demonstrated that the major contribution formed the first hairpin loop containing the QVVAG sequence *[Turk (1985), Ohkubo et al. (1984)]* .According to the model of N-terminal segment of cystatin which is more flexible bridges over the active site Cys 25 residue of papain without completely burying it and additionally the side chain of Leu 8 binds to the S_2 subsite of papain which determines the substrate specificity of papain *[Drenth et al. (1976) Asboth et al. (1988)].* The side chain groups of QLVSG segment makes the most intimate contact with putative S_1 subsite of papain ***[Fig. 7].*** This explains the presence of Gly 9 in close vicinity of Cys 25 of papain but not in direct contact with it. Removal of Leu 7 and Leu 8 leads to approximately 5000 fold lower affinity of papain. This was supported by *Brzin et al. (1984)* who demonstrated that the truncated form of human cystatin C starting with Leu-Val before Gly-11 (corresponding to Gly-9 of chicken cystatin) has virtually the same affinity for papain as the full length form whereas the truncated form starting with Gly 12 has been reported to be 1000 fold weaker inhibitor *[Abrahamson et al. (1987)].* However, *Nycander and Bjork (1990)* emphasized the role of trp-104 in the inhibition of CP. According to his model, Trp 104 of cystatin interacts primarily with two tryptophan side chains (Trp 177 and Trp 181) in the active site cleft of papain in such a manner that the indole ring of Trp 104 stacks on the side chain of Trp 177 and its edge lies on the indole ring of Trp 181.

A two step mechanism of inhibition of the lysosomal CP, cathepsin B by its endogenous inhibitor, cystatin C was observed by *Nycander et al. (1998).* An initial weak interaction in which N-terminal of the inhibitor binds to the proteinase is followed by a conformational change. Subsequently, the occluding loop of the proteinase that partially obscures the active site is displaced by the inhibitor, bringing about another conformational change. The presence of occluding loop of cathepsin renders it much more susceptible to inhibition by cystatins as compared to other proteinases. A similar two step binding of cystatin A to the CP was suggested by *Estrada and Bjork (2000)* which shows that the flexible N-terminal region of the cystatin binds independently to the target proteinases after hairpin loops.

Fig-7 Scheme of proposed model for the interaction of chicken cystatin with papain

Interaction of chicken cystatin with papain has been illustrated


CYSTATIN
PAPAIN
V12
R52
Q53
K59
L58
L54
G57
V55
S56
P11
Q116
Q107
I102
N106
P103
W104
L105
C25
A137
A136
H159
Q19
W177
W181
G9
G65
L8
L7
Y67
W69
V 133
V157
A160
F207
S208
F204
HNH

1.10 PHYSIOLOGICAL FUNCTIONS OF CYSTATINS

The present knowledge of serine proteinases and their endogenous inhibitors at the molecular level is much more detailed than our understanding of the structure and functions of thiol proteinases. Since, cystatins constitute a powerful regulatory system for endogenous cysteine proteinases, which are often secreted or leaking from the lysosomes of dying or diseased cells *[Ekiel et al. (1997)]* which otherwise may cause uncontrolled proteolysis and tissue damage. It is established that imbalance between these endogenous inhibitors and thiol proteinases have been associated with various diseases. The activity of thiol proteinases cannot normally be measured in body fluids, but can be detected extracellularly in conditions like endotoxin induced sepsis *[Abrahamson (1994)]*, metastasizing cancer *[Koppel et al. (1994) , Lee et al. (2008)]* and local inflammatory processes such as rheumatoid arthritis *[Trabandt et al. (1991)]* , purulent bronchiectasis, *[Buttle et al. (1990)]* peridontitis and renal failure *[Kabanda et al. (1995), Servais (2008)]* Alzheimer's disease *[Nakamura) et al. (1991), Levy (2008)]*, multiple sclerosis *[Bever et al. (1995)]* and muscular dystrophy *[Sohar et al(1998)]* which indicates that a tight enzyme regulation by cystatins is a necessity in the normal state.In general, cystatin concentrations are elevated in patients suffering from inflammatory diseases and autoimmunity *[(Barrett (1986) , Hansen et al. (2000), Mangge et al. (2000)]*. Cystatin B (β) serves a general protective role. The abundance of cystatin A selectively in squamous epithelial cells and in PMN leucocytes contrasts strongly with the general distribution of cystatin B and suggests a specific function connected with these types of cells *[Kotyza and Krepla (2002)]*. These cells are in the front line defense against invading pathogens and parasite, many of which use CP for entering in the body. A cysteine proteinase produced by *Leishmania* spp. is inhibited by human cystatin A, B C and kininogens. *[North (1982)]*.

Apart from defensive role, it is possible that cystatin A is involved directly in the physiology and pathology of the skin. *Jarvinen et al. (1978)* suggested that the inhibitor may contribute to the keratinisation process and *Hibino et al. (1980)* demonstrated production of cystatin α in the upper spinous or granular cells that became keratinized. *Fukuyama et al. (1982)*

have also suggested that cystatin α is involved in the differentiation of epidermis on the basis of electron microscope immunolocalization work. *Hopsu-Havu et al. (1981)* demonstrated antibodies to cystatin A in patients with a wide variety of dermatological conditions like dermatitis and psoriasis. Auto antibodies may be produced when the inhibitor leaks into the blood stream following damage to the epidermis and dermis. Cystatins have also been implicated in other inflammatory reactions in the skin *[Udaka and Hayashi et al. (1965)]*.

Alaviakko et al. (1985) reported that one or two cases of AIDS showed profound alterations of dendritic reticulum cells revealed by localization of cystatin A. A role of type I cystatins has also been considered in connection with the control and turnover of myofibrils in the cytoplasm of muscle cells it is probably mediated by CP and can be modified by low MW inhibitors. *Spanier and Bird (1982)* indicated that levels of cystatins decrease in the guinea pigs with a nutritional muscular dystrophy induced by vitamin E deficiency.

Cystatin A level increases in patients with cardiovascular disease, sarcoidosis and hepatic cirrhosis *[Hopsu-Havu et al. (1983b)]*. The concentration of cystatin B increases in patients suffering from inflammatory diseases and kidney failure.

Some reports tend to implicate the role of lysosomal CP in the malignancy suggesting that they may contribute to the nutrition of tumour cells, to the destruction of connective tissue elements, in the invasion and sloughing off the metastatic cells into the circulation *[Strauli et al. (1990), Sloane and Honn (1984)]*. Furthermore the role of cathepsins and their inhibitors in various types of cancers has been investigated *[Sloane et al. (1994), Mirtii et al. (2003)] Nishida et al. (1984)* have reported that cells of one human melanoma cell line released cystatin like inhibitor into their culture medium. On the contrary, a deficiency state in which the levels of the intracellular cystatin B are lowered due to mutations has been shown to segregate with a form of progressive myeclonus epilepsy *[Pennacchio et al. (1996)]*. Recent observations indicate that some pathogens produce proteins homologous to cystatins that are capable of suppressing the host immune response against the pathogens through interference with the MHC class II – restricted antigen processing in the antigen present cells of infected host. This

type of pathogen induced immune suppression is exemplified by Bm-CPI-2 protein, a cystatin homologue secreted by filarial parasite *Brugia malayi*, which inhibits the MHC class II-restricted antigen processing via direct inhibition of multiple lysosomal cysteine proteases including cathepsins S, L, B and Legumain. The concentration of cystatin C may be elevated in the serum of patients suffering from autoimmune diseases, lupus erythematosus and glomerulonephritis *[Brzin et al. (1984)]*.

Certain genetic disorders are linked to mutation in cystatin gene. In Icelandic hereditary cystatin C amyloid angiopathy, a mutation in the cystatin C gene (CSTC) causing a single substitution (L68Q) in cystatin C gene is coupled to decreased concentration of this major CPI in CSF, and leads to its amyloid deposition in almost all tissues including the cerebral arteries *[Pennacchio et al. (1998), Grubb (1990)]*.

This deposition results in fatal cerebral haemorrhages in young non-hypertensiveadults. Another pathological condition has been implicated due to a decrease in CSF cystatin C level. That is Guilain-Barr syndrome, chronic inflammatory demyelinating poly-neuropathy and muscular sclerosis. In these groups of patients, high cathepsin B activity but not cathepsin H was observed in CSF *[Nagai et al. (2000)]* *Kos et al. (1997)* pointed out that cystatin C level in the sera of advanced melanoma patients increases owing to higher levels of cathepsin B and H in these patients. Differently truncated forms of cystatin C are found in the urine of patients with nephrological diseases *[Popovic et al. (1990)]*.

Moreover, cystatins play additional roles in human defence by being the members of non-immune defence system of the body against microbial invasion. Cell culture shows that chicken cystatin C and D inhibit the replication of polio *[Korant et al. (1985)]* herpes simplex virus *[Bjork et al. (1990)]* and corna virus *[Collins and Grubbs (1991)]*, respectively. Antibacterial activity of peptidyl derivative like cyclopeptides structurally based upon the inhibitory centre of human cystatin C were reported by *Kasprzykowshi et al. (2000)* against *Streptococcus pyogenes* and *Staphylococcus aureus*. *Takahashi et al. (1994)* also reported the inhibition of

Staphylococcus aureus V8 by phosphorylated cystatin α in the skin cornified envelop of *Staphylococcus aureus*.

However, the results obtained by *Blankenvoorde et al. (1998)* suggested that the growth of *porphyromonas gingivalis* is due to the antibacterial sequences present in chicken cystatin and cystatin S and that it does not depend on the inhibition of *P. gingivalis* CP. Antifungal role of cystatin from plant sources has also been reported *[Soares-costa et al. (2002)]*. Cystatin 11 was also found to have antimicrobial activity against Escherichia coli. Different reports suggest possible clinical application of these proteins for prediction of prognosis *[Duffy et al. (1996)]*. Serum cystatin C can be used as a marker of GFR in patients with various renal diseases *[Newmann et al. (1995), Servais (2008)]*.

1.11 FUTURE SCOPE

Cysteine proteinases inhibitors of the cystatin super family are ubiquitous in the mammalian system. Uncontrolled proteolysis and damage are avoided by regulating the activities of endogenous proteinases. Thus they play a protective role.Enormous amount of progress has been made in the efforts to understand the structure, functions and evolutionary relationship of cystatin and their target enzymes. There is rapidly growing information about the importance of these biologically active substances in many pathological events. Serum cystatin C concentration has been reported to independently predict brain and cardiovascular diseases, heart failure, peripheral arterial disease and mortality *[Shlipak et al. (2005), Sarnak et al.(2005), O Haire et al. (2005), Shipak et al. (2006)]*.

Cystatin are crucial for proper brain functioning. Alzheimer's disease is one of the most prevalent diseases of the ageing population which is characterized by amyloid plaques. It has been investigated that lysosomal proteinase (cathepsins) and their endogenous inhibitors (cystatins) have been closely associated with senile plaques, cerebrovascular amyloid deposits and neurofibrillary tangles in Alzheimer's disease *[Bernstein (1996), Levy (2008)]*. Cystatin C is also found to be linked with epileptogenesis and epilepsy along with other neurodegenerative diseases *[Mc Geer (1995)]*. Thus a detailed

investigation of cystatins will help in understanding about the mechanisism of these diseases

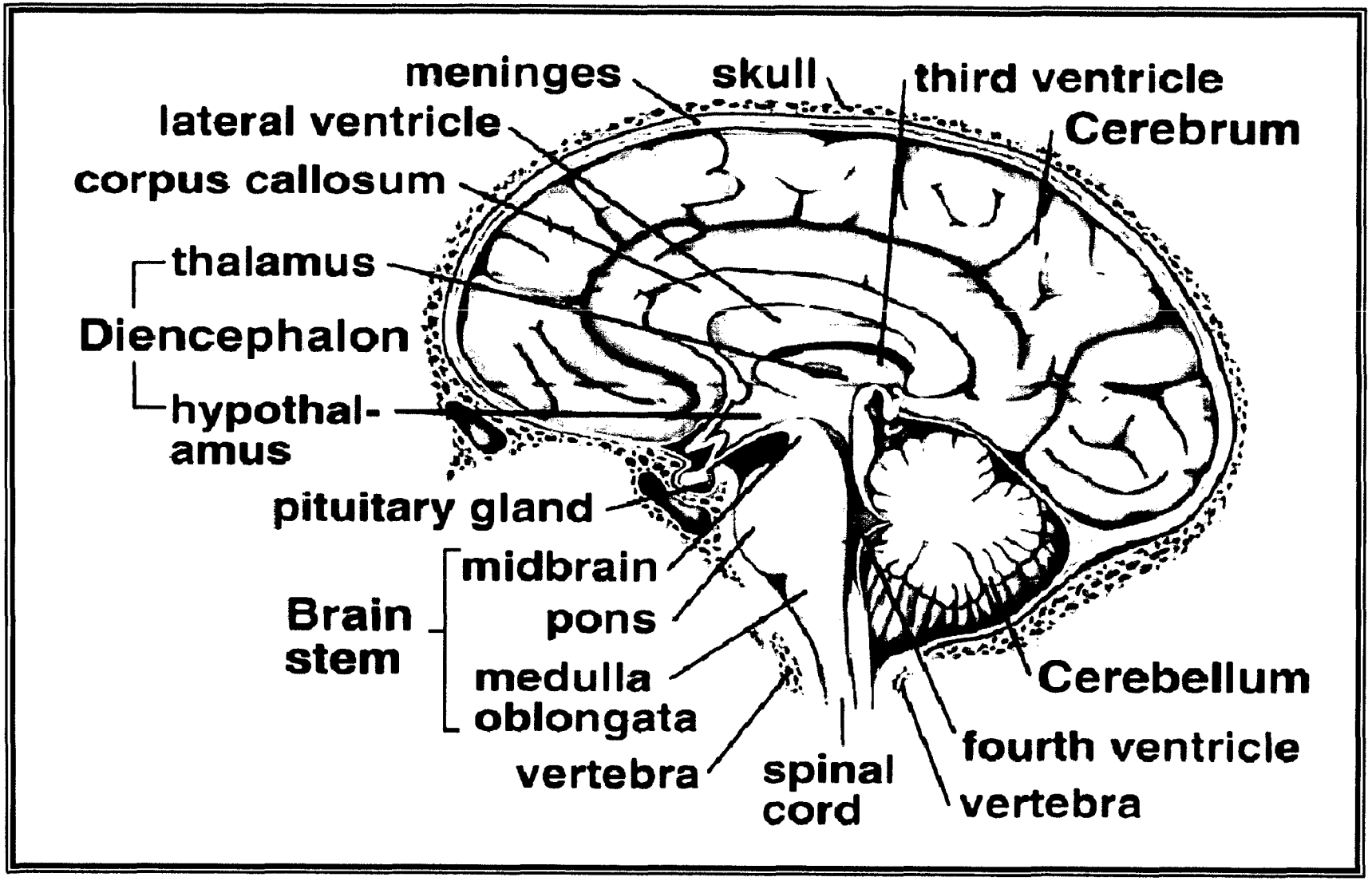
1.12 BRAIN

Brain is the most important organ of the mammalian body. It is a large soft mass of nerve tissue contained within the cranium. The brain consists of five parts: the cerebrum, cerebellum, pons varilii, medulla oblongata and the midbrain. It is the center for regulating and coordinating body activities ***[Fig-8].*** A review of the existing literature showed that Cystatins from mammalian sources have species as well as organ differences, they differ not only in size but in several other properties. Therefore, in view of the reported differences in molecular and functional properties of cystatin from various species and organs it was thought desirable to isolate and purify cystatin from a hitherto uninvestigated source and to characterize it on physicochemical basis. The current study has been focused on identification and purification of cystatin from animal source *Bubalus bubalis* (buffalo) in a multistep procedure including alkaline treatment, ammonium sulphate fractionation and gel filtration chromatography. The procedure has been found to be efficient and simple with a high percent yield and fold purification as compared to previous studies. The work has involved characterization of the isolated protein.

Studies on the isolated cystatin include molecular mass determination, kinetics pH and temperature stability studies, carbohydrate content and sulphydryl group determination, fluorescence and UV spectral analysis. The work gives comprehensive information about the properties of a new animal thiol proteinases inhibitor and its characterization on the basis of its interaction of free radicals and neurological drugs. Conformational studies of isolated cystatin have also been done, this study therefore might be instrumental in finding the relevance of the various pathophysiological associations made with the thiol proteinase inhibitor *cystatins*.

1.13 INTERACTION OF FREE RADICALS WITH BRAIN CYSTATIN

Oxidation and production of free radicals are an integral part of human metabolism. Oxygen is the ultimate electron acceptor in a closely linked electron flow system that produces energy in the form of ATP. Under certain


meninges
skull
third ventricle
lateral ventricle
Cerebrum
corpus callosum
thalamus
Diencephalon
hypothal-
amus
pituitary gland
midbrain
Brain
stem
pons
medulla
oblongata
Cerebellum
fourth ventricle
vertebra
spinal
cord
vertebra

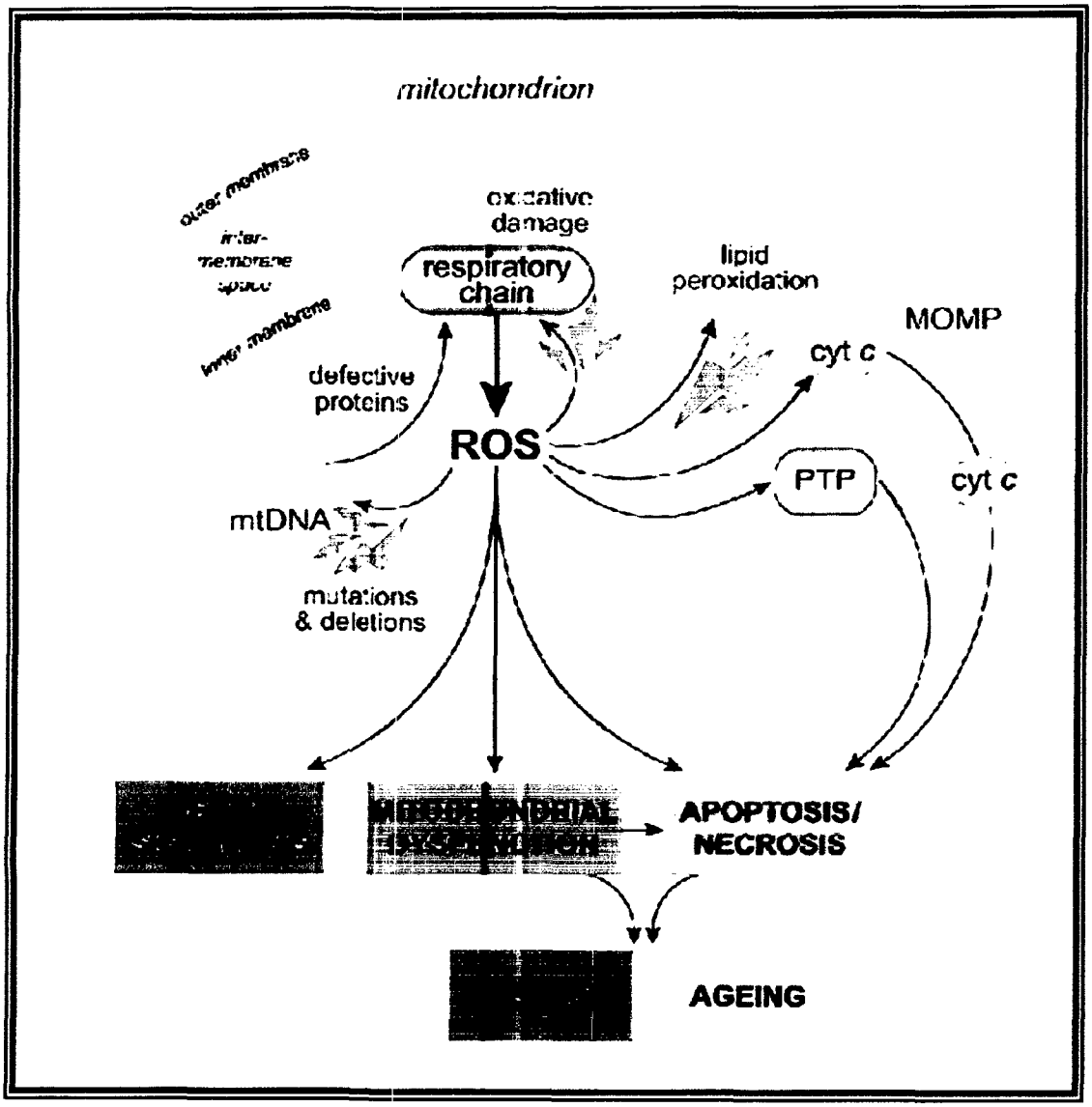
conditions, the electron flow become uncoupled, leading to production of free radicals, which are molecules with unpaired electrons and thus show high reactivity.They oxidize other molecules to gain electrons and stabilize themselves. The reaction produces another free radical, initiating a domino effect of free radical stabilization and formation *[Machlin and Bendich (1987), Uddin and Ahmad (1995)].* The oxidative damage created by free radicals is referred to as oxidative stress, and has been associated with several degenerative diseases, including cardiovascular and inflammatory diseases, cancer, aging, and stroke *[Machlin and Bendich (1987), Uddin and Ahmad (1995)].* Free radicals are produced in the cells and in the their environment as normal product of cellular metabolisim Examples of free radicals include superoxide ($O^{\bullet}_2$), hydroxyl ($OH^{\bullet}$), peroxyl ($RO^{\bullet}_2$), alcoxyl radical ($RO^{\bullet}$), oxides of nitrogen($NO^{\bullet}$, $NO^{\bullet}_2$) and thiyl ($RS^{\bullet}$) *[Halliwell et al. (1995), Andrens (1996)].* In addition, free radicals may be induced by reactive oxygen species generated in biological systems such as phagocyte activation, lipid oxidation, the arachidonate pathway, autoxidation of cathecolamines, reduced flavins, hemoprotein and iron mediated reaction *[Halliwell et al. (1995), Thomas (1995),].* Moreover other molecules like H_2O_2 and hypochlorous acid although not themselves free radical can lead to generation of free radical . It is well known that free radicals cause cell damage through mechanisms of covalent binding and lipid peroxidation with subsequent tissue injury *[(Albina (1998).* The highly reactive species refered to as ROS causes cellular damage by oxidizing proteins, membrane lipids, and DNA.

Mitochondria are an important source of ROS (reactive oxygen species) within most mammalian cells *[Andreyev et al. (2005), Adam and Chinopoulos (2006),].* The ROS production contributes to mitochondrial damage in a range of pathologies *[Balaban et al. (2005),].* Consequently, knowledge of how mitochondria produce ROS is vital to understand a range of currently important biomedical problems ***[Fig-9]***.

The first report that the respiratory chain produced ROS came in 1966 *[Jensen (1966)],* followed by the pioneering work of Chance and colleagues who showed that isolated mitochondria produces H_2O_2 *[Chance et al. (1979)].*Later, it was confirmed that this H_2O_2 arose from the dismutation of superoxide ($O_2^{\bullet -}$) generated within mitochondria *[Loschen et al. (1974)].*

Fig-9 Overview of mitochondrial ROS production

ROS production by mitochondria can lead to oxidative damage to mitochondrial proteins, membranes and DNA, impairing the ability of mitochondria to synthesize ATP and to carry out their wide range of metabolic functions, Mitochondrial oxidative damage can also increase the tendency of mitochondria to release intermembrane space proteins such as cytochrome c (cyt c) to the cytosol by mitochondrial outer membrane permeabilization (MOMP).


mitochondrion
outer membrane
inter-membrane space
inner membrane
oxidative damage
respiratory chain
lipid peroxidation
MOMP
cyt c
defective proteins
ROS
PTP
cyt c
mtDNA
mutations & deletions
APOPTOSIS/ NECROSIS
AGEING

Antioxidant agents of natural origin have attracted special interest because they can protect human body from free radicals *[Osawa et al (1990), Houghton et al (1995].* The production of these reactive species in healthy organisms is approximately balanced by antioxidant defense systems. However, an organism may be suffering from so–called 'oxidative stress' while experiencing disturbance in the pro-oxidant–antioxidant balance in favor of the former, leading to potential damage *[(Halliwell and Gulteridge, 1999)].* When an excessive amount of oxygen free radicals are produced or defense mechanisms are impaired, oxidative damage may occur and this appears to be important in contributing to several pathological conditions like aging, carcinogenesis and stroke. Oxidative damage to membrane lipids causes changes in the structure of the membrane which usually result in a more rigid membrane that will cause changes in the activity of essential membrane proteins such as Na/K-APTase enhanced oxidative stress seems to have an important contribution to brain aging in general and neurodegenerative diseases in specific. Therefore it becomes more and more important to develop neutraceuticals drugs that possibly are neuroprotective.The brain is the most susceptible organ to oxidative damage due to its high oxygen demand *[Commenges et al. (2000),* Elevated oxygen consumption may lead to oxidative stress. The pathology of aging-associated neurodegeneration is increasingly linked to oxidative and nitrosative stress *[Eckert et al (2003), Barja (2004)].*

Cystatins the natural inhibitor of cystiene proteases have received much attention in the last two decades due to their potential in regulating the function of cystiene proteinases. Alteration in proteinases to their inhibitor ratio contribute to the progression of several pathological conditions for example Cystatins are involved in Alzheimer disease, inflammation tumor growth and metastasis these are typical examples of pathological processes that are the result of imbalance between enzymes and their physiological inhibitors *[(Blankenvoorde et al. .2000)]* There is now good evidence that proteins are major targets for oxidants and the protein oxidation plays a very important role in several pathological conditions. Thus in view of this present study was carried out to see the effects of oxidative stress produced by

photosensitized riboflavin, hydrogen peroxide nitric oxide radicals and hypochlorous acid on purified brain cystatin.

NITRIC OXIDE INDUCED MODIFICATIONS OF BC

Nitric oxide is a gas synthesized by a family of enzymes present in the cells of the body. NO is a molecule with pleiotropic effects in different tissue. Nitric oxide (NO) in biological tissues is catalysed by specific enzymes nitric oxide synthases (NOS) in a reaction leading to the oxidation of L -arginine to L-citrulline via five electron oxidative reaction *[Ghafourifar and Cadenas (2005), and Virginia et al. (2003)]* NO synthases (NOS), is a family of enzymes with four major types: endothelial, neuronal, inducible and mitochondrial. They can be found in almost all the tissues and they can even coexist in the same tissue. NO is a good vasorelaxant agent, it works as a neurotransmitter when produced by neurons *[Bredt and Snyder (1994)].* it is also involved in defence when produced by immune and glial cells *[Guix et al. (2005)]* NO is thermodynamically unstable molecule and tends to react with other molecules especially proteins resulting in their oxidation, nitration and nitrosylation, with the concomitant effects on many cellular mechanisms. However, under pathophysiological conditions NO has damaging effects ***[Fig.10].*** NO is found to be involved in neural disorders involving oxidative stress such as Alzheimer's disease *[Tran et al. (2003)]* ischemia *[Thiyagarajan et al (2004)]* Parkinson's disease *[Torreilles et al. (1999)]* Huntington's disease *[Butterfied et al (2001)]* and multiple sclerosis *[Khal et al. (2003)]* over production of reactive nitrogen species (RNS) is called nitrosative stress *[Klat and Lamas (2000) and Ridnour et al. (2004)].*

This may occur when the generation of reactive nitrogen species in a system exceeds the system's ability to neutralize and eliminate them. Nitrosative stress may lead to nitrosylation reactions that can alter the structure of proteins ***[Fig-11]*** and so inhibit their normal function.Reactive nitrogen intermediates, such as nitric oxide (NO.), peroxynitrite ($ONOO^-$) and nitrogen dioxide (NO_2)has also been shown to play an important role in the Inflammatory processes *[Clancy and Abramson (1995)].*

Peroxynitrite ($ONOO^-$) is a powerful oxidant species, which can be formed in vivo by the non enzymatic reactions of nitric oxide (NO) and

Fig-10 Formation of NO in the vasculature and the effects of SOD

The (shear stress) activates endothelial NOS in endothelial cells (EC), resulting in the synthesis of NO from arginine. The NO is freely diffusible and enters vascular smooth muscle cells (VSMC), where it binds reversibly to soluble guanylate cyclase (sGC), resulting in the formation of cGMP and ultimately vessel relaxation. In competition with the binding to NO is the reaction of NO with $O_2^{\bullet-}$ which is inhibited by SOD or results in the formation of peroxynitrite (ONOO−).

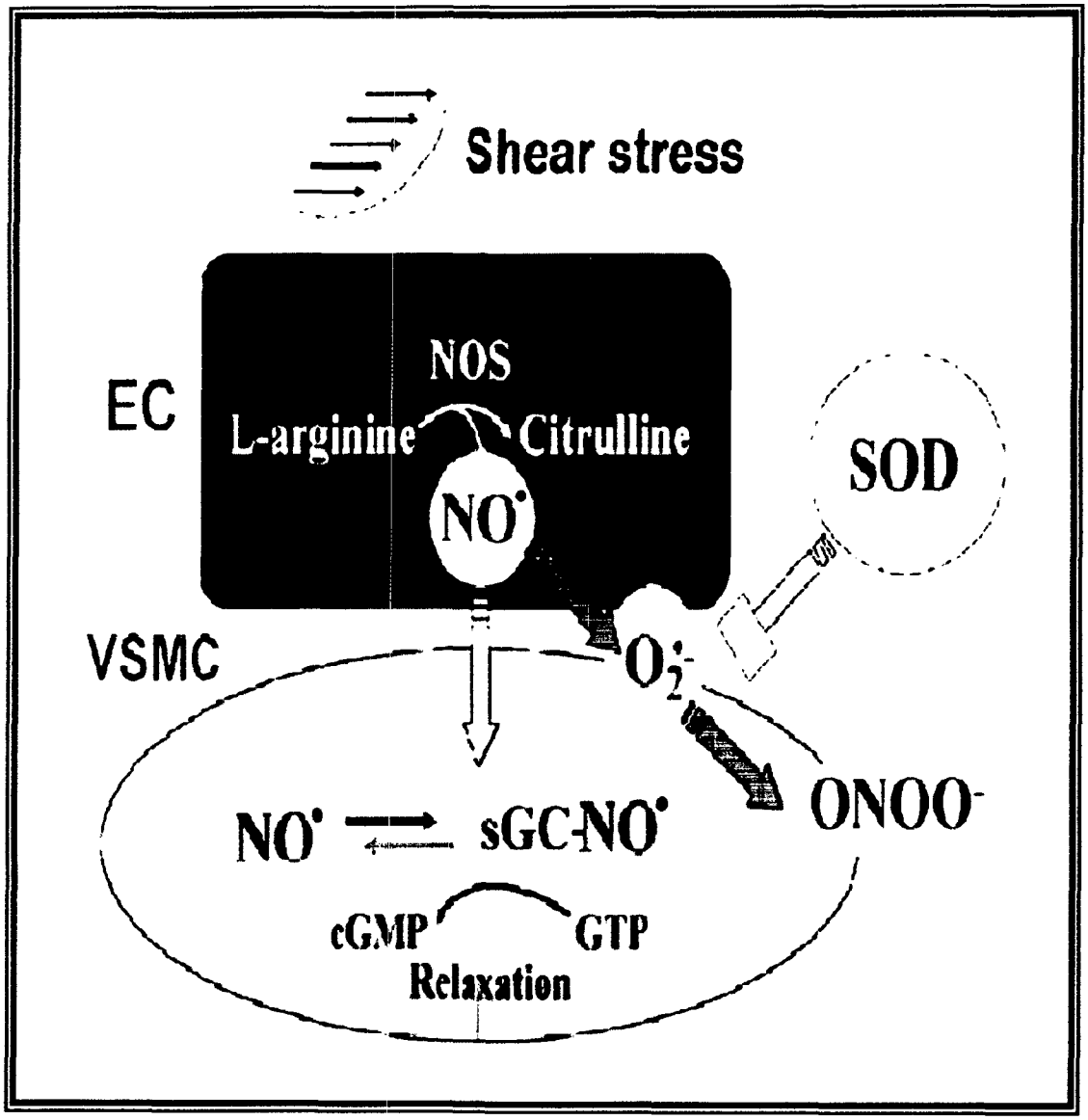
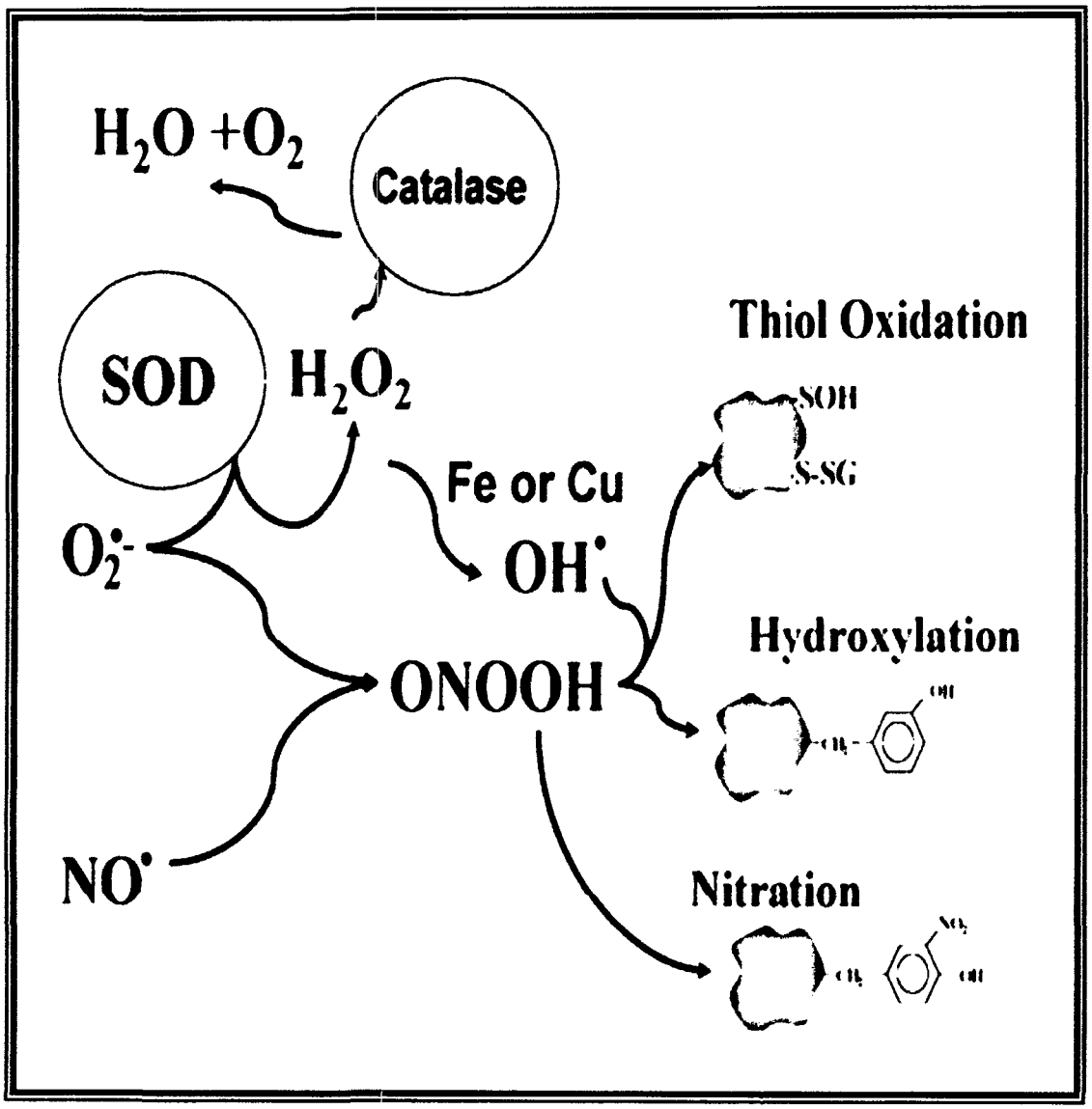

Shear stress
EC
NOS
L-arginine
Citrulline
NO·
SOD
VSMC
$O_2^{\cdot-}$
ONOO⁻
NO· ⇌ sGC-NO·
cGMP
GTP
Relaxation

Fig-11 Comparison of the oxidation reactions mediated by the Fenton reaction and ONOOH

Before the proposal that ONOOH (peroxynitrous acid) was produced biologically, the predominant mechanism thought to cause oxidation and hydroxylation was the Fenton reaction. This is shown in this simplified scheme in which H_2O_2 is shown to be the source of the powerful $OH^{\cdot}$ radical which had been shown to mediate many oxidative reactions, including thiol oxidation and phenol hydroxylation. NO and $O_2^{\bullet-}$ react together to form ONOOH .which is then capable of mediating oxidation and hydroxylation reactions which do not require the participation of transition metals, and is inhibited by SOD but not catalase. A reaction which is not mediated by the Fenton reaction, but is catalysed by ONOOH, is nitration of tyrosine residues.


$H_2O + O_2$
Catalase
SOD
H_2O_2
Thiol Oxidation
SOH
S-SG
Fe or Cu
$O_2^{\bullet-}$
$OH^{\bullet}$
Hydroxylation
ONOOH
$NO^{\bullet}$
Nitration

superoxide anion at an extremely rapid rate limited only by diffusion. At physiological pH ONOO is protonated form peroxynitrous acid which rapidly decomposes forming highly reactive oxidant species especially in the presence of carbon dioxide. *[Ferdinandy (2006) Beckman et al. (1990) Saren et al. (1990)].*

$$NO^{\bullet} + O2^{\bullet-} \longrightarrow ONOO^{-}$$

$$ONOO^{-} + H^{+} \longrightarrow ONOOH$$

$$ONOOH \longrightarrow OH + NO_2^{-}$$

HYPOCHLOROUS ACID [HOCl]

It is a chlorinating agent and potent oxidant generated in neutrophiles by the reaction of chloride ion with hydrogen peroxide catalyzed by myeloperoxidase (MPO) *[Foote et al. (1983)]*

$$H_2O_2 + Cl \xrightarrow{MPO} HOCl + OH^{\bullet}$$

HOCl is believed to be the major oxidant produced by neutrophils under physiological conditions *[Kettle and Winterboum (1997)]* It reacts with a wide variety of biological molecules and is known to cause tissue damage *[Winterboum (1985)]* Reaction of HOCl with amino acids, peptides and proteins has been the subject of extensive study, and it has been demonstrated that such processes are important in both bacterial cell killing and human disease *[Hazel et al. (1994), Yang et al. (1997)].* Active myeloperoxidase has been identified in human atherosclerotic plaque tissue *[Daugherty et al. (1994)]*, as have HOCl-modified apolipoprotein B-100 [from low-density lipoproteins (LDLs)] and other proteins *[Hazel et al. (1996)].*

Treatment of proteins with HOCl results in direct oxidative damage to the protein (alteration of amino acid side-chains, protein fragmentation and dimerization) and renders the protein more susceptible to degradation by proteolytic enzymes *[Davis et al. (1993) ,Hazel et al. (1994), Yang et al. (1997),Hazel et al. (1996), Vissers and Winterboum (1991)]* For example, exposure of fibronectin to HOCl results in changes to both the primary and tertiary structure of the protein and increases the susceptibility of the protein to degradation by elastase *[Vissers and Winterboum (1991)]*.

Certain amino acids are particularly susceptible to modification by HOCl. Tyr and Phe (free amino acids or in proteins) undergo ring chlorination, and these materials have been employed as markers of HOCl-induced damage *[Kettle (1996), Leeuwenburgh (1997)]*.

Protein aggregation by HOCl could contribute to inflammatory tissue injury, including the early stages of atherosclerosis.The mechanism of HOCl-mediated protein aggregation, and the properties that make some proteins more susceptible than others.

HYDROGEN PEROXIDE [H_2O_2]

Hydrogen peroxide (H_2O_2) is a very pale blue liquid, which appears colourless in dilute solution. It is a weak acid, has strong oxidizing properties, and is a powerful bleaching agent. It is used as a disinfectant, antiseptic, oxidizer, in organisms hydrogen peroxide is naturally produced as a byproduct of oxygen metabolism, virtually all enzymes catalyzing this reaction are called as peroxidases, which harmlessly and catalytically decompose low concentrations of hydrogen peroxide to water and oxygen. Scientists found that hydrogen peroxide is released after tissues are damaged.Antimicrobial levels of ROS are produced by the mammalian host defense to kill invading bacteria and limit bacterial colonization thiol proteins are the main target of H_2O_2 upon oxidative stress.

Oxidatively modified thiol groups appear to be specifically sensitive to either hydrogen peroxide or NaOCl stress. These results indicate that individual oxidants targets distinct proteins in vivo. H_2O_2 can freely cross membrane and in the presence of iron or copper it is prone to participate in fenton reaction.

H_2O_2 is able to induce oxidative stress and it can cause. oxidative damage to proteins relatively small concentration of hydrogen peroxide fractions of (mM) are sufficient to carry out the reactions *[Germat (1982)]* H_2O_2 in addition produces highly reactive molecules through hydroxyl radical or peroxidase action can exert a number of direct effect on cell organelles and enzymes *[Ramasarma (1990)]* Wide range of free radical are generated in the living system ***[Table -3]***

TABLE -3 FREE RADICALS OF THE LIVING SYSTEM

S.No	Name	Formula	Comments
1	**Hydrogen atom**	$H^{\bullet}$	The simplest free radical
2	**Superoxide**	$O_2^{\bullet-}$	An oxygen centered radical
3	**Hydroxyl**	$OH^{\bullet}$	An oxygen centered radical :the most highly reactive oxygen radical
4	**Peroxyl, alkoxyl**	$RO_2^{\bullet}$, $RO^{\bullet}$	An oxygen centered radicals formed during the break down of organic peroxidase
5	**Oxides of nitrogen**	$NO^{\bullet}$, $NO_2^{\bullet}$	Both are free radicals $NO^{\bullet}$ is formed in vivo from the Amino acid L –arginine , $NO_2^{\bullet}$ is made when $NO^{\bullet}$ reacts with O_2 and is found in polluted air and smoke from burning organic materials

EFFECT OF RIBOFLAVIN ON CYSTATIN

Riboflavin also known as vitamin B2 is an easily absorbed micronutrient with a key role in maintaining health in humans and animals.It is the central component of the cofactors FAD and FMN, and is therefore required by all flavoproteins. Riboflavin is yellow or yellow-orange in color and in addition to being used as a food coloring it is also used to fortify some foods. *[Yang and Mccornick (1965)].* some substrate (typically amines amino acids and some aromatic compounds) are apparently oxidized *[Frisell Chung and Mackenzie (1959)]* photosensitized destruction of amino acids and nucleotide bases has been used to induce chemical modification in protein and nucleic acids *[Simon (1967)]*.

SUPEROXIDE

Superoxide may serve important biological function such as intracellular signaling and cell growth regulation. Auto oxidation reactions in which compounds such as catecholamine ascorbic acid are alleged to react directly with O_2 to form $O_2^{\bullet-}$. Extra cellular $O_2^{\bullet}$ is produced in vivo by several cell types other than phagocytes including lymphocytes fibroblast Generation of $O_2^{\bullet-}$,HOCl and H_2O_2 by phagocytes is known to play important part in the killing of several bacterial cell and fungal strains.

$O_2^{\bullet}$- combines with $NO^{\bullet}$ the resulting peroxynitrite may be directly cytotoxic examples by oxidizing essential - SH groups on proteins *[Radi et al. (1990)].* Peroxynitrite can also decomposes to generate a range of toxic species including $OH^{\bullet}$, $NO_2^{\bullet}$ and NO_2^{+} *[Beckman et al. (1990)]*

$$O_2^{\bullet} + NO^{\bullet} \longrightarrow ONOO^{-}$$

$$ONOO^{-} + H^{+} \longrightarrow OH^{\bullet} + NO_2^{\bullet}$$

Because $NO^{\bullet}$ is a vasodilator agent the ability of $O_2^{\bullet}$ to remove it can produce vasoconstrictor effects *[Moncada et al. (1989)].*

FLAVONOIDS

Flavonoids are natural antioxidants derived from plants and commonly found in foods, such as fruits and vegetables. They are the most commonly known compounds for their antioxidant activity. Flavonoids are commonly

referred to as bioflavonoid. They are biological in origin. There are several subclasses of flavonoids; flavanols, flavanones, flavones, isoflavones, anthocyanidins, and flavonols *[Hollman and Katan (1997)].*The divisions in flavonoid subclasses are based on structural properties. The flavanols are found in red grapes and red wine, flavanones are in citrus foods, flavones are in green leafy spices, isoflavones are found in soy foods, anthocyanidins are in berries, and flavonols are found in almost all foods *[Beecher (2003)].* Flavonoids have been referred to as natural biological response modifier because of their inherent ability to modify the body reactions. They show anti allergic, anti-inflammatory, anti microbial and anticancer activity *[Yamammoto and Gaynor (1989)].*

EFFECT OF CURCUMIN AS SCAVENGER

The rhizome of turmeric is widely used in indigenous medicine. *[Nadkarni (1954)]* A paste made from powdered rhizome of Curcuma longa Linn, mixed with slaked lime applied locally, an ancient household remedy for sprains, muscular pain and inflamed joints. It is also applied in poultices to relieve pain and inflammation. Curcumin is yellow colored phenolic pigment, ***[Fig-12a]***. *[Cooper et al. (1994)]* obtained from powdered rhizome of C. longa Linn. *(Family -Zingiberaceae).*

It is extensively used in the traditional Indian medicine and used to treat a wide variety of diseases. Its therapeutic effects well studied, anti-inflammatory, *[Arora et al. (1971)]* antibacterial, *[Negi et al. (1999)]* antiviral, *[Bourne et al. (1999)]* antifungal, *[Apisariyakul et al. (1995)]* antitumor, *[Kawamori et al. (1999)]* antispasmodic *[Itthipanichpong (2003)]* and hepatoprotective.*[Park et al.(2000)]* Recently, its potential utility in autoimmune deficiency syndrome (AIDS) has been demonstrated.*[Mazumder et al. (1996), James (1993)]* Curcumin acts as a free radical scavenger and antioxidant, inhibiting lipid peroxidation. Curcuminoids are potent inhibitors of cytochrome P450 *[Shukla (2003)].* Curcumin was found to be a very potent antioxidant.*[Sharma (1976), Iqbal et al. (2003)]* It was found to generate hydroxyl radicals through the Fenton reaction by reducing Fe3+ to Fe2+.*[Elizabeth, Rao (1989)].*

SCAVENGING EFFECT OF QUERECITIN ON BC DAMAGE

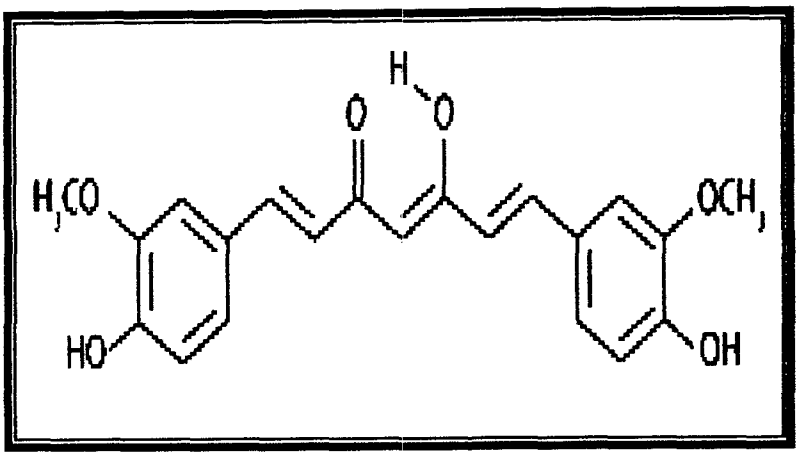
Quercetin, a major representative of the flavonol subclass, has received considerable attention. Dietary Sources : Fruits and vegetables – particularly citrus fruits, apples, onions, parsley, tea, and red wine – are the primary dietary sources of quercetin ***[Fig-12b].***Quercetin and its glucosylated forms represent 60-75% of flavonoid intake *[Bouktaib et al. (2002)].* Quercetin has displayed the ability to prevent the oxidation of low-density lipoproteins (LDL) by scavenging free radicals and chelating transition metal ions. As a result, quercetin may aid in the prevention of certain diseases, such as cancer, atherosclerosis and chronic inflammation and brain disease. *[Murota and Terao (2003)].* The oxidation of low-density lipoproteins (LDL) can result in the formation of atherosclerotic plaques, leading to cardiovascular disease *[Hollman and Katan (1997)].* However, several studies have illustrated quercetin's ability to inhibit LDL oxidation. The vulnerability of brain lipid membranes to lipid peroxidation thought to lead neurodegenerative disease, such as Alzheimer's and Parkinson's disease *[Balazs and Leon (1994)]. Balazs and Leon (1994)* found that oxidative stress occurring in the brain membrane lipids is associated with the extracellular accumulation of amyloid beta-peptide, which precedes neural losses in Alzheimer's patients. Yet, formation of amyloid plaques can be prevented by taking antioxidants *[Ansari et al. (2008), Harman et al. (1976)].*

In this situation, quercetin does not only stop the propagation of lipid peroxidation, but also increases glutathione (GSH) levels *[Ansari et al. (2008)].* GSH is part of the neuron's defense against oxidative damage.

When the superoxide radical is formed, the radical can be converted to the hydrogen peroxide radical by superoxide dismutase; however, GSH can convert hydrogen peroxide to oxygen and water, preventing the formation of free radicals *[Balazs and Leon (1994)].*

Quercetin can also reduce inflammation by scavenging free radicals. Quercetins, and other flavonoids, have the structure to act as powerful antioxidants, and have often proven so *in vitro*. Quercetin, being a major constituent of the flavonoid intake, could be key in fighting several chronic degenerative diseases *[Bouktaib et al. (2002)].*

a



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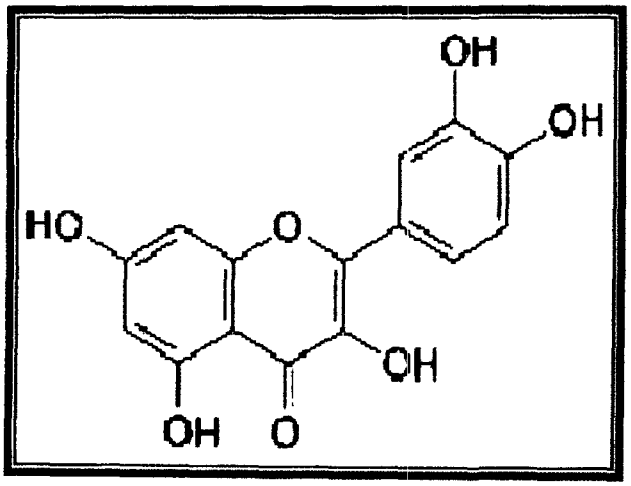


GLUCOSE:

It is found in considerable concentration in the fruits and in honey. Glucose is very important for the body especially for the brain and red blood cells. Glucose act as scavenger of OH. In physiological concentration and therefore may serve an antioxidant role in the biological systems *[Sagone (1983)]*.

Mannitol:	scavenger of hydroxyl radical
Sodium azide:	scavenger of singlet oxygen
Thiourea:	scavenger of hydroxyl radical
Potassium iodide:	scavenger of flavin triplet
Sodium benzoate:	scavenger of hydroxyl radical

1.14 ANTIDEPRESSANT DRUG

An antidepressant is a psychiatric medication used to alleviate mood disorders, such as major depression. Drugs including the monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs), tetracyclic antidepressants (TeCAs), selective serotonin reuptake inhibitors (SSRIs), and serotonin-norepinephrine reuptake inhibitors (SNRIs) are most commonly associated with the term. Despite the name, antidepressants are often used to treat other conditions, such as anxiety disorders, obsessive compulsive disorder, eating disorders and chronic pain.

AMITRIPTYLINE HYDROCHLORIDE:

Amitriptyline Hydrochloride is a tricyclic antidepressant drug. It is a white, odorless, crystalline compound which is freely soluble in water; it is usually dispensed in tablet form. In terms of its mechanism of action, amitriptyline inhibits serotonin and noradrenaline reuptake almost equally. Amitriptyline is approved most commonly for the treatment of major depression. It is used to treat various forms of depressions like pain associated with the nerves (neuropathic pain), and to prevent migraine

headaches. ***[Fig-13a]*** Amitriptyline may be prescribed for other conditions also such as insomnia *[Endep Consumer Medicine Information (2005)]*, rebound headache, chronic pain, chronic cough, postherpetic neuralgia (persistent pain following a shingles attack), diabetic peripheral neuropathy, and neurological pain Typically lower dosages10 to 50 mg daily are required for pain control *[MedlinePlus Drug Information (2008)]*.

Common side effects of using amitriptyline are mostly due to its anticholinergic activity, including weight gain, dry mouth, loss of appetite, drowsiness, muscle stiffness, nausea, constipation, nervousness, dizziness, blurred vision and urinary retention and insomnia. Some rare side effects include tinnitus, lip and mouth ulcers and hepatic toxicity.

FLUOXETINE:

Fluoxetine (trade name Prozac) is an antidepressant of the selective serotonin reuptake inhibitor (SSRI) class. ***[Fig-13b]*** Fluoxetine is approved for the treatment of major depression (including pediatric depression), obsessive-compulsive disorder (in both Adult and pediatric populations), anorexia nervosa, panic disorder and premenstrual dysphoric disorder.*[Prozac Pharmacology, Pharmacokinetics, Studies, Metabolism (2007)]*. The bioavailability of fluoxetine is relatively high (72%), and peak plasma concentrations are reached in 6 to 8 hours. It significantly binds to plasma proteins, mostly albumin. Fluoxetine is metabolized in the liver by isoenzymes of the cytochrome P450 system, only one metabolite of fluoxetine, norfluoxetine (demethylated fluoxetine), is biologically active. The extremely slow elimination of fluoxetine and its active metabolite norfluoxetine from the body distinguishes it from other antidepressants. Fluoxetine has been associated with related movement disorders *[Leo (1996); Gerber, Lynd (1998); Caley (1997)]* Fluoxetine taken during pregnancy also increases rate of poor neonatal adaptation. *[Expert Panel Report on Reproductive and Developmental Toxicity of fluoxetine (2004)]*.

Fluoxetine and norfluoxetine if taken in higher doses inhibit many isozymes of the cytochrome P450 system that makes drug metabolism possible *[Sandson et al (2005) Joy, Adams, Lawrie (2006)]* The simultaneous use of fluoxetine with serotonergic agents triptans and tramadol can result in

rare, but potentially life-threatening adverse drug reaction called serotonin syndrome.

HALOPERIDOL

Haloperidol is a typical antipsychotic drug.***[Fig-13c]***.It is an older antipsychotic drug used in the treatment of schizophrenia and more acutely, in the treatment of acute psychotic states and delirium. Due to its strong central antidopaminergic action, it is classified as a highly potent neuroleptic drug and possesses a strong activity against delusions and hallucinations, the peripheral antidopaminergic effects of haloperidol account for its strong antiemetic activity.

The drug is rapidly absorbed. Plasma-levels reach their maximum within 20 minutes after injection. The bioavailability is 100% and the very rapid onset of action is seen within about ten minutes. The duration of action is 3 to 6 hours. Plasma levels of 4 micrograms per liter to 20 micrograms per liter are required for therapeutic action. A comprehensive review of haloperidol has found it to be an effective agent in treatment of symptoms associated with schizophrenia. *[Joy, Adams, Lawrie (2006)]* Haloperidol is also used in the control of the symptoms of acute psychosis, hyperactivity and aggression. The risk of the facial disfiguring is around 4% per year in younger patients. *[Joy, Adams, Lawrie (2006)].*

DONEPEZIL

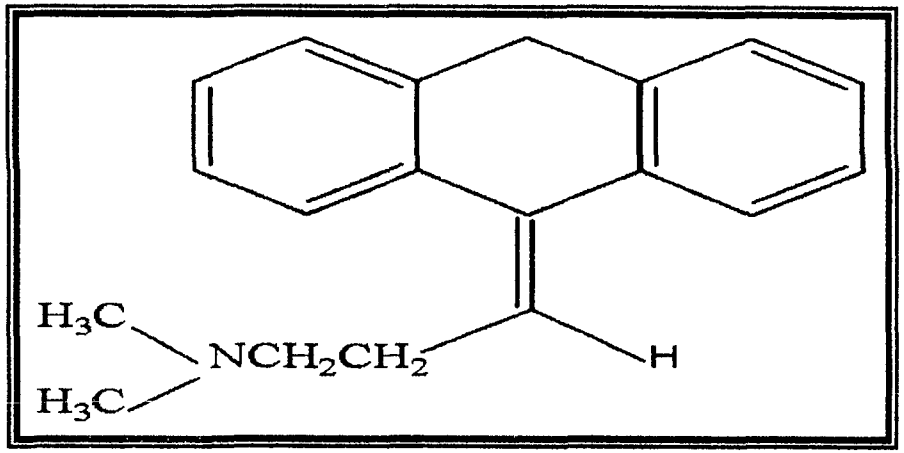
Donepezil, marketed under the trade name Aricept,is a reversible acetylcholinesterase inhibitor.*[Birks, Harvey (2006)].* Its main therapeutic use is in the treatment of Alzheimer drug where it is used to increase cortical acetylcholine. It has an oral bioavailability of 100% and easily crosses the blood-brain barrier. It has a half life of about 70 hours. Alzheimer disease is the most common cause of dementia and is a primary degenerative disease of the brain of unknown cause. Onset is usually late in life with increasing impairment of memory, developing gradually into a global impairment of cognition, orientation, linguistic ability and judgement. Acetylcholine is an important neurotransmitter associated with memory, and abnormalities in cholinergic neurones (including cell loss) are among the many neurological

and neurochemical abnormalities that develop in Alzheimer disease. One approach to lessening the impact of these abnormalities is to inhibit the breakdown of acetylcholine by blocking the relevant enzyme. Tacrine *(another Alzheimer drug)* was the first compound approved as a treatment for Alzheimer disease (AD) in the US and worked for the disease, but caused severe side effects. donepezil, is a second generation cholinesterase inhibitor and appears to be highly specific, with relatively few side effects *[Birks , Melzer (2000)].*Donepezil. **[*Fig-13d*]** and tacrine can bind proteins, donepezil is metabolized by CYP3A4 and CYP2D6. *[Jann et al. (2002)].*

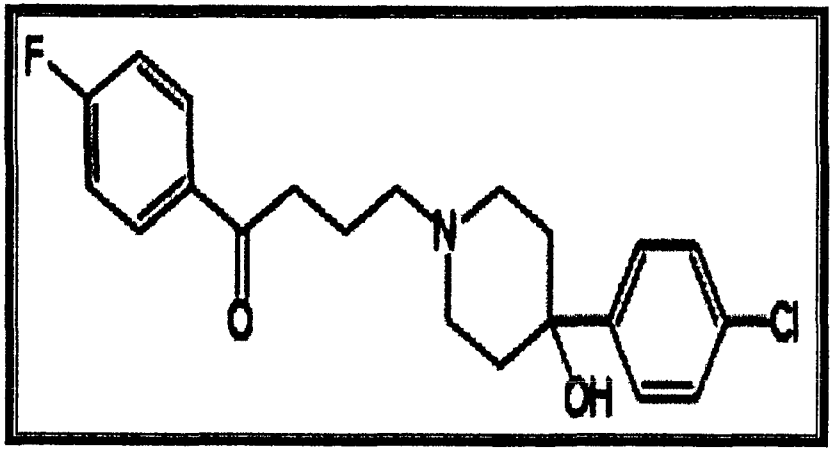
Even small structural differences in protein conformation can lead to drastic changes in functional parameters. *[Sneppen, Zocchi, (2006)]* Addition of small molecules such as substrates, coenzymes, inhibitors and activators that bind especially to the native state and can alters the delicate balance of various interaction in protein. In this regard, many drugs, particularly those with local anesthetic, tranquillizer, and antidepressants exert their activity by interaction with biological membranes. These drugs are carried to their sites of action by means of protein carriers at which they bind with different affinities. Accumulation of drug molecules at certain sites in the body causing a localized high concentration, Adverse drug reactions *[Wen, Ye (1993)]* and ligand induced protein structure conformational changes *[Takeda et al (1988)]* are major problems complicating drug medical therapy. Therefore, studies which combine the study of the conformational changes in proteins through variations of external parameters such as pH, temperature and the analysis of the binding mechanism between proteins and antidepressant drugs and the structure of the resulting complexes are of particular interest. These studies enable to elucidate how ligand affinity is regulated and how the protein conformation is altered upon omplexations *[Sneppen, Zocchi (2006)]* which are of crucial importance in a vast range of important biochemical phenomena.

1.15 NEUROTRANSMITTERS

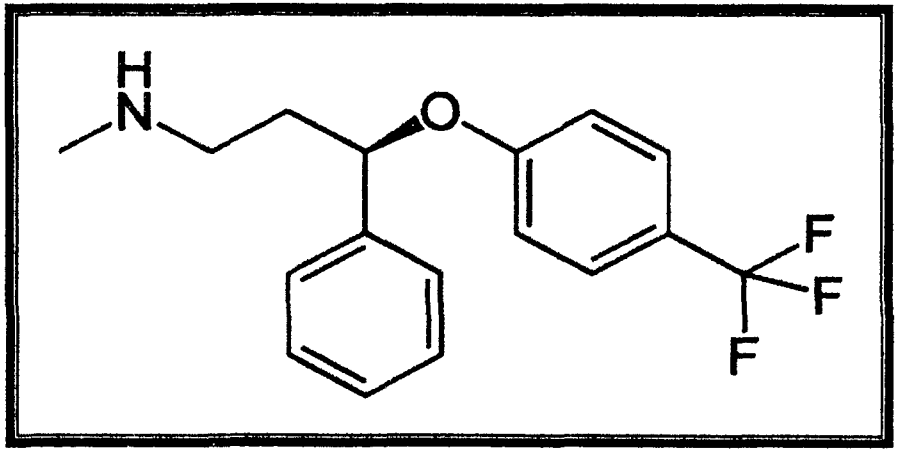
Neurotransmitters are endogenous chemicals which relay, amplify, and modulate signals between a neuron and another cell. *[Di Chiara, Loddo, Tanda (1999)]* They are packaged into synaptic vesicles that cluster beneath



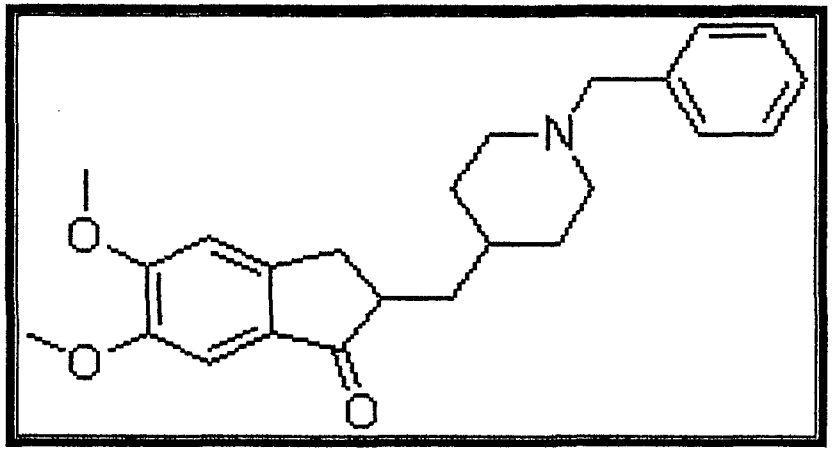
AMYTRIPTYLINE



HALOPERIDOL



FLUOXETINE



DONEPEZIL

the membrane on the presynaptic side of a synapse, and are released into the synaptic cleft, where they bind to receptors in the membrane on the postsynaptic side of the synapse. Release of neurotransmitters usually follows arrival of an action potential at the synapse, but may follow graded electrical potentials. Low level "baseline" release also occurs without electrical stimulation. Amino acids, peptides, and monoamines are included into neurotransmitters. Major neurotransmitters are amino acids and peptides glutamate, aspartate, serine, glycine and γ-amino butyric acid (GABA), Monoamines like Dopamine (DA), Norepinephrine (noradrenalin) epinephrine (Adrenaline), serotonin, melatonin. Others neurotransmitters include acetylcholine (ACh), adenosine and histamine

AMINO ACIDS NEUROTRANSMITTER

An amino acid neurotransmitter is a chemical substance which is able to transmit a nerve message across a synapse. Neurotransmitters (chemicals) are packaged into vesicles that cluster beneath the axon terminal membrane on the presynaptic side of a synapse in a process called endocytosis. Amino acid neurotransmitter are released by the mechanism of exocytosis is dependent upon calcium (Ca^{2+}) concentration and is a presynaptic response. There are inhibitory amino acids (IAA) and excitatory amino acids (EAA). The IAA include GABA, Glycine, β-Alanine, and Taurine. The IAA depresses the activity of post-synaptic cells. *[Hugo (2002)]*. The EAA are L-Glutamate, L-Aspartate, L-Cysteine, and L-Homocysteine. These neurotransmitter systems will activate post-synaptic cells.

MONOAMINE NEUROTRANSMITTER (DOPAMINE)

Dopamine, the Monoamine neurotransmittersare neurotransmitters and neuromodulators that contain one amino group that is connected to an aromatic ring by a two-carbon chain ($-CH_2-CH_2-$). All monoamines are derived from aromatic amino acids like phenylalanine, tyrosine and tryptophan. Among organic compounds, there are six monoamines classified as neurotransmitters in addition to non-monoamines such as acetylcholine, purines, certain fatty acids, and around fifty peptides including some hormones The six monoamine neurotransmitters include:

Dopamine, produced from phenylalanine and tyrosine, Norepinephrine or noradrenaline, Epinephrine or adrenaline, Serotonin, also known as 5-hydroxytryptamine or 5HT, Melatonin ,Histamine, produced from histidine

Monoamines are moved into or out of cells via a class of proteins called monoamine transporters.After release into the synaptic cleft, monoamine neurotransmitter action is ended by reuptake into the presynaptic terminal. There, they can be repackaged into synaptic vesicles or degraded by the enzyme monoamine oxidase (MAO), which is a target of monoamine oxidase inhibitors, a class of antidepressants.

Antidepressants and psychoactive drugs often affect the monoamine transporters rather than the monoamine itself. A monoamine neurotransmitter relays, amplifies, and modulates electrical signals between two neurons. MAOIs [Monoamine oxidase inhibitors or antidepressants] prevent breakdown of monoamine neurotransmitters, therefore increasing the concentrations of neurotransmitters in the brain. In the present study two most important neurotransmitters that is dopamine and serotonin have been investigated for their interaction with cystatin.

DOPAMINE

Dopamine abbreviated is "DA.". ***[Fig-14a]*** has the chemical formula $C_6H_3(OH)_2$-CH_2-CH_2-NH_2. Its chemical name is "4-(2-aminoethyl) benzene-1,2-diol" They are formed in the brain by the decarboxylation of dopa and essential for the normal functioning of the central nervous system. A reduction in its concentration within the brain is associated with Parkinson's disease and its high concentration leads to schizophrenia. Dopamine is manufactured inside dopamine neurons in a controlled manner from the amino acid precursor L-tyrosine, which mammals obtain through the normal diet. Dopamine is then stored in vesicles within the nerve terminals, which may fuse with the cell membrane to release dopamine into the synapse. ***[Fig-15].***

Dopamine has many functions in the brain, including important roles in behavior and cognition, voluntary movement, motivation, inhibition of prolactin production (involved in lactation), sleep, mood, attention, and learning. Dopaminergic neurons (i.e., neurons whose primary neurotransmitter is

dopamine) are present chiefly in the ventral tegmental area (VTA) of the midbrain. ***[Fig-16]***

SEROTONIN

Serotonin (5-hydroxytryptamine or Serotonin). ***[Fig-14b]*** is a monoamine neurotransmitter. It is found extensively in the gastrointestinal tract of animals, and about 80 to 90 percent of the human body's total serotonin is located in the gut, where it is used to regulate intestinal movements. *[Berger et al. (2009)]* The remainder is synthesized in serotonergic neurons in the central nervous system (CNS) where it has various functions, including control of appetite, mood and anger.

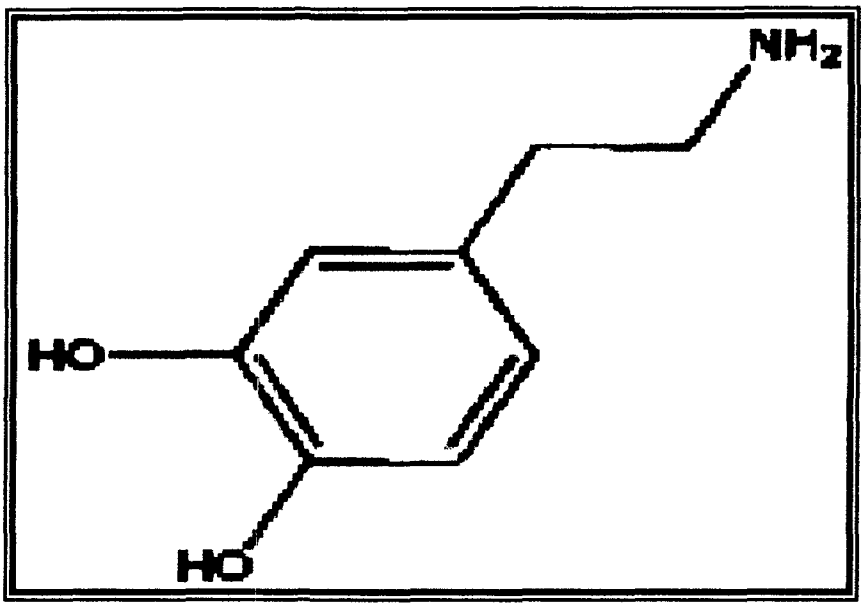
Serotonin is not only found in animals, but also in fungi and plants,*[Kang et al. (2009)]* including fruits and vegetables. Serotonergic action is terminated primarily via uptake of Serotonin from the synapse. This is through the specific monoamine transporter for Serotonin, SERT, on the presynaptic neuron. This chemical is found in very low amounts in people diagnosed with depression compared to normal individuals. Serotonin works as a neurotransmitter and helps with the modulation of things such as anger, appetite, sleep, and mood. People with depression often have impaired serotonin genes. Selective serotonin (Serotonin) reuptake inhibitors (SSRIs) are efficacious in depression because of their ability to increase Serotonin neurotransmission. The MAOIs (Antidepressants) prevent the breakdown of monoamine neurotransmitters (including serotonin), and therefore increase concentrations of the neurotransmitter in the brain. The tricyclic antidepressants (TCAs) inhibit the re-uptake of both serotonin and Dopamine. Amytriptyline, flouxetine and Haloperidol have fewer side-effects and fewer interactions with other drugs. Therefore these antidepressants have been chosen to investigate their effect on cystatin. If neurons that make serotonin - (serotonergic neurons) - are abnormal in infants, there is a risk of sudden infant death syndrome (SIDS). *[Paterson et al. (2006)]* Low levels of serotonin may also be associated with intense spiritual experiences. *[Lars and Jacqueline (2003)]*.

It is known that the distribution, free concentration and the metabolism of various drugs are strongly affected by drug–protein interactions in the blood

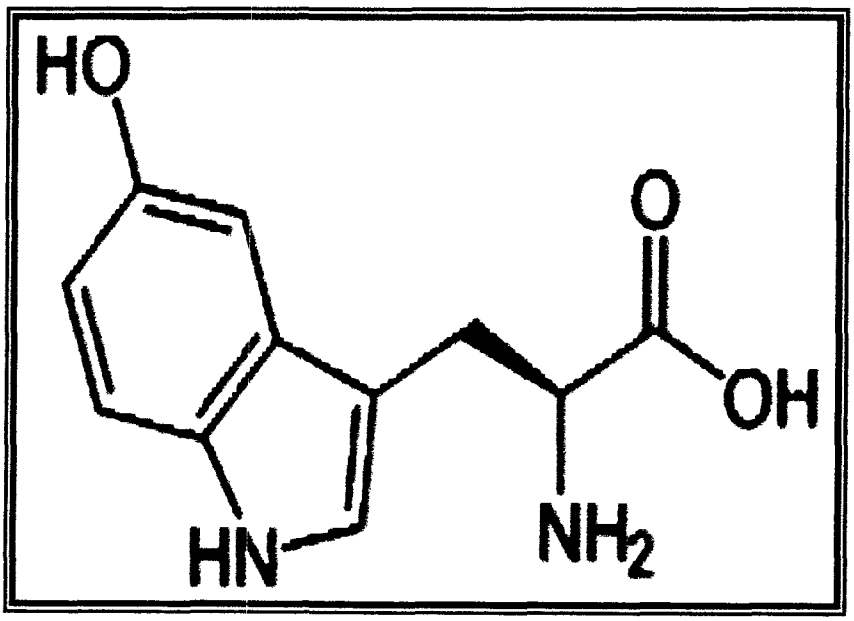
FIG-14 CHEMICAL STRUCTURE OF NEUROTRANSMITTERS

a) DOPAMINE

b) SEROTONIN



DOPAMINE



SEROTONIN

Fig-15 Illustration of the major elements in a prototypical synapse.

Synapses are gaps between nerve cells. These cells convert their electrical impulses into bursts of chemical relayers, called neurotransmitters, which travel across the synapses to receptors on adjacent cells, triggering electrical impulses to travel down the latter cells.

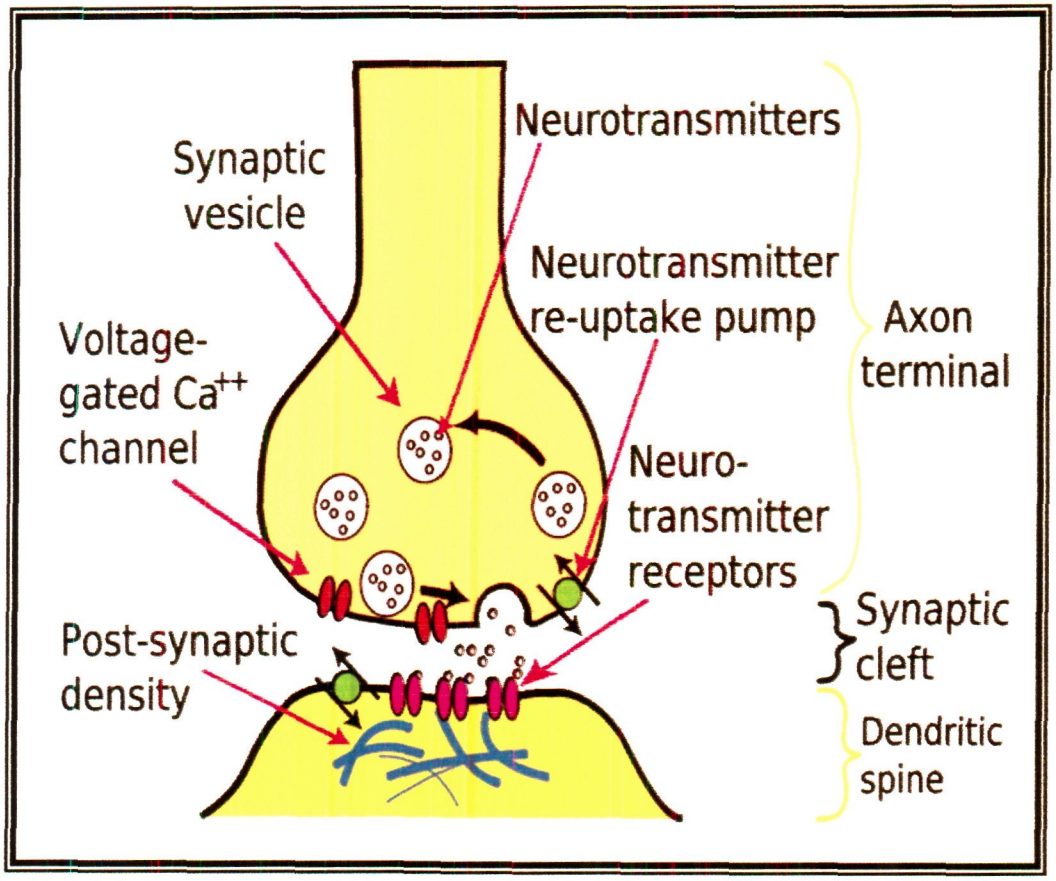
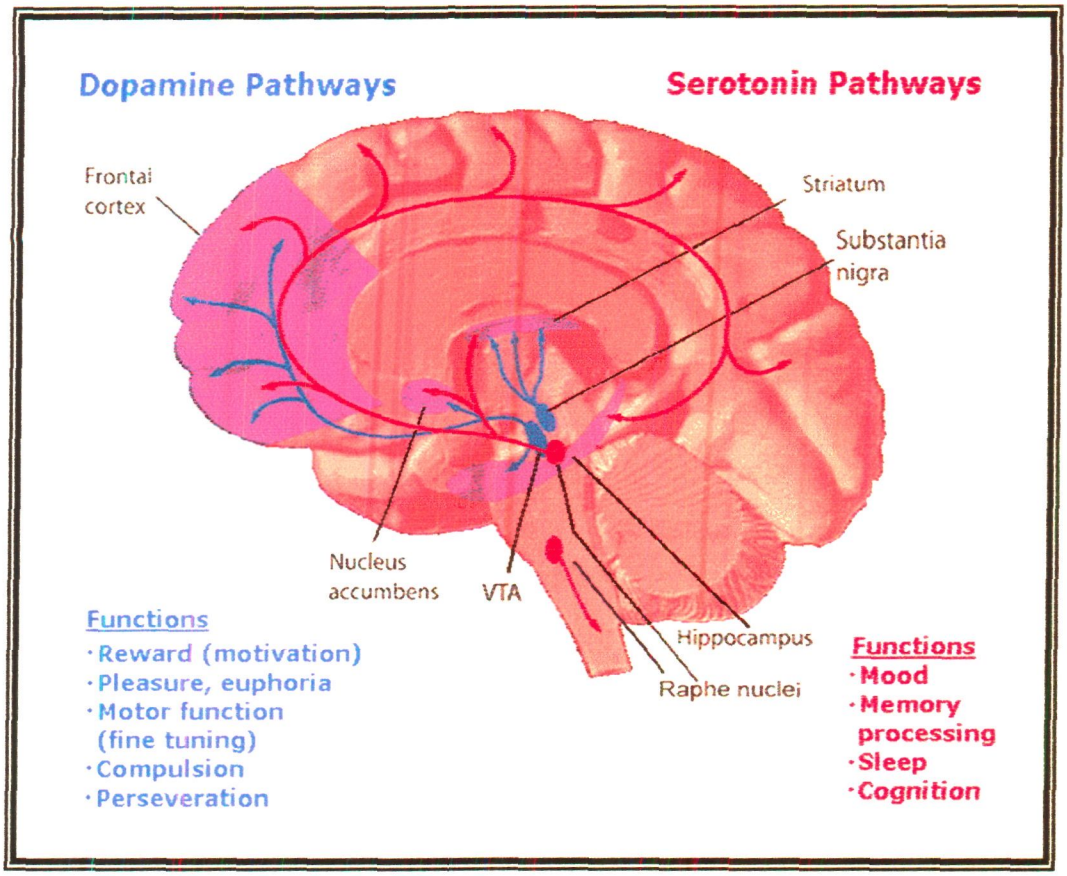

Neurotransmitters
Synaptic vesicle
Neurotransmitter re-uptake pump
Axon terminal
Voltage-gated Ca++ channel
Neuro-transmitter receptors
Synaptic cleft
Post-synaptic density
Dendritic spine

FIG-16 SEROTONIN SYSTEM, CONTRASTED WITH DOPAMINE SYSTEM.


Dopamine Pathways
Serotonin Pathways
Frontal cortex
Striatum
Substantia nigra
Nucleus accumbens
VTA
Hippocampus
Raphe nuclei
Functions
·Reward (motivation)
·Pleasure, euphoria
·Motor function (fine tuning)
·Compulsion
·Perseveration
Functions
·Mood
·Memory processing
·Sleep
·Cognition

effectiveness carries the risk of side effects and an increase of therapeutic action of free fractions of drugs. This can be dangerous when exhibiting toxic effect *[Hu et al. (2005)]*. Regulation of cysteine proteinases and their inhibitors are of utmost importance in neurodegenerative diseases like Alzheimer and Parkinsons. Protease - antiprotease imbalance accelerates disease progresssion. In the present study, the effects of antidepressants and neurotransmitters have been studied to explore the drug induced protein dysfunction.

1.16 THE MECHANISM OF ACTION OF ANTIDEPRESSANTS AND NEUROTRANSMITTERS

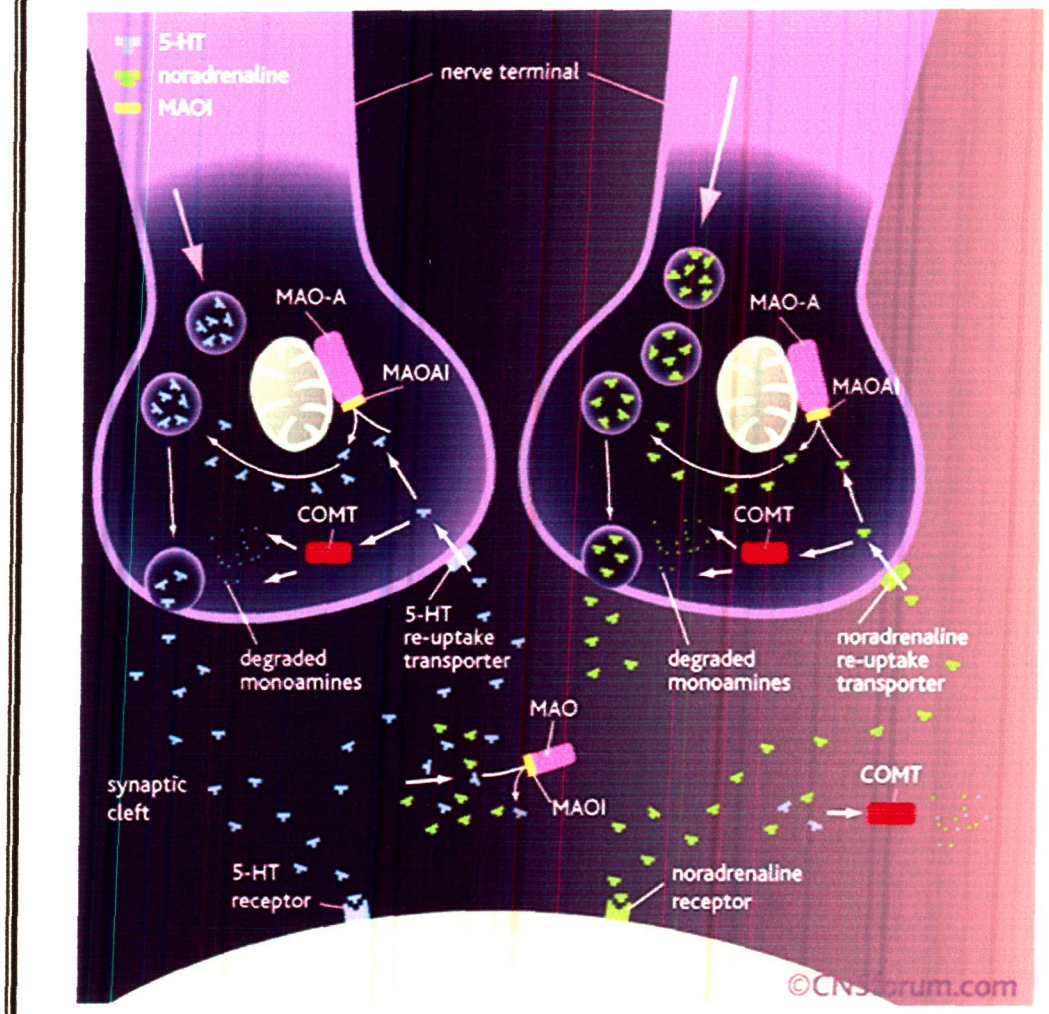
Antidepressants, the tricyclics were first intro-duced in the late 1950s, the number of new classes of antidepressant medication used to treat major depressive disorders have grown dramatically. Newer classes of drugs Include MAOA inhibitorsalso called as the selective serotonin reuptake inhibitors (SSRIs) *[Amytriptyline and flouxetine]*, serotonin Dopamine reuptake inhibitors (SNRIs) *[Haloperidol]* and selective Dopamine reuptake inhibitors (NRIs).Compared with traditional antidepressant drugs, newer drug classes such as SSRIs and SNRIs offer improved tolerability to therapy with a high level of efficacy. Drugs have been classified by their origin or source, by physiologic effects, therapeutic use, site of action, chemical structure, or mechanism of action. *[Jacobs, Fehr (1987)]*. MAO are enzymes that catalyze the oxidation of monoamines They are found to be bound to the outer membrane of mitochondria in most cell types in the body. In humans there are two types of MAO: MAO-A and MAO-B. Both are found in neurons and astroglia. Outside the central nervous system there function is to catalyze the oxidative deamination of monoamines because of the vital role that MAOs play in the inactivation of neurotransmitters, MAO dysfunction (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 inhibitors are one of the major classes of drug prescribed for the treatment of depression Monoamine oxidase A (MAOA) is an enzyme involved in the metabolism of the monoamines, like serotonin and dopamine. It converts the monoamines into their corresponding carboxylic acid via an

aldehyde intermediate. MAOA regulates both the free intraneuronal concentration and the releasable stores of serotonin.

MAOA inhibitors, such as antidepressants Fluoxetine, amytriptyline and Haloperidol bind and inhibit MAOA, prevent monoamine degradation thus reducing depression. This results in greater stores of monoamines available for release. MAOA inhibitors (antidepressants) are used in the treatment of depression. People with depression have lower than normal levels of the monoamines and MAOA inhibitors restore the levels to within the normal range and helps in the cure of depression ***[Fig-17].***

Specifically, the effect of antidepressants is based on the number and nature of the binding sites that recognize the drug, the concentration of the drug at these sites, and whether the drug functions as an agonist or antagonist. How an antidepressant drug recognizes and interacts with its receptor or transporter helps to determine its activity and efficacy. A drug may directly act on a receptor as full or partial agonist or may block the binding of other substances at this site (i.e., a receptor antagonist). Drugs that exert their action via Serotonin pathways affect Serotonin–containing neurons that are located in the brainstem (pons and medulla), primarily the raphe nuclei. Serotonin neurons project into the neocortex, limbic system, hypothalamus, and cerebellum. There are at least 14 Serotonin receptor subtypes. Because Serotonin receptors are found to be important for sleep, mood, motoneuron function, sensory transmission, and autonomic functions, a drug that acts on a Serotonin receptor can affect sleep, mood, pain, movement, and endocrine function. The therapeutic effects of antidepressants SSRIs and SNRIs [ie: Amytriptyline, flouxetine and Haloperidol] act by inhibition of the reuptake of serotonin via blockade of the high-affinity serotonin transporter .Dopamine - containing neurons are located in the same region as Serotonin–containing neurons – (the pons and medulla of the brainstem). There are at least 11 Dopamine receptor subtypes, although most of the effects are associated with 2 subfamilies, the α and $\beta 1$ receptors. Dopamine is associated with a broad range of effects, including effects on attention, appetite, reinforcement, mood, arousal, and blood pressure regulation. While both SSRIs [ie: Amytriptyline and Flouxetine] and SNRIs [Haloperidol] elevate the synaptic levels of

Fig-17 The mechanism of action of antidepressants (monoamine oxidase a inhibitors- selective serotonin reuptake inhibitors)


5-HT
noradrenaline
MAOI
nerve terminal
MAO-A
MAOAI
COMT
5-HT
re-uptake
transporter
degraded
monoamines
MAO
MAOI
synaptic
cleft
5-HT
receptor
MAO-A
MAOAI
COMT
degraded
monoamines
noradrenaline
re-uptake
transporter
COMT
noradrenaline
receptor
©CNSforum.com

Serotonin, SNRIs also elevate synaptic levels of Dopamine *[Delgado (2000)]*. Therefore, in theory, an antidepressant classified as an SNRI should be able to affect the processes linked to the hallmark symptoms of depression associated with the function of Serotonin, such as depressed mood and sleep disturbances, as well as the areas of the brain associated with attention, arousal, and motivation linked to dopamine. In the present investigation the properties of SSRIs and SNRIs (Amytriptyline, flouxetine and Haloperidol) look at how the drug binds to the receptor (via receptor binding assays), the drug's effect on reuptake, and the relative effect of the drug on different receptors. Binding studies are used to determine the ability of a drug to attach to a particular site (affinity) it can be evaluated using various drug concentrations which also gives information about the number of binding sites.

Material & Methods

2. MATERIAL AND METHODS

A) MATERIALS

Chemicals uses for the present study were obtained from the sources indicated.

S.no	Source/Company	Chemicals
1.	**Genei Bangalore, India**	Molecular weight markers – MWM,
5.	**Sigma Chemicalsco. USA**	Bovine serum albumin, Bromelain, Chymotrypsin, Curcumin, Griess reagent, Ficin, hydrogen peroxide, Ovalbumin, Papain, riboflavin, Sephadex G-75, Sodium nitrite, Soyabean trypsin inhibitor, Trypsin, 2-mercaptoethanol, sodium azide, Blue dextran, Dopamine and serotonin
6.	**Qualiegens Fine Chemicals, India**	ammonium sulphate, bromophenol blue, disodium hydrogen phosphate, ethyl alcohol, , glacial acetic acid, glycerol,hydrochloric acid, mannitol, monosodium dihydrogen phosphate, potassium iodide, sodium benzoate, sodium carbonate, sodium chloride, sodium hydroxide, sodium potassium tartarate, sulphuric acid, thiourea, trichloroaceticacid, TEMED, Tris (hydroxymethyl amninomethane).
7.	**Sisco Research Lab (SRL), India**	Acrylamide, N'N' methylene bis-acrylamide, casein, L-cysteine, glucose, commassie brilliant blue- R250, EDTA, Folin Ciocalteau's phenol reagent, glycine, phenol and sucrose.
8	**Drugs**	Amytriptyline (Merind manufactured by work hardt limited) ,Fluoxetine (Cadila pharmaceutical limited),Haloperidol (Torrent pharmaceutical ltd) Donepezil (Eisai pharmaceutical limited)

B) METHODS

2.1 PURIFICATION OF BRAIN CYSTATINS

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 in a Beckman J-21 cooling centrifuge residue was discarded and the supernatant was further processed.

Alkaline Treatment

The supernatant obtained was subjected to alkaline pH 11.0 by 1 M NaOH and incubated for 15 minutes at 4^0C to remove unwanted proteins. After incubation, the pH of the supernatant was brought back to pH -7.5 with glacial acetic acid. The precipitated proteins were removed by centrifugation at 11000rpm for 30 minutes at 4^0C in a Beckman J-21 cooling centrifuge.

Ammonium Sulphate Fractionation

The supernatant was fractionated with 20% of ammonium sulphate saturation with gentle stirring at 4^0C. After 4hrs precipitate was removed by centrifugation at 11000rpm for 30 minutes at 4^0C and the supernatant thus collected was made 60% saturated with ammonium sulphate. The solution was allowed to stand for 4hrs at 4^0C, the precipitate thus obtained by centrifugation at 11000 rpm for 30 minutes at 4^0C was dissolved in minimum amount of 0.05M sodium phosphate buffer pH 7.5 containing 1% NaCl. The precipitated protein were extensively dialyzed to remove ammonium sulphate against several volume of same buffer at 4^0C containing 1% NaCl.

Gel Filtration Chromatography

A Sephadex G-75 column was prepared as recommended by *Peterson and Sober (1962).* The gel was allowed to swell in sufficient amount of distilled water for 3hrs in a boiling water bath. The gel fines were removed by suspending the gel in two to four fold excess of 0.05 M sodium phosphate buffer, pH 7.5 and allowing 90-95% of the gel to settle down. A glass column mounted on a sturdy vertical support, was filled to one third of its length with

operating buffer in order to check leaks and flush air bubbles from the dead space. The dearerated gel slurry was then poured with the help of a glass rod into column with care to avoid generating air bubbles. The column was left standing overnight. Flow rate was increased gradually and after accomplishing a constant flow rate higher than that required for final elution, the column was adjusted to the required flow rate. The packed column was thoroughly washed with two bed volumes of operating buffer (0.05 M sodium phosphate buffer, pH 7.5). In order to check uniform packing and to determine the void volume of the column, 2% (w/v) solution of blue dextran in 0.05 M sodium phosphate buffer, pH 7.5 was passed through the column. The volume of blue dextran and protein solution applied was not more than 2-3% of the total bed volume. Five milliliter fractions were collected and assayed for protein and cystatin activity. Homogeneity of the preparation was analyzed by 7.5% PAGE.

2.2 COLORIMETRIC ANALYSIS

Determination of Protein Concentration

Protein was estimated by the method of *Lowry et al (1951).* Aliquots of protein solution were taken in a set of test tubes and final volume was made up to 1ml with distilled water. Five ml of alkaline copper reagent containing one part of 1% (w/v) copper sulphate and 2% (w/v) sodium potassium tartarate in 1% (w/v) sodium hydroxide and sodium carbonate was added followed after 10 minutes of incubation at room temperature then 0.5 ml of 1:1 Folin Ciocalteau's phenol reagent added. The tubes were instantly vortexed. The color development was read after 30minutes at 660nm against a reagent blank. A standard curve was prepared using BSA as standard. Protein in the column fraction was also monitored at 280nm Camspec spectrophotometer Model M330B.

Carbohydrate Estimation

The procedure described by *Dubois (1956)* was followed.for carbohydrate estimation .Two milliliter aliquot containing 10-70 µg of protein was pipetted in two set of test tubes and 1ml of 5% phenol was added. This

was followed by the addition of concentrated sulphuric acid. The tubes were allowed to stand for 30 minutes at room temperature. The color intensity was measured at 490nm for the quantization of hexose content. Glucose was used as standard.

Thiol Group Estimation

The procedure described by *Ellman (1959)* was followed for estimating the thiol groups of cystatins. SDS and β-mercaptoethanol induced appearance of free thiol group in the cystatin was followed by titration with DTNB reagent. An appropiate aliqouts of 0.2 ml of native and SDS treated inhibitor were mixed with 0.1ml of DTNB reagent (prepared by dissolving 40mg in 100ml of 20mM Tris-EDTA buffer, pH 8.2) in a total volume of 3.6 ml. The absorbance was read after 30 minutes at 412nm in Camspec Spectrophotometer Model M330 B .Free thiol concentration was calculated from the absorbance using molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$ for the released thionitrobenzoic acid. A plot was prepared using L- cysteine as standard.

Assay of cystatin inhibitory activity

Inhibitory assay of cystatin was performed as described by the *Kunitz (1947).* BC was examined for its ability to prevent thiol proteinases from digesting casein. For determination of inhibitory activity, papain was activated in the presence of 0.14M cysteine and 0.047 M EDTA for 10 minutes prior to incubation of papain cystatin complex for 30 minutes at 37^0C in 0.05 M sodium phosphate buffer, pH 7.5. The enzyme inhibitor complex was further incubated with casein for 30 minutes at 37^0C in same buffer and the reaction was stopped by addition of 10% TCA. Acid insoluble material was removed by centrifugation at 2500rpm for 15 minutes. The supernatant was analyzed for acid soluble peptides with Folin phenol reagent as described by *Lowy et al. (1951).* Ficin inhibition was also assayed by similar method.

Assay of caseinolytie activity of Bromelain

The proteolytic activity of bromelain was measured according to the method of *Murachi and Neurath (1960).* The enzyme was activated at 37^0C

for 10 minutes in the presence of 0.14M cysteine. Then the volume was made upto 1 ml by 0.05 M sodium phosphate buffer pH 7.5. One ml of 0.5% casein was added and incubated for 30 minutes at 37^0C. The reaction was stopped by addition of 1.0ml of 5% TCA. Acid insoluble material was removed by centrifugation at 2500rpm for 15 minutes. The supernatant was analysed for acid soluble peptides by Folin phenol reagent by the method of *Lowry et al. (1951).*

2.3 SLAB GEL ELECTROPHORESIS

Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoresis was performed by the method of *Laemmli (1970)* using the slab gel apparatus manufactured by Biotech, India. Concentrated stock solution of 30% acrylamide containg 0.8% N'N' methylene bis-acrylamide and 1.5M Tris, pH 8.8 were mixed in appropriate portion to give the desired concentration of gel. It was then poured in to the mould formed by the glass plates (8.5 x 10 cm) separated by 1.5 mm thick spacers. Bubbles and leak were avoided. A comb providing template for seven wells was inserted into the stacking gel solution before the polymerization began. The polymerization was complete in about 30 minutes after which the comb was removed and wells overlaid with running buffer. Routinely, 7.5% and 12.5% gels were used. Samples containing 40-60µg of protein was mixed with equal volume of sample buffer 62.5mM Tris HCl pH 6.8, 10% (v/v) glycerol and 0.001% bromophenol as tracking dye was applied to the wells. Electrophoresis was performed at 100V in the electrophoresis buffer containing 192 mM glycine was added and 25mM Tris-HCL buffer of pH 6.8 was used until the tracking dye reached the bottom of the gel.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was essentially performed by the Tris-glycine system of *Laemmli (1970)* using slab gel electrophoresis apparatus. Concentrated stock solution of 30% acrylamide containing 0.8% N'N' methylene bis-acrylamide and 1.5M Tris, pH 8.8 were mixed in appropriate proportions to give the desired percentage of gel. Protein

samples were prepared in solution containing 62.5 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercatoethanol and 0.001% (w/v) bromophenol blue. The samples were incubated at 100^0C for 5 minutes. Electrophoresis was performed at 100 V till the tracking dye reached the bottom of the gel. Running buffer used during electrophoresis contained 1% SDS in addition to 192 mM glycine and 25 mM Tris-HCL, pH 6.8.

2.4 STAINING OF THE GEL

Commassie Blue Staining

After electrophoresis the gels were stained with five gel volumes of 0.25% commassie brilliant blue R-250 dye in 50% methanol and 10% acetic acid for at least 4hrs. For distaining, the gels were incubated with shaking in 5% methanol and 7.5% acetic acid at room temperature.

2.5 MOLECULAR WEIGHT DETERMINATION

The molecular weight of brain cystatin were determined under native, SDS PAGE denaturing conditions and by gel filtration chromatography.

Molecular weight determination by gel filtration chromatography

The molecular weight of native BC was computed from its elution volume on Sephadex G-75 column (1.1 x100 cm), the column was calibrated by determining the elution volume of the following marker proteins 1- Lysozyme (23 KDa) 2- Trypsin (20.1 KDa) 3- Pepsin (35 KDa) 4- Oval albumin (45 KDa) 5- BSA (68 KDa) This data was analyzed according to the theoretical treatment of *Andrews (1964)* method. The linear plot between Ve/Vo and log M was used in calculating the molecular weight of brain cystatin where Ve is the elution volume of the protein and Vo is the void volume of the column determined by using blue dextran.

Molecular weight determination by SDS-PAGE

Molecular weight of cystatins under denaturing conditions was calculated by the procedure of *Weber and Osborn (1969)* using SDS PAGE. The mobilities of marker proteins determined under identical conditions were plotted against the logarithms of molecular weight. The standard proteins

used were phosphorylase b (97KDa), bovine serum albumin (68 KDa), ovalbumin (45KDa), carbonic anhydrase (29 KDa) and soyabean trypsin inhibitor (20 KDa). The analyses of data indicate a linear relationship between logM and relative mobility (Rm) and the plot was used for calculating the molecular weight of cystatins.

2.6 KINETICS OF INHIBITION

Stoichiometery of proteinases inhibitor

Papain was used for the titeration of BC.The inhibitory Activity of cystatin was assessed by its ability to inhibit caseinolytic activity of papain by the method of *Kunitz (1947)*. The concentration of papain was varied from 0.01-0.06µM. whereas the inhibitor concentration was fixed at 0.06µM. papain was activated on incubation with 0.14M cysteine and 0.047 M EDTA for 10 minutes at 37^0C. The inhibitor was added and the volume was diluted to 1 ml by 0.05M sodium phosphate buffer, pH 7.5 and further incubated for 30 minutes for at 37^0C. 1 ml of 2% casein was added and again incubated for 30 minutes at 37^0C. The reaction was stopped by adding 1 ml of 10% trichloroacetic acid. After centrifugation (2500rpm for 15 minutes) the acid soluble peptides were quantitated by the method of *Lowry et al. (1951)* Identical experiments were carried out for the BC with other proteinases, ficin and bromelain using casein as substrate *[Kunitz (1947) , Murachi and Neurath (1960]*.

Inhibition constant (Ki) determination

Ki determination was carried out after lowering the enzyme and inhibitor concentration to obtain a non linearity of dose response curves. Thus papain was used at a concentration of 0.06µM to react with inhibitor varying from 0.01-0.24µM and measurement of residual activity was made as described by *Kunitz (1947)* using casein as substrate. Four different substrate concentrations were used (0.5 Km, 1Km and 2Km with Km= 2.4mM) and the results were analyzed by the steady state equation of *Krupka and Laidler (1959)*. The linear equation is presented as follows *[Henderson, 1972]*.

$$\frac{I_0}{1-\frac{V_i}{V_0}} = k_i\left[1+\frac{[S]_0}{k_m}\right]\frac{V_i}{V_0} + E$$

Where $[I_o]$, [E] and $[S]_0$ are initial concentrations, enzyme and substrate respectively. Vo is the velocity without inhibitor. The plot of $[I]_0$/ (1-Vi/Vo) against Vo/Vi is a straight line, the slope of which gives.

$$k_i(app) = k_i\left[1+\frac{[S]_0}{K_m}\right]$$

True Ki was obtained from a replot of Ki (app) against $[S]_0$ Similar experiments for Ki determination were done for ficin and bromelain with BC using their respective substrates.

2.7 SPECTRAL ANALYSIS

Absorption Difference Spectra

Ultraviolet absorption difference spectra were measured for BC (80µg/ml) along with activated papain at a molar ratio of 1:1 at 25±20C.Spectra were recorded by measuring the absorption between 200-350nm on Cintra-5 spectrophotometer in a cuvette of 1 cm path length. Appropriate Controls of the solvent buffer were run and corrections were made wherever necessary.

Fluorescence Spectroscopy

Fluorescence measurements for papain BC along with their complexes with papain were performed at 25+ 1OC in Shimadzu spectrofluorimeter model RF-540 equipped with a data recorder DR-3. The excitation wavelength was 250nm (λ_{max}) and emission wavelength range was 300-400nm. 2µM BC and 2µM papain in a total volume of 1ml was used for the study. Cells with 1cm path length were used and samples were continuously stirred during measurements. Corrected emission spectra were recorded with an excitation

emission band width of 5nm. Appropriate controls were run and corrections were made wherever necessary.

2.8 OXIDATIVE STRESS INDUCED CYSTATIN DAMAGE BY FREE RADICALS

EFFECT OF NITRIC OXIDE [NO•] ON CYSTATIN

Nitric oxide production from sodium nitroprusside:

Nitric oxide was generated from sodium nitroprusside and was measured by the Griess reaction. Sodium nitroprusside in aqueous solution at physiological pH generates spontaneously nitric oxide which interacts with oxygen to produce nitrite ion which can be estimated by the use of Griess reagent *[Green et al. (1982) and Marcocci et al. (1994)].* 100mM of SNP was prepared by dissolving the powder in phosphate buffer saline *[PBS]* pH 7.4. The reaction mixture [2ml] containing 100mM SNP (0.2ml, final concentration 10mM) and PBS (1.8ml) was incubated at 25^0C for 180 min. At 30 min intervals. 1 ml aliquots were withdrawn from the incubation and diluted with 1ml of GR.

Griess Reaction

The Griess reaction relies on diazotization. The GR consist of 1% sulphanilamide and 0.1% naphthylene diamine dihydrochloride in 2%. H_3PO_4. The plot between the concentration of nitrite and incubation time exhibited the best incubation time for nitrite production from SNP.In this method, nitrite is first treated with a diazotizing reagent e.g. sulfanilamide in acidic media to form a transient diazonium salt. This intermediate is then allowed to react with a coupling reagent, N-naphthyl-ethylenediamine to form a stable azo compound. The intense purple color of the product allows nitrite assay with high sensitivity and can be used to measure nitrite concentration as low as ~0.5 mM level. The absorbance of this adduct at 540 nm is linearly proportional to the nitrite concentration in the sample.

Treatment of Brain Cystatin with NO•

To see the effect of nitric oxide on BC it was incubated with nitric oxide BC (1µM) was incubated with 0.05 mM, 1mM and 10mM SNP for 30 min or with 0.05mM SNP for varying time interval (0-180 min) in 50mM Sodium Phosphate Buffer pH 7.5 in a final reaction volume of 1ml at Room temperature following the incubation samples were subjected to fluorescence spectroscopy and assay of antiproteolytic activity.

Effect of natural antioxidants on NO induced modification of BC

1µM of Cystatin incubated with 0.05mM of SNP for 30 min in the presence of (1-50µM) curcumin and (50-250 µM) quercetin in 0.050M sodium phosphate buffer (pH 7.5) in a final reaction volume of 1 ml at Room temperature. After incubation samples were subjected to fluorescence spectroscopy and assay of antiproteolytic activity.

Fluorescence spectroscopy

Intrinsic fluorescence measurement was carried out on shimadzes spectroflourimeter model RF-540 equipped with data recorder DR-3 25±0.1^{0}C. The fluorescence was recorded in the wavelength range 300-400 nM after exciting the protein solution at 280nM for total protein fluorescence. The path length of the samples was 1 cm.An appropriate controls containing the oxidants used for the treatment were run and corrections were made wherever necessary .Each spectrum was the average of three scans.

Antiproteolytic activity of Cystatin

Functional activity of BC was assessed on the basis of its ability to inhibit the caseinolytic activity of papain as described by *Kunitz (1947).* Cystatin was incubated along with Riboflavin NO, HOCL and H_2O_2 with varying concentration and for varying time interval showed.

REACTION OF HYPOCHLOROUS ACID (HOCl) WITH CYSTATIN

HOCl concentration was quantified immediately before use It was determined spectrophotometrically at 290 nM (pH 12 €=350/M/cm] *[Marries*

(1966)] HOCl was diluted in ice cold water to made a stock solution of 10 mM and stored no longer than 1min. *[Whitman et al. (2003)]*

Treatment of Cystatin with HOCl

BC (1µM) was incubated with HOCl (1-5µM) for 30 min or with 5µM HOCl for varying time intervals (0-30 min) in 0.05M sodium phosphate buffer (pH 7.5) in a final reaction volume of 1.5 ml at Room temperature. At the end of incubation, HOCl was quenched with 150µl (2mM) reduced glutathione. The treated protein samples were subjected to fluorescence measurement and assay of thiol proteinase inhibitory activity by the method of *kunitz (1947).*

Effect of Scavenger's and Natural Antioxidants on HOCl Induced Modification of Cystatin

BC (1µM) was incubated with 5µM of HOCl in the presence of 25mM sodium benzoate, mannitol, glucose and 120 µM of each quercetin and curcumin in a final reaction volume of 1.5 ml then the treated protein samples were subjected to fluorescence measurement and assay of thiol proteinase inhibitory activity.

REACTION OF HYDROGEN PEROXIDE WITH BRAIN CYSTATIN

The concentration of H_2O_2 was estimated prior to experiment by the absorbance at 230 nM and € = 0.081 mM^{-1} cm^{-1} *[Andrease (1955)].*

Treatment of Brain Cystatin with H_2O_2

BC (1µM) was incubated with (1mM – 250mM) H_2O_2 in 0.05M sodium phosphate buffer pH 7.5 in a final reaction volume of 1.5 ml at Room temperature for 30 min. BC (1µM) was also incubated with 250mM of H_2O_2 for varying time intervals (0-120 min) in a final reaction volume of 1.5 ml at Room temperature. The treated protein was subjected to fluorescence measurement and thiol proteinase inhibitory activity.

Effect of scavenger's and natural antioxidants on H_2O_2 induced modifications of Cystatin

1µM of BC was incubated with 250mM of H_2O_2 in presence of Scavengers 25mM mannitol, sodium benzoate 120µM of quercetin and

curcumin in a final reaction volume of 1.5ml for 30 min. The treated proteins samples were subjected to fluorescence measurements and 1 ml of final reaction volume was used for assay of thiol proteinase inhibitory activity.

REACTION OF PHOTOSENSITIZED RIBOFLAVIN WITH BRAIN CYSTATIN

BC (1μM) was photo illuminated with increasing concentration of riboflavin (5-50μM) in a final volume of 1ml at Room temperature for 30 min. Riboflavin was freshly prepared at 2mM concentrations in 0.05M sodium phosphate buffer (pH - 7.5) .

In another experiment Cystatin (1μM) was also photo illuminated for different time interval of (0-60 min) with 50μM of riboflavin in a final reaction volume of 1ml at Room temperature , for light incubation tubes were set at a distance of 1cm from 40 watt cool fluorescent lamp. These treated protein samples (1.5 ml) were subjected to fluorescence measurement and assay of thiol proteinase inhibitory activity.

Effect of scavenger's on Riboflavin induced modification of Purified Cystatin

To validate the type free radical involved in riboflavin induced BC modification different scavengers were used. BC (1μM) was photo illuminated in the prescence of 50μM of riboflavin for 30 min in presence of 25mM of potassium iodide, Glucose, sodium oxide, mannitol, thiol Urea and sodium benzoate. The treated protein samples were subjected to fluorescence measurement and thiol proteinase inhibitory activity.

2.9 EFFECT OF DRUG AND NEUROTRANSMITTER ON BRAIN CYSTATIN

Interaction of Drugs (Antidepressants: Amytriptyline, Flouxetine Haloperidol and Donepezil: An Alzhimer drug) and neurotransmitter (Dopamine and Serotonin) with the Cystatin.

2.10 SPECTROCOPIC STUDIES

Fluorescence spectra of brain cystatin with drugs and neurotransmitter

Brain cystatin (BC) (1μM) was incubated for 30 min with increasing concentration of Drug 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 region 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

$$\frac{F_0}{F} = 1 + Ksv\,[Q]$$

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)

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)]*

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

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.**

UV spectra of cystatin in the presence of antidepressants and neurotransmitters

The UV measurement of brain cystatin in the presence and absence of antidepressants were made in the range of 200-300 nm and the inhibitor (Cystatin) concentration was fixed at 1μM while the drug concentration was varied for different drugs 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 drugs (Amytriptyline, Flouxetine, Haloperidol and Donepezil) and neurotransmitter (Dopamine and Serotonin)

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 [Kunitz (1947)].* The inhibitor (1 μM) was incubated with increasing concentrations of drugs at 25^0C for 30 min before the activity was measured.Acivity of untreated BC was taken as 100%.

Result & Discussion

Results

Chapter- 1

Purification and Characterization of Buffalo Brain Cystatin

3.1 RESULT

PURIFICATION AND CHARACTERIZATION OF BUFFALO BRAIN CYSTATIN

3.1.1 PURIFICATION

In the present study cystatin has been purified from buffalo brain by the modification of the method of *Bige (1985).*As described in the methods section the procedure involved a combination of alkaline treatment (pH 11.0), ammonium sulphate fractionation and gel filtration chromatography. The proteins obtained after alkaline treatment were precipitated between 40-60% ammonium sulphate saturation.The precipitate obtained thus obtained was dissolved in 0.05 M sodium phosphate buffer pH - 7.5 and was dialyzed with several changes against the same buffer containing 0.15 M NaCl. This resulted in fold purification of 53.51 and with a yield of 71.4% ***[Table -4]***

3.1.2 GEL FILTRATION

Protein sample after dialysis was chromatographed on a column of sephadex G-75 (1.1 x 100 cm) equilliberated with 0.05 M sodium phosphate buffer pH 7.5 . Elution profile showed two protein peak one major and one minor called as peak-I and peak-II ***[Fig-18]***, peak-I corresponding to high molecular weight buffalo brain Cystatin had significant inhibitory activity however peak-II with insignificant proteins concentration and low inhibitory activity was not taken into consideration, for further studies Peak-I named as BC was 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

3.1.3 HOMOGENEITY

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-19]***

TABLE-4

PURIFICATION TABLE OF BUFFALO BRAIN CYSTATIN

Steps of purification	Volume of homogenate (ml)	Protein (mg/ml)	Total protein content (mg)	Activity* units/ml	Total activity (units)	Specific activity (Units/mg)	Fold purification	% yield
Crude	300	32	9600	0.2912	87.36	0.0091	1	100
Alkaline treatment	250	27	6750	0.32	80	0.0118	1.296	91.57
40-60% Ammonium Sulphate cut	16	8	128	3.9	62.4	0.487	53.51	71.4
Gel filtration	20	0.8	16	2.8015	56.0	3.501	384.72	64.13

*1 unit of inhibitor enzyme activity is defined as the amount of inhibitor in bringing about 0.001 change in O.D/min/ml

Fig-18 Gel filtration chromatography on sephadex G-75

The protein obtained by precipitation between 40-60% ammonium sulphate fractionation was dissolved and dialyzed against 0.05 M sodium phosphate buffer pH 7.5 containing 0.15M NaCl at 4^0C . The sample was applied on sephadex G-75 column (1.1 x 100 cm) and the fractions were eluted with the same buffer at the rate of 30ml /hr. Fractions of 5 ml each were analyzed for their inhibitory activity against papain, absorbance was taken at 660nm.

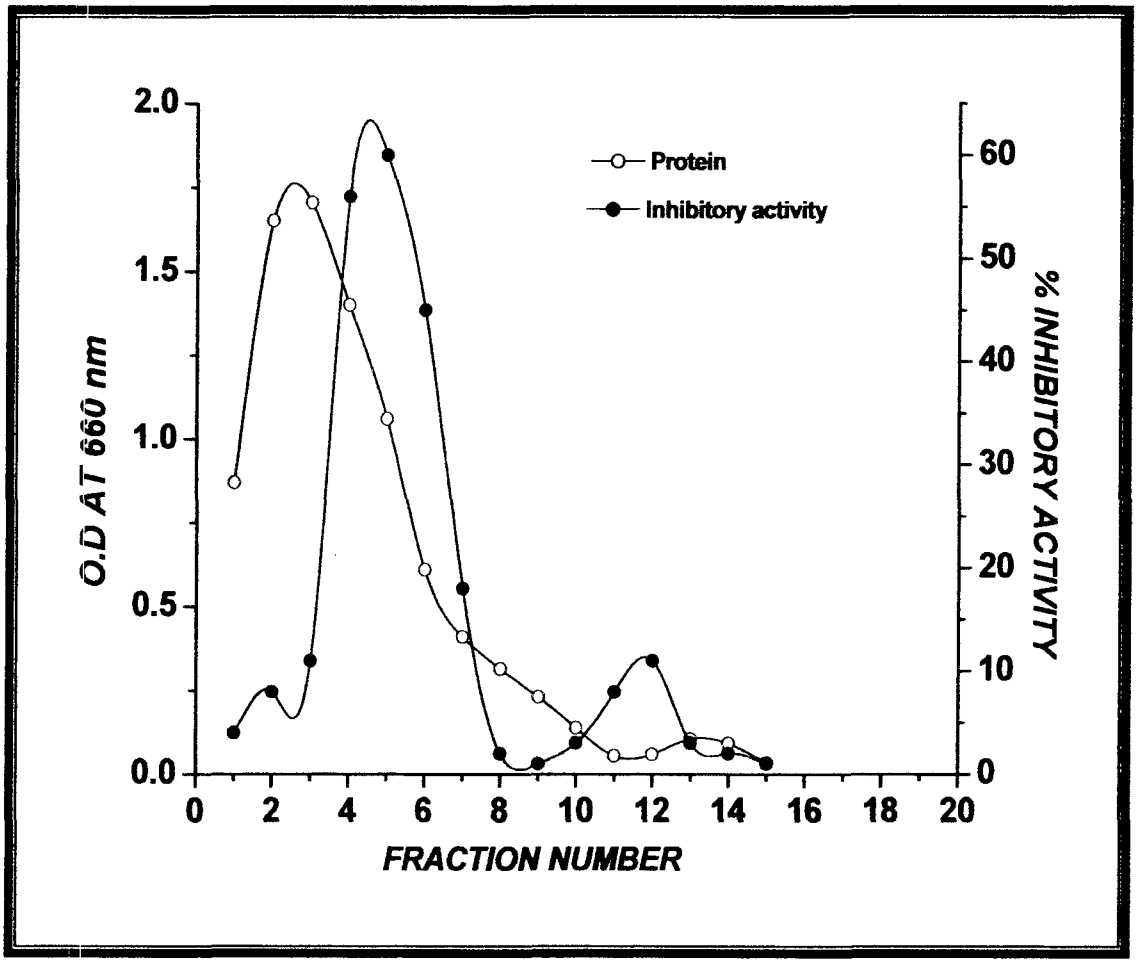

Protein
Inhibitory activity
O.D AT 660 nm
% INHIBITORY ACTIVITY
FRACTION NUMBER
2.0
1.5
1.0
0.5
0.0
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20

Fig-19 Gel electrophoresis of Brain Cystatin during various stages of purification

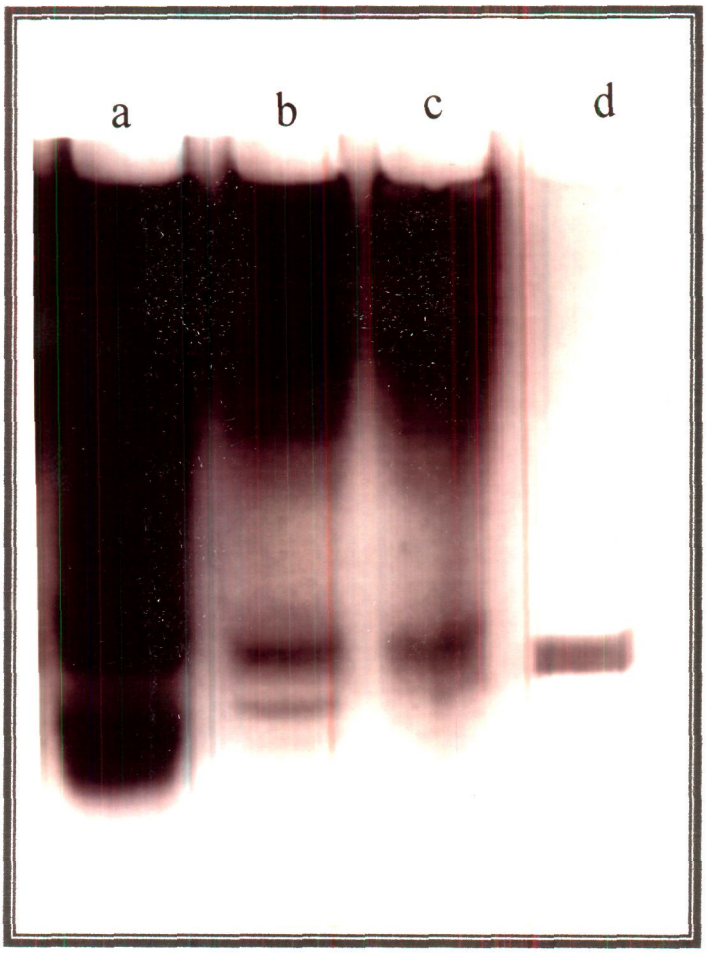
Electrophoresis was performed on 7.5% gel as described in the methods section at 25^0 C

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

Laned: 60µg of purified fraction after gel filteration chromatoraphy on sephadex G-75


a
b
c
d

3.1.4 REDUCING AND NON REDUCING SDS PAGE

Purified cystatin was also analyzed by SDS – PAGE under reducing conditions by the method of *weber and Osborn (1969)* (in the presence of β-mercaptoethonal) and non reducing conditions (in the absence of β-mercaptoethonal) ***[Fig-20]***. BC migrated as two band with different motilities in both the conditions suggesting a two subunit structure which are held together by non-covalent forces

3.1.5 PROPERTIES OF PURIFIED BRAIN CYSTATIN

Molecular Weight Determination

The molecular weight of BC was determined under denaturing conditions by the method of *Weber and Osborn (1969)*.The relative mobility (Rm) of each marker protein was determined. The markers used were 1-phosphorylase b , 2- B.S.A , 3- oval albumin , 4- carbonic anhydrase, 5-Soyabeen trypsin inhibitor , 6- lysozyme was plotted against log molecular weight. ***[Fig-21]*** .Positions of the two subunit corresponding to molecular weight of 31.62 kDa and another of 12.58 kDa were obtained. The least square analysis of the data indicated a linear relation ship between log M and relative mobility which gave the molecular weight as 44.2kDa

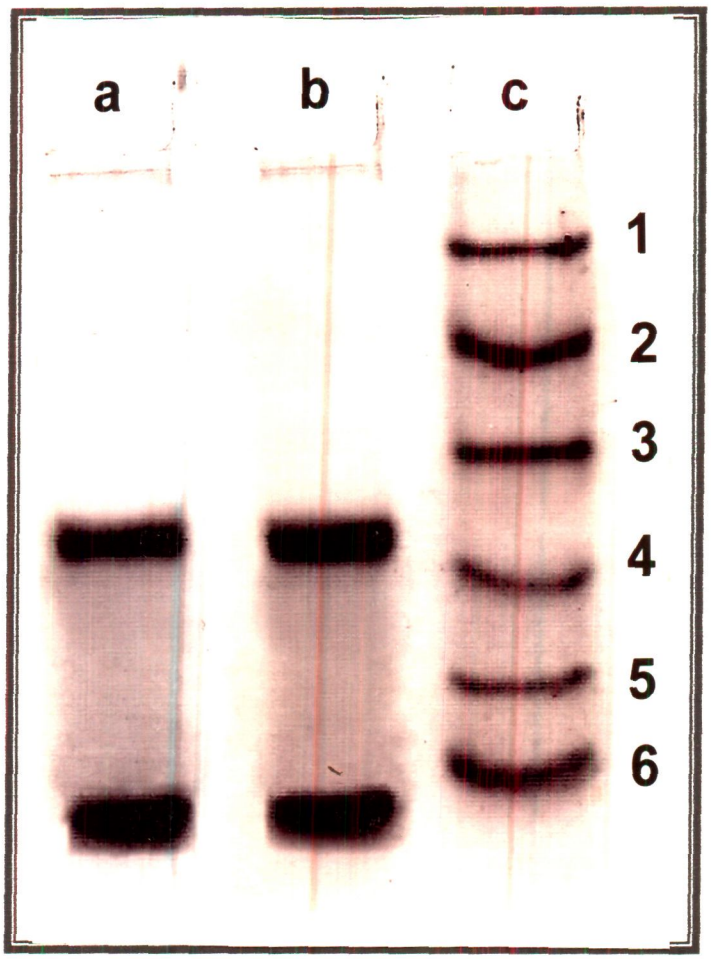
Molecular weight by Gel filteration

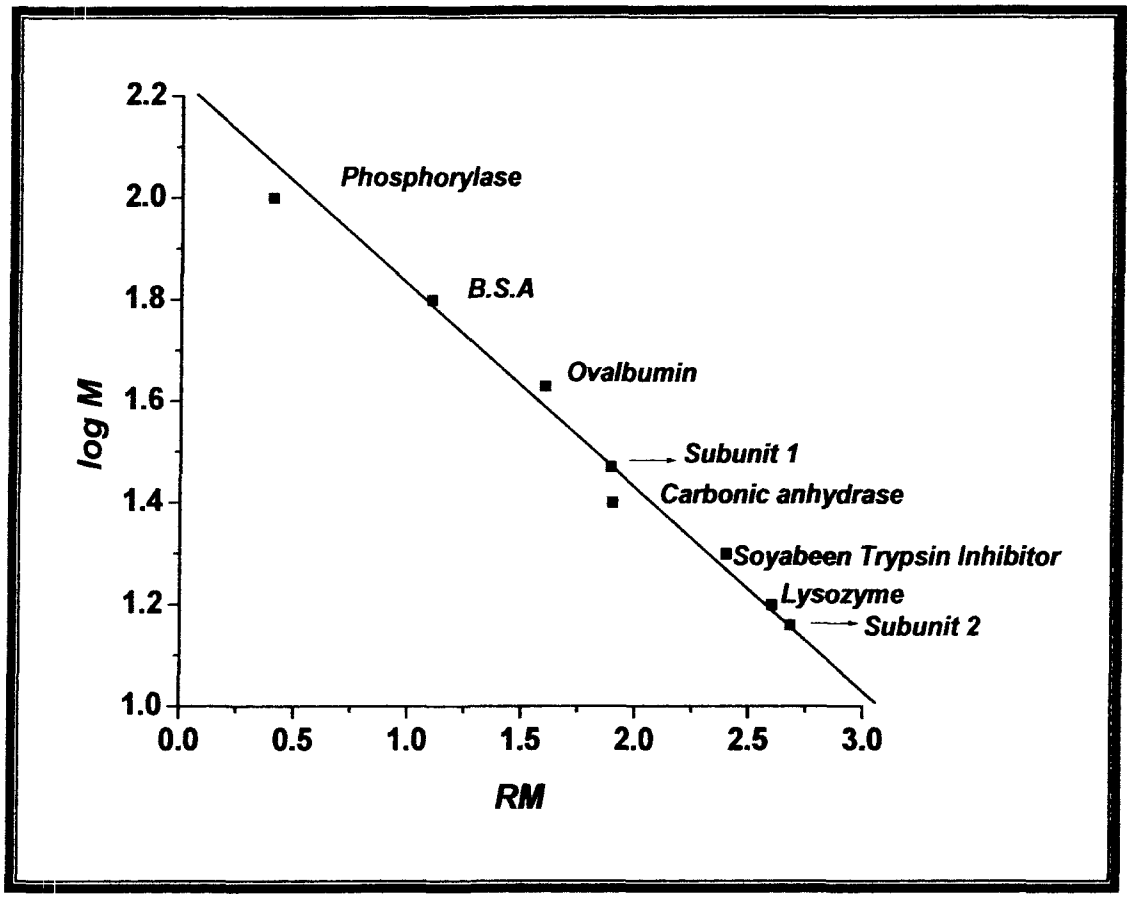
Molecular weight of Cystatin in native condition was determined by using gel filtration chromatography on sephadex G-75. The marker proteins were 1- Lysozyme (23 KDa) 2- Trypsin (20.1 KDa) 3- Pepsin (35 KDa) 4-Oval albumin (45 KDa) 5- BSA (68 KDa) . these proteins were chromatographed after equilibration of the column sephadex G-75 (1.1x100 cm) with 0.05 M sodium phosphate buffer pH -7.5 and then elution volume was determined by passing them from column. Analysis of the data indicated linear relation ship between log M and V_e / V_0 by the method of *Andrews (1964)*.where Ve is the elution volume and V_0 is the void volume of the column ***[Fig -22]*** The V_e / V_0 of the native cystatin corresponds to molecular weight of 43.6 kDa.

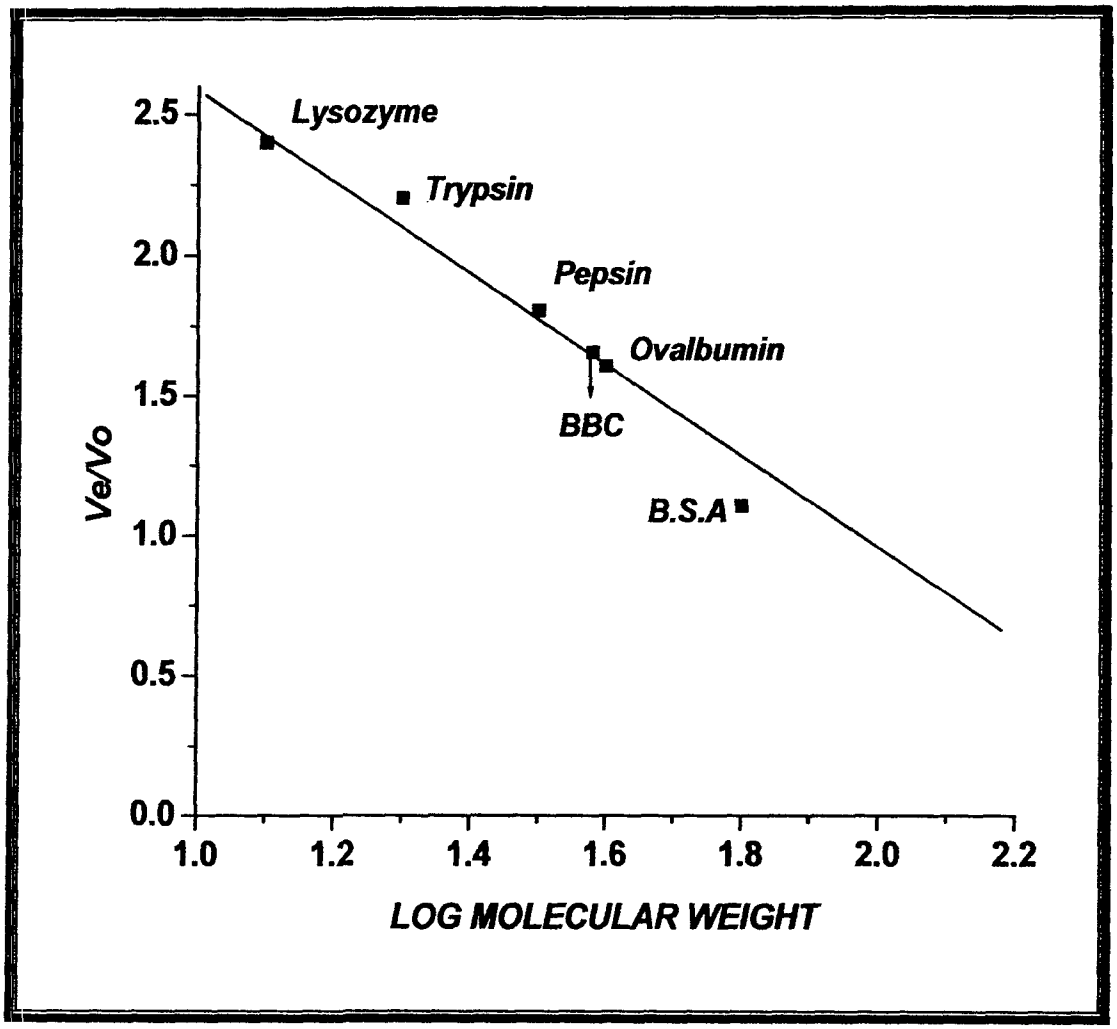
Fig-20 SDS PAGE of the Brain Cystatin under reducing and non reducing conditions

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)


a
b
c
1
2
3
4
5
6


2.2
2.0
1.8
1.6
1.4
1.2
1.0
log M
0.0
0.5
1.0
1.5
2.0
2.5
3.0
RM
Phosphorylase
B.S.A
Ovalbumin
Subunit 1
Carbonic anhydrase
Soyabeen Trypsin Inhibitor
Lysozyme
Subunit 2


Lysozyme
Trypsin
Pepsin
Ovalbumin
BBC
B.S.A
Ve/Vo
2.5
2.0
1.5
1.0
0.5
0.0
1.0
1.2
1.4
1.6
1.8
2.0
2.2
LOG MOLECULAR WEIGHT

Determination of stokes radius and diffusion coefficient

The stokes radius of a protein correlates well with its elution behavior from gel filteration column. The stokes radius of BC was determined by method of *Andrews (1964)* using sephadex G-75 column (1.1x 100 cm) with 0.05M sodium phosphate buffer pH-7.5 using markers proteins of known stokes radii. The coloumn was caliberated by determining the elution volume of proteins such as albumin 35.5 A^0 ovalalbumin 27.3 A^0 , trypsin 20.2 A^0, pepsin 22.9 A^0 The data was analyzed according to the equation. *Laurent and Killander (1964)* ***[Fig -23]***

$$k_{av} = \frac{V_e - V_0}{V_t - V_0}$$

V_e = Elution volume V_0 = Void volume

V_t = Total volume k_{av} = Partition coefficient

The linear plot between the stokes radius and $[-\log k_{av}]^{1/2}$ of the marker proteins was used for the calculation of stokes radius . its value for BC was found to be 27 ^{0}A.

Diffusion Coefficient

The diffusion coefficient (D) of the BC corresponding to their respective stokes radius were computed to be *8.1 $\times 10^{-7}$ cm^2/ sec* with the help of equations given below

$$D = \frac{kT}{f}$$

Where ,

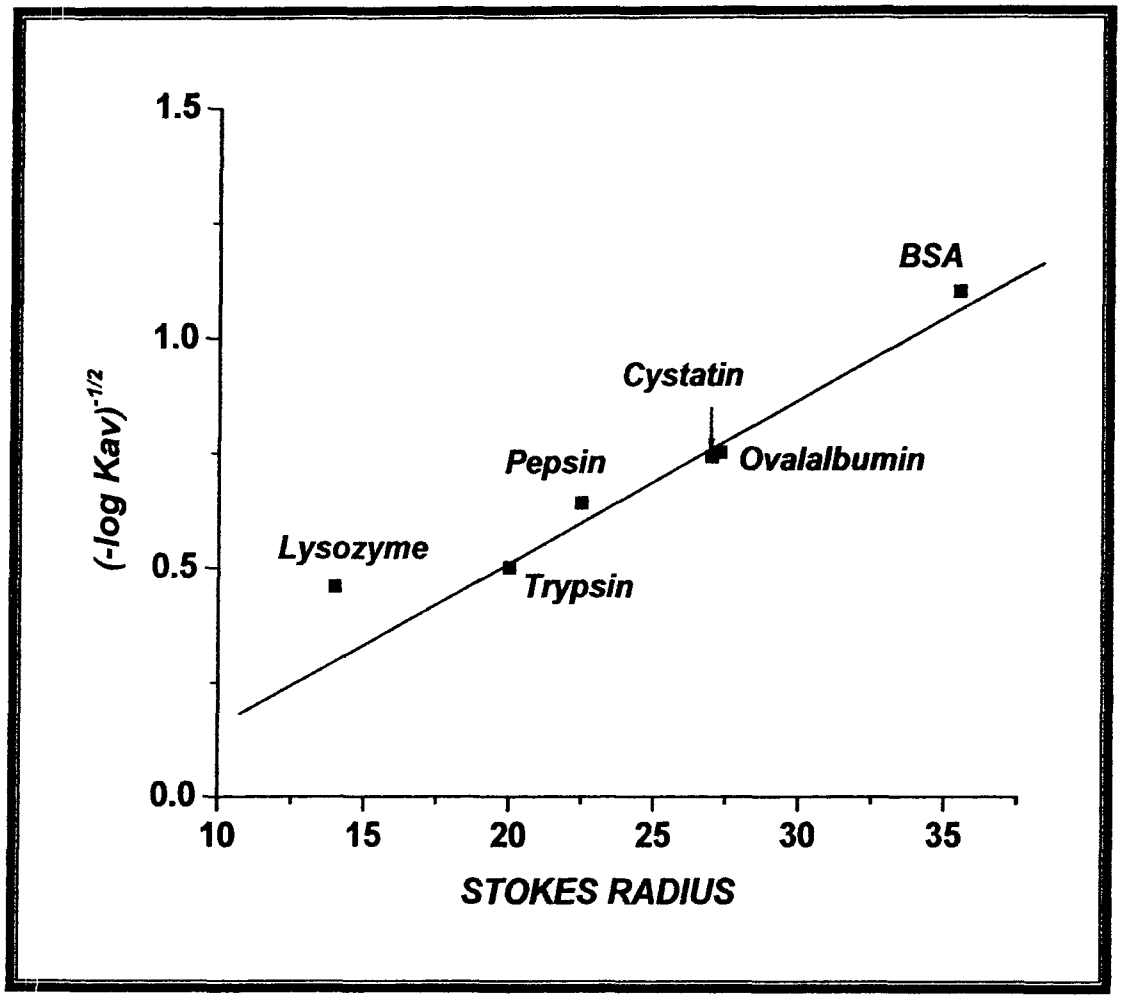
$$f = 6\pi\eta r$$

f is the frictional coefficient that depends upon shape and size of protein

r = radius of the protein (determined accurately by gel filtration)

η = is coefficient of viscosity of the medium (0.01 gm/cm sec for water and dilute and aqueous salt solutions at 20°C)

k=1.38 $\times 10^{-6}$ erg/deg is *Boltzmann's constant*, T is the absolute temperature


1.5
1.0
0.5
0.0
(-log Kav)^-1/2
10
15
20
25
30
35
STOKES RADIUS
BSA
Cystatin
Ovalalbumin
Pepsin
Lysozyme
Trypsin

Carbohydrate content

The cystatin was found to be devoid of any carbohydrate content which is the property of class -II cystatin family.

Sulphydryl content

The sulphydryl groups of cystatin was titrated against DTNB as described in the methods section *Ellmans (1959)* colorless solutions were obtained indicating that no free sulphydryl groups are present in the purified protein.

pH stability of Cystatin

Cystatin was found to be quite stable in the pH range 6-8 when incubated with varying pH range (3-10). At pH 3 BC retained its 84% of inhibitory activity. However at pH 9 and pH 10 it shows more than 88-92 % inhibitory activity ***[Fig-24]***

Temperature stability of BC

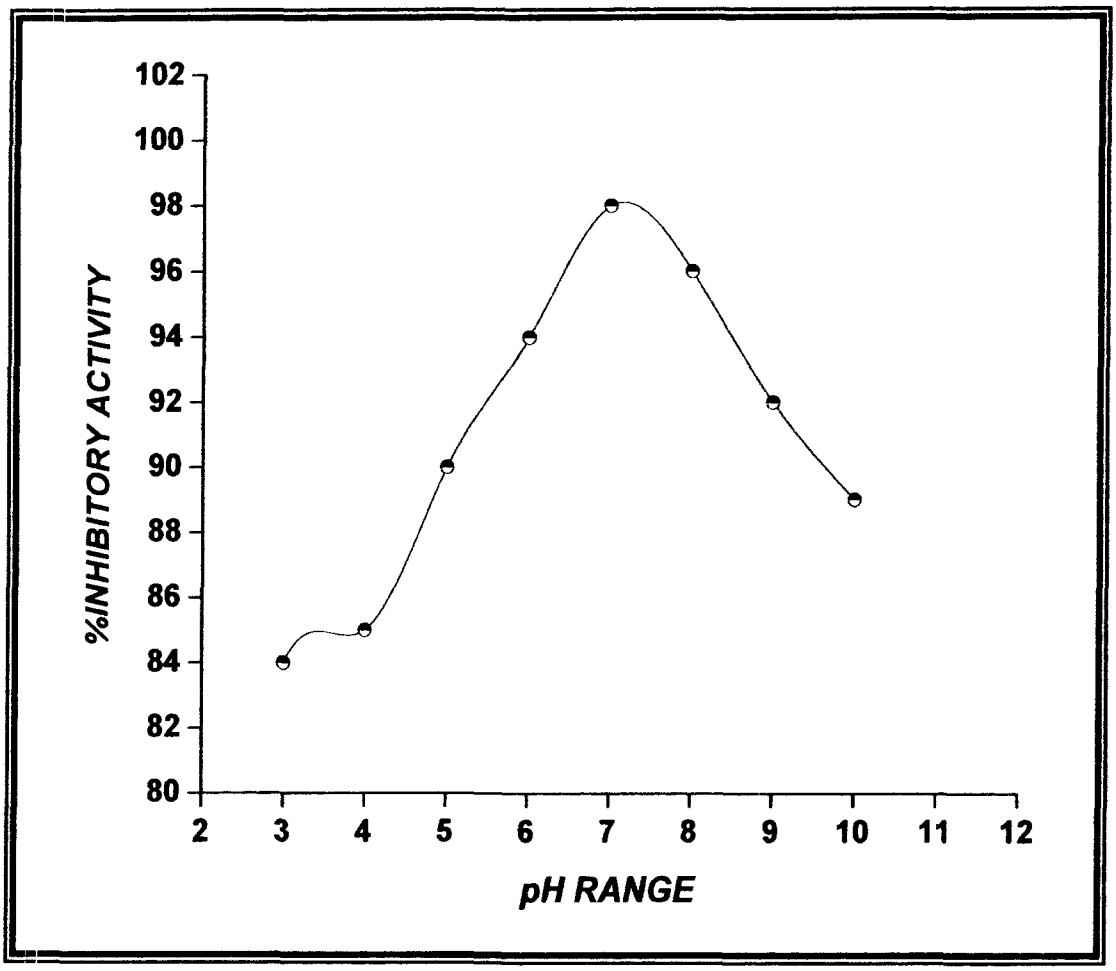
When BC was kept at different temperatures it was found to be maximally active at the temperature range of 25- 60^{0}C. At temperature higher than 80 ^{0}C it showed 25% loss in inhibitory activity ***(Fig-25)***

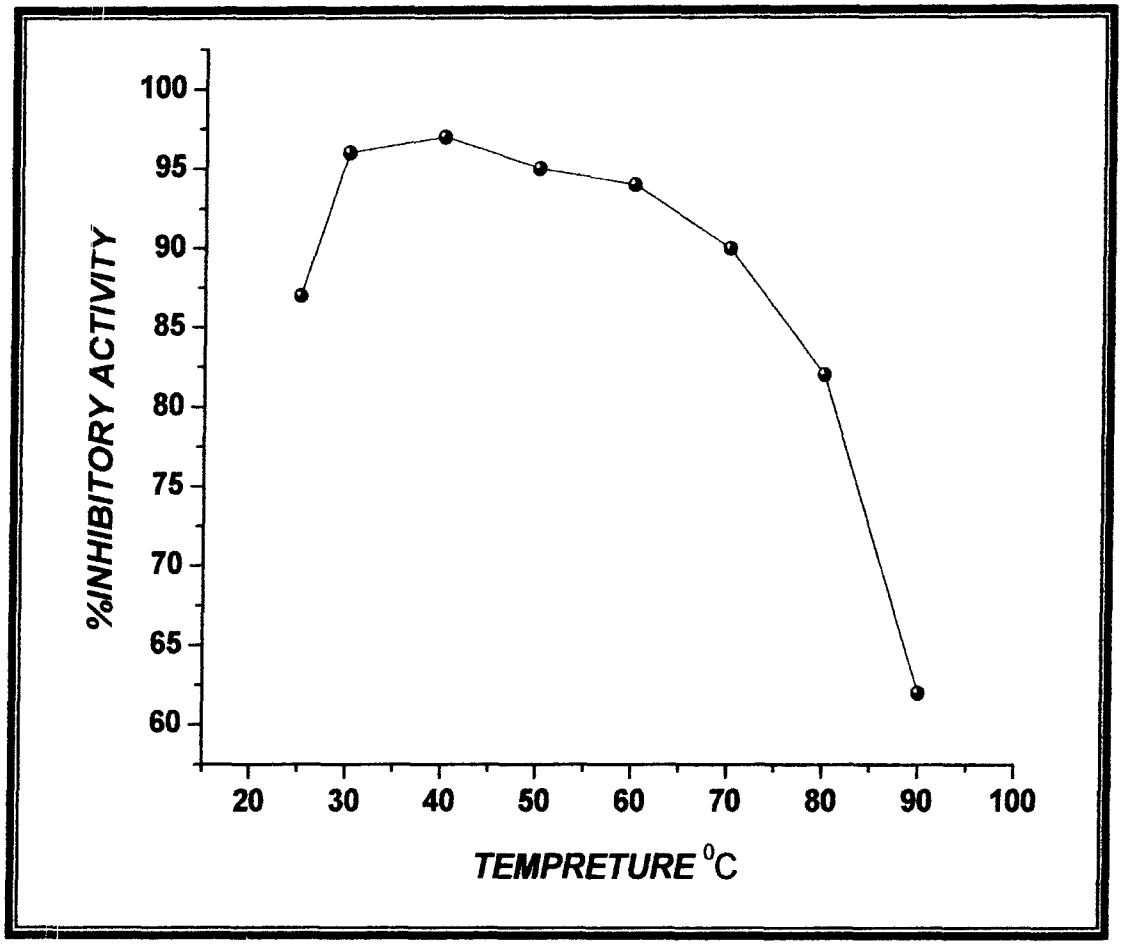
Thermal denaturation

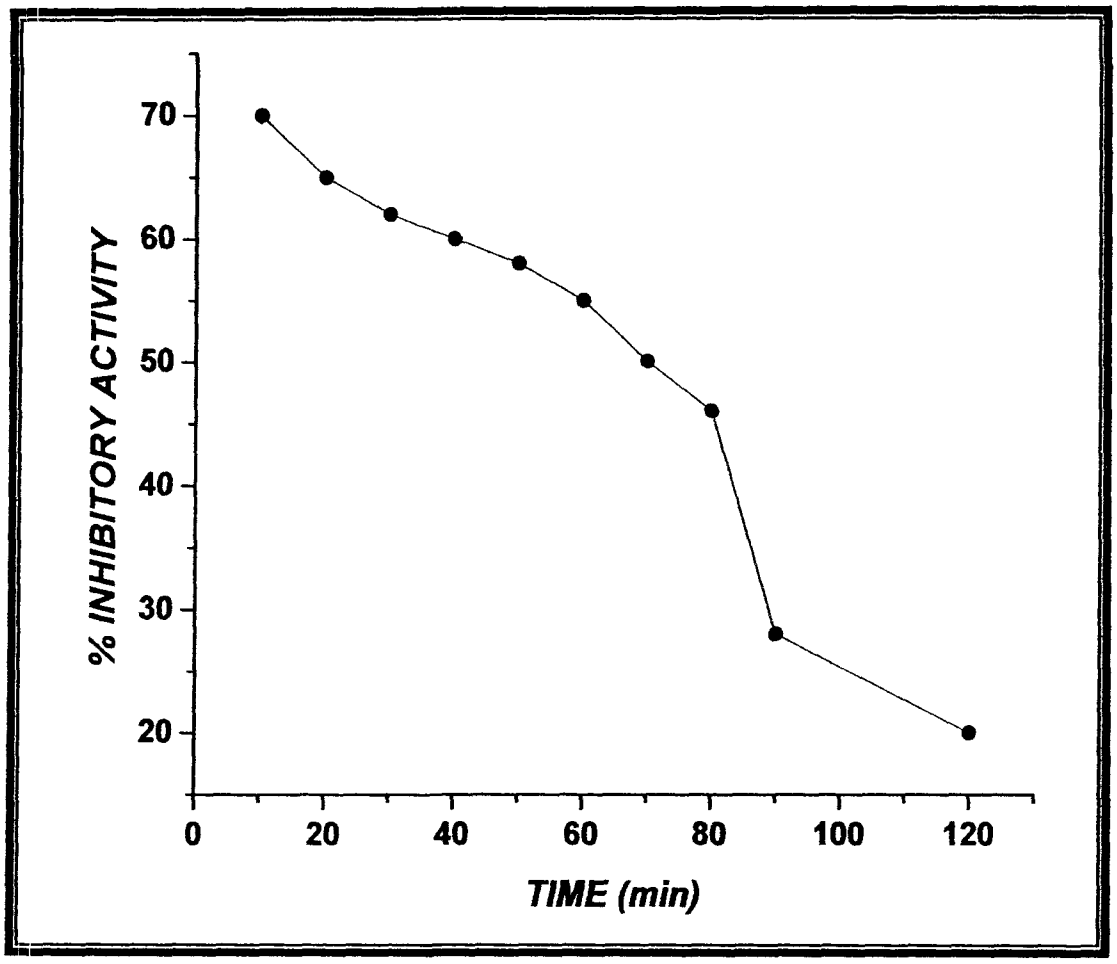
When BC was exposed to 90 ^{0}C for different time intervals and rapidly cooled , it was found to be stable upto 40 min. Above 40 min it retained below 55 % of its activity, however upto 80 min of incubation at 90 ^{0}C more than half of its activity was reduced . ***(Fig-26)***

3.1.6 SPECTRAL ANALYSES

Changes in the secondary structure of BC on binding with papain were studied by UV absorption difference spectra and fluorescence emission spectra


%INHIBITORY ACTIVITY
102
100
98
96
94
92
90
88
86
84
82
80
2
3
4
5
6
7
8
9
10
11
12
pH RANGE


%INHIBITORY ACTIVITY
100
95
90
85
80
75
70
65
60
20
30
40
50
60
70
80
90
100
TEMPRETURE ^{0}C


% INHIBITORY ACTIVITY
70
60
50
40
30
20
0
20
40
60
80
100
120
TIME (min)

U.V absorption

The ultraviolet difference spectra of BC with papain showed maxima around 285 nm and minima around 245nm. The peak around 245nm is partly due to phenylalanine residues and may also be due to contribution from some aromatic amino acids residues. Maxima around 285nm indicates changes around tyrosine residues ***[Fig-27]***

Fluorescence emission spectra of BC in complex with papain

Fluorescence emission spectra showed maxima at 340nm for Cystatin. Upon papain inhibitor complex formation intensity decreased with 10nm red shift these changes are indicative for alteration in the conformation of either one or both the proteins involved in complex formation. ***[Fig-28]***

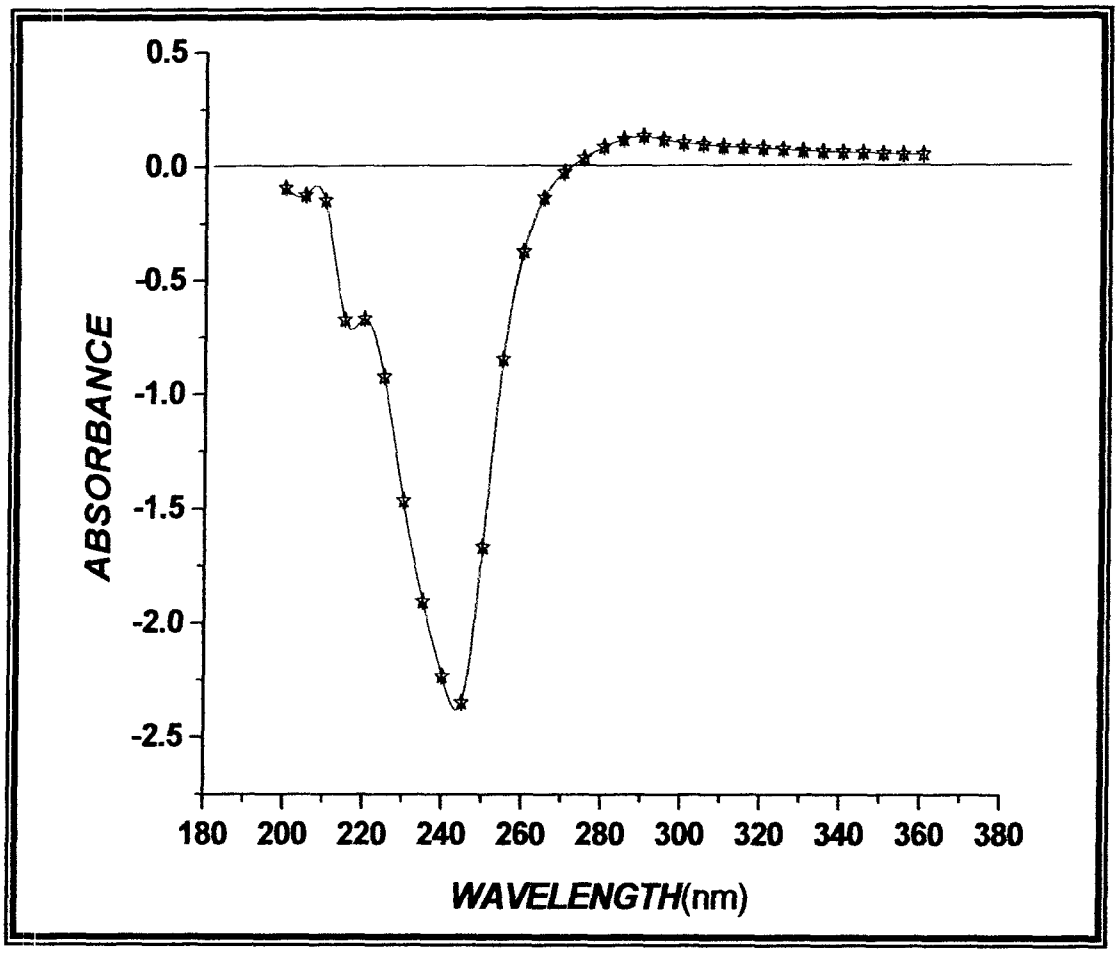
Inhibition of proteinases by BC

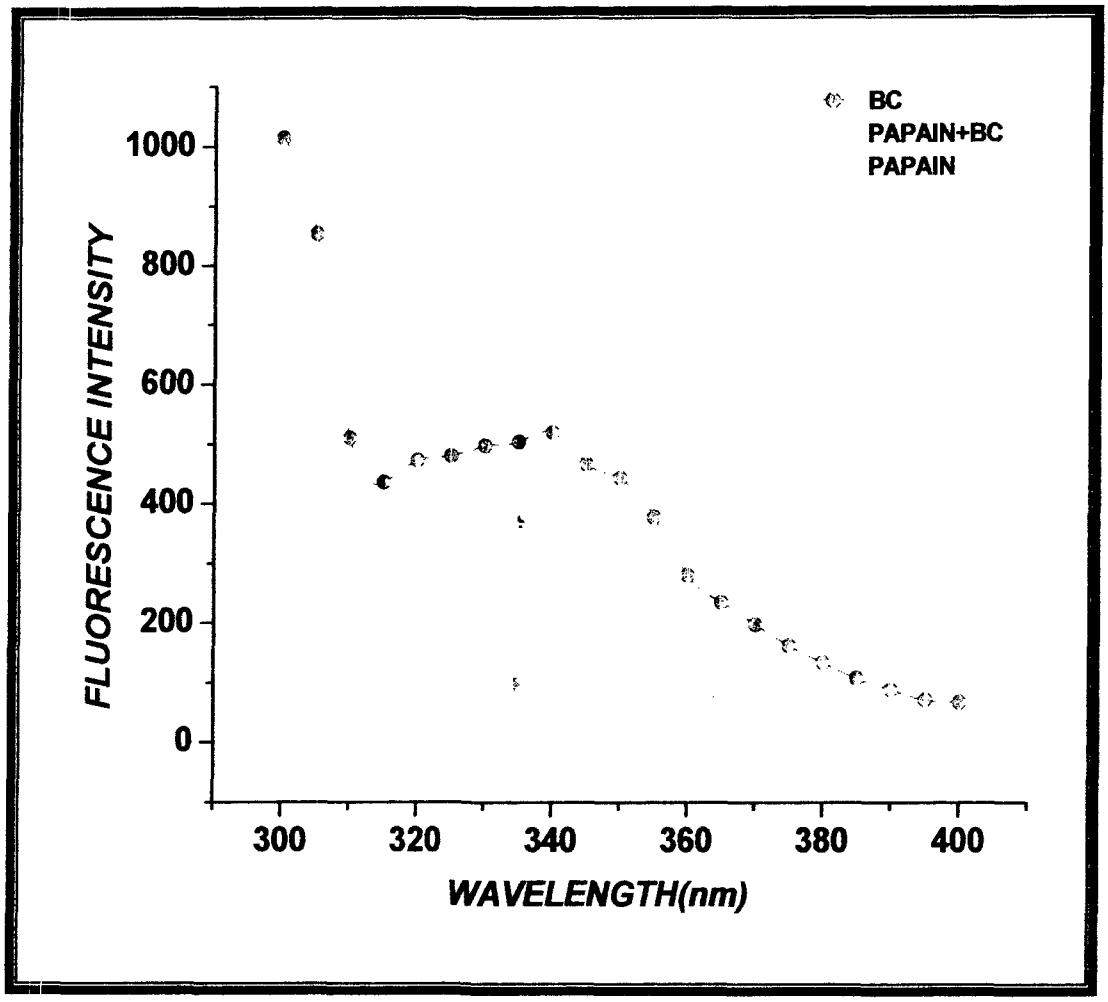
The inhibitory activity of BC towards thiol proteinases like papain , ficn, bromelain and serine proteinases, trypsin and chymotrypsin was examined using casein as substrate by the method of kunitz (1947). The cystatin inhibited thiol proteinases in the order papain> ficin > bromelain while it did not show significant inhibition of serine proteinases trypsin and chymotrypsin ***[Fig-29]***

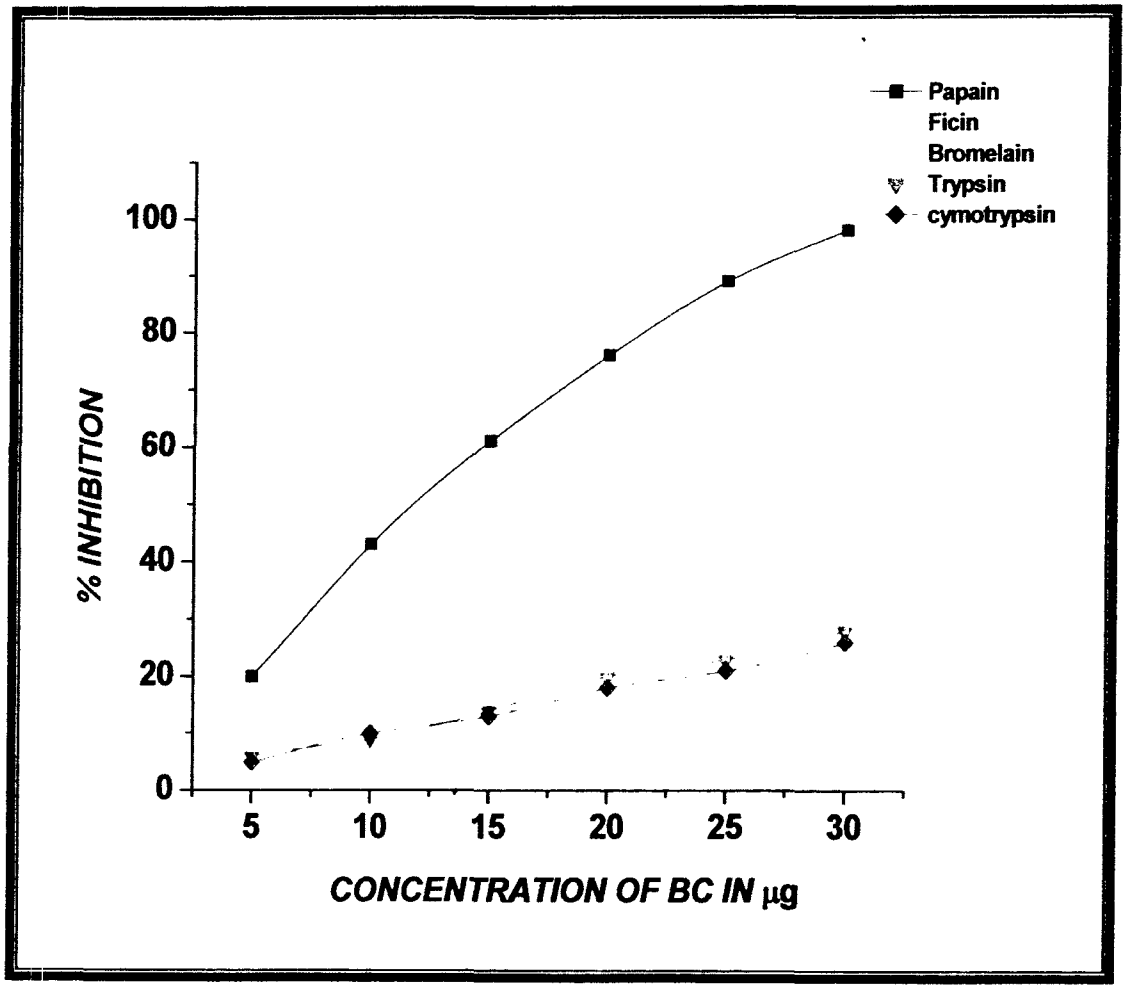
3.1.7 KINETICS OF INHIBITION

Stiochiometry of inhibition

Purified cystatin was found to be highly specific for thiol proteases with no specificity for serine proteinase.The inhibition of proteinase was also studied by varying the molar concentration of proteinase at a fixed molar concentration of cystatin .The remaining activity of proteinase showed that as the concentration of proteinase was increased from 00.1-0.06μM it is progressively inhibited by the cystatin.concentration of BC (0.06 μM) results showed the stiochiometric ratio of 1:1 showing that one molecule of BC inhibits 1 molecule of active papain.


0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
ABSORBANCE
180
200
220
240
260
280
300
320
340
360
380
WAVELENGTH(nm)


BC
PAPAIN+BC
PAPAIN
FLUORESCENCE INTENSITY
1000
800
600
400
200
0
300
320
340
360
380
400
WAVELENGTH(nm)


Papain
Ficin
Bromelain
Trypsin
cymotrypsin
% INHIBITION
0
20
40
60
80
100
5
10
15
20
25
30
CONCENTRATION OF BC IN µg

Ki determination

Ki values have been determined after lowering the papain and inhibitor concentration, which favour the dissociation of the complex. Ki values were determined using the steady state equation derived by *krupka and laidler (1959)*

$$\frac{I_0}{1-\frac{V_i}{V_0}} = k_i\left[1+\frac{[S]_0}{k_m}\right]\frac{V_i}{V_0}+E$$

Where I_0, E_0 and S_0 are the initial concentrations of inhibitor, enzyme and Substrate, respectively

V_0 is the velocity without inhibitor

V_i is the velocity in the presence of inhibitor

The plot of $\frac{I_0}{1-\frac{V_i}{V_0}}$ against $\frac{V_0}{V_i}$ is a straight line

The slop of which gives

$$k_i(aap) = k_i\left[1+\frac{[S]_0}{K_m}\right]$$

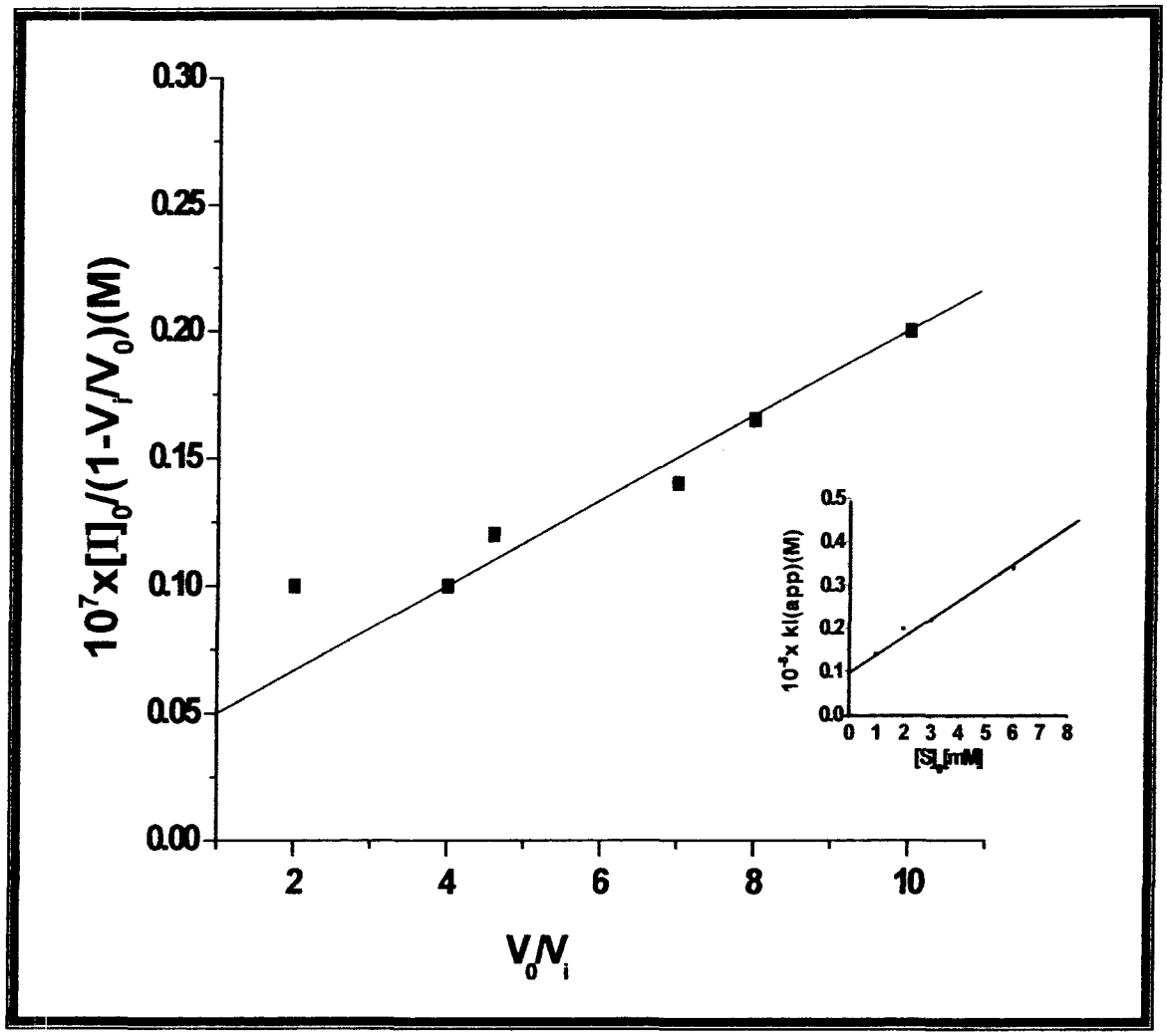
True Ki was obtained from a replot of Ki (aap) against $[S]_0$

Ki (aap) increase as a function of the substrate concentration. The true Ki value may be obtained from replot of Ki (aap) against [S].

The Ki values obtained from the plot were 1 nm for papain, ficin 1.85nm and for bromelain 2.25nm ***(Fig: 30-32)*** hence papain showing highest specificity for BC from other proteinase results summerised in ***Table-5***

IC_{50} values

IC_{50} is the concentration of the inhibitor at which 50% of the enzyme is inhibited. The IC_{50} value obtained with various thiol proteinases have been summarized in the ***Table -5*** the value obtained with various thiol proteinases papain ficin and bromelain showed that papain (0.09 μM) has maximum affinity for BC while ficin (0.12 μM) and bromelain (0.15 μM) showed the less affinity towards BC


0.30
0.25
0.20
0.15
0.10
0.05
0.00
$10^7 x [I]_0/(1-V_i/V_0)(M)$
2
4
6
8
10
V_0/V_i
0.5
0.4
0.3
0.2
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10^{-5} x kI(app)(M)
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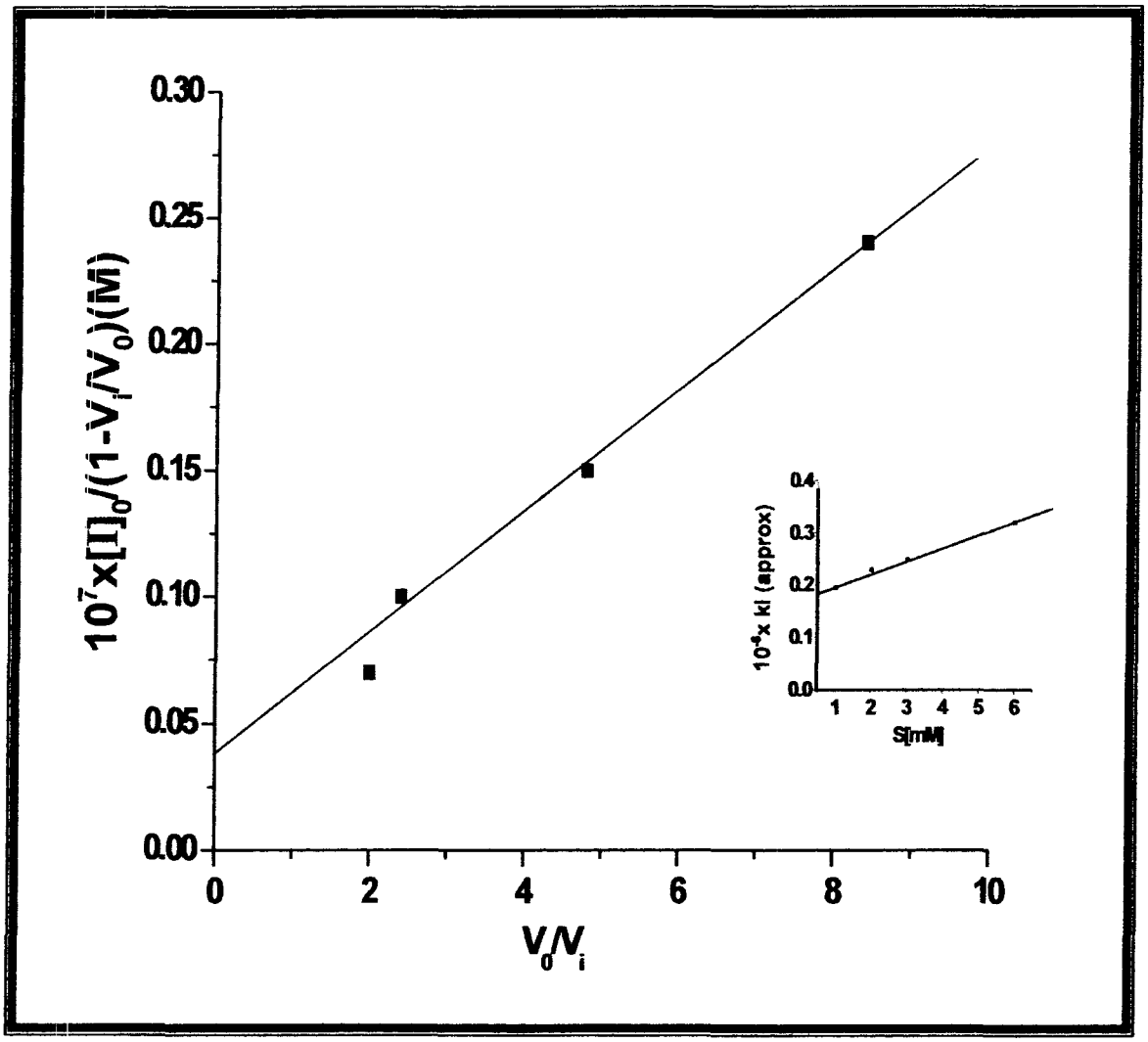
$[S]_0$[mM]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$10^7 x[I]_0/(1-V_i/V_0)(M)$
V_0/V_i
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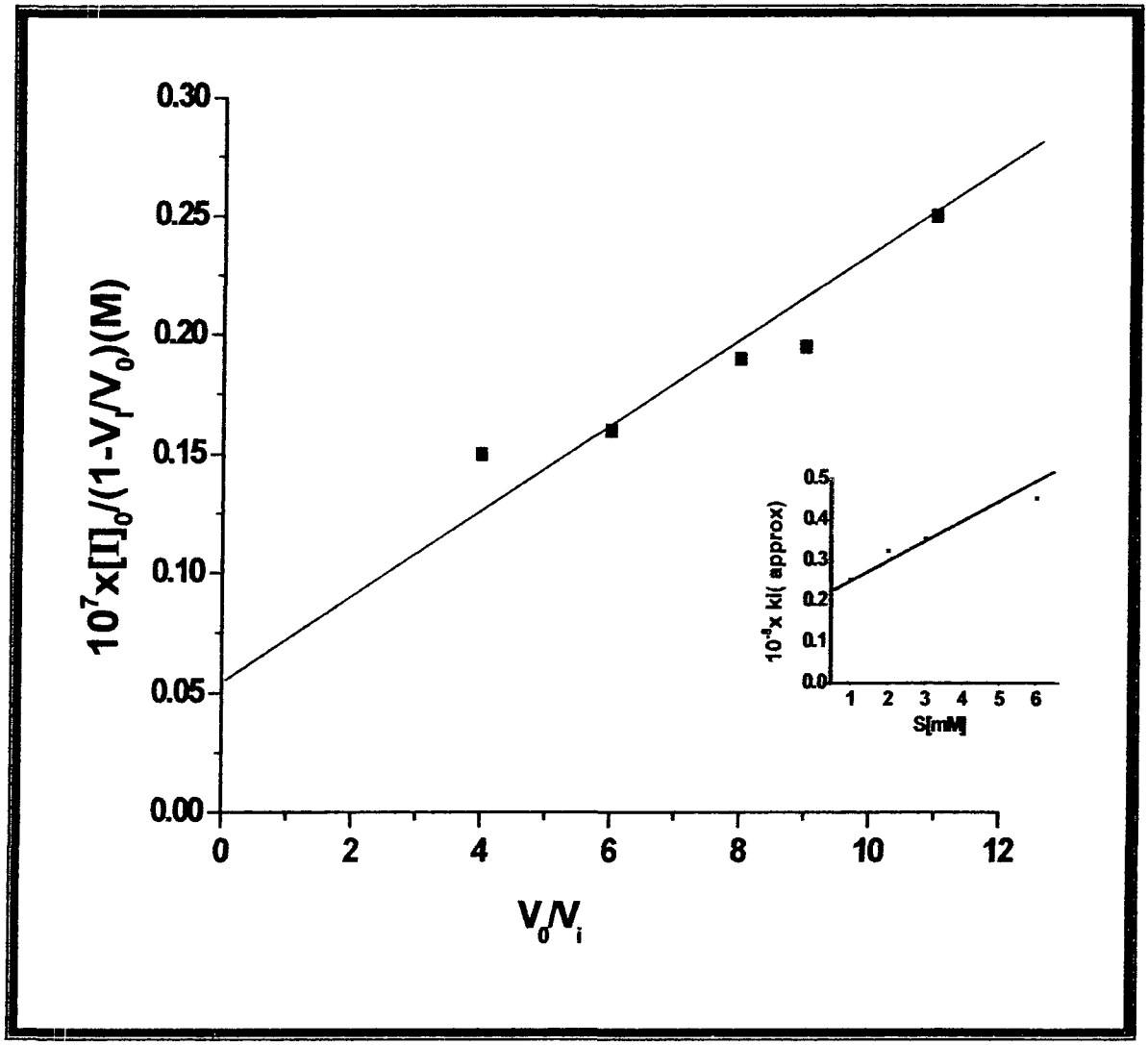
1 2 3 4 5 6
S[mM]
10^4x ki (approx)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$10^7 x[\Pi]_0/(1-V_i/V_0)(M)$
V_0/V_i
0.5
0.4
0.3
0.2
0.1
0.0
1
2
3
4
5
6
10^{-4}x ki(approx)
S[mM]

TABLE-5 KINETICS CONSTANT FOR INTERACTION OF BC WITH DIFFERENT PROTEINASES

Proteinases	Ki values	IC_{50}
Papain	1.0 nM	0.09 µM
Ficin	1.85 nM	0.12 µM
Bromelain	2.25 nM	0.15 µM

Discussion

Chapter- 1

DISCUSSION

Endogenous thiol proteinase inhibitors cystatins constitute the powerful regulatory system for overall cellular activity of cysteine proteinases *[Sotiropoulou et al.(1997)]*.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.

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 *[Delaisse et al. (1991)]*, renal failure , cardiovascular and cancer diseases *[Kabanda et a.l (1995) , Servais (2008)]*, resulting due to imbalance of endogenous cysteine proteinases and their inhibitors.

Extensive work has been carried out for the purification and characterization of these inhibitors using various isolation procedures. Cystatins have been purified and characterized from various sources including bovine muscle *[Bige et al. (1985)]*, goat kidney *[Zehra et al. (2005)]*, human spleen *[(Jarvinen M (1982)]* human liver *[Green et al. (1984)]*, amyloid fibrils *[Cohen et al. (1983)]*, human placenta *[Rashid et al. (2006)]* and sheep plasma *[Baba et al. (2005)]*, The CPIs have been isolated in multiple forms from several organs. However, brain being the key organ of the mammalian system was chosen for the present study as lacunas exist in the purification and physic-chemical characterization of cystatin from buffalo brain. Thus, it was envisaged that a thorough and systematic study of CPI from this source will be helpful in understanding in-depth about the cystatins of mammalian system and to compare its properties with other known mammalian cystatins.

The progress of a typical purification is summarized in ***[Table-4]***. The procedure adopted for purification of cystatin from buffalo brain is slightly modified from that reported by *Bige (1985)*.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 .The precipitated protein obtained after 40-60 % ammonium sulphate fractionation was dialyzed and loaded on sephadex G-75 column. A major protein peak with significant papain inhibitory activity was obtained ***[Fig-18]***

The fractions corresponding to the peak-I with significant inhibitory activity were pooled and lyophilized for further analyses. The procedure is efficient and simple with a fold purification of 384.72 and percent yield of 64.13. Purification of CPIs from other sources has been reported using a combination of several techniques like affinity chromatography, chromatofocussing, gel filtration and ion exchange chromatography *[Anastasi et al. (1983) , Evans and Barrett (1987) , Rashid et al. (2006) Baba et al. (2005)].* 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)].* ***[Table -1]***

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-20]***. The molecular weigth obtained by SDS PAGE as shown in ***Fig-21*** .The relative mobility of each marker protein was plotted against their log molecular weight .The molecular weight obtained by SDS PAGE for two the subunits were 31.62 kDa and 12.58 kDa which is equivalent to was 44.2 kDa.It was further determined by gel filteration under native condition by passing marker proteins through Sephadex G-75 column. The V_e/V_0 ratio for each protein obtained from gel filteration was plotted against Log M of marker proteins ***[Fig-22]***. Molecular weight obtained from gel filteration chromatograghy was found to be 43 .6 kDa.Cystatins from tissues are usually small inhibitors having Mr in the range of 11 to 25 KDa *[Rashid et al. (2006), Green et al. (1984) , Zabari et al. (1993) , Turk et al. (1995)].* Now contrasted with these reports on low molecular mass. *Ylonen et al. [1999]* isolated and purified high molecular mass thiol proteinase inhibitors *(of 43 kDa and ~52 kDa)* from the skin of Atlantic salmon. Cystatin isolated from goat kidney was reported to have a molecular mass of 67 kDa *[Zehra et al.*

(2005)]. Recently, the work from our lab, has reported the purification of a high molecular mass cystatins from goat lung *[Khan and Bano (2009)]* and a cystatin of 70.8 kDa isolated from goat brain *[Sumbul and Bano (2006)].* Generally type I and type II cystatins isolated from tissues lack carbohydrate content. However,the presence of carbohydrate content has been reported in some type II cystatins like rat cystatin C isolated from urine is slightly glycosylated *[Esnard et al. (1990)]* and cystatin isolated from goat kidney has also been found to be slightly glycosylated *[Zehra et al. (2005)]. Ni et al. (1997)* found carbohydrate attachments in cystatin E. Glycosylation has also been demonstrated in cystatin F from the immune cells and in cystatin M isolated from primary tumor cell lines *[Ni et al. (1998) , Sotiropoulou et al. (1997)].* Presence of carbohydrate chains is a distinguishing feature of type III cystatins, the kininogens *[Salvesen et al. (1985) , Gounaris et al. (1984) , Baba et al. (2005)].*Analysis of all the results showed BC possessed high molecular weight negligible amount of CHO content and no sulphydryl groups. Hydrodynamic parameter for BC as determined from gel filtration behavior suggested a stokes radius 27.3 A^0 ***[Fig-23]*** and diffusion coefficient of 8.1 $\times 10^{-7}$ $cm^2/$ sec. The values of stokes radius and S_{max}/S ratio can be used to predict the shape of the protein molecule *[Schurmann et al (2001)].* Globular proteins typically have S_{max}/S ratio of 1.2-1.3 (for example catalase and serum albumin have S_{max}/S of 1.20 and 1.29), and the ratio increases to 1.6-2.0 or more for elongated proteins *[Erickson (1982)].* For BC S_{max}/S ratio was calculated to be 1.2. The values of stokes radius and S_{max}/S ratio for BC are in close agreement with those of ovalbumin, suggesting that BC is a globular in shape.

Effect of pH on BC activity was examined at various pH values ***[Fig-24]*** results show that the purified inhibitor is stable in the pH range 3- 10. Stability of BC was also investigated as a function of temperature in the range of 30-90^0C in 0.05M sodium phosphate buffer of pH- 7.5 by monitoring its papain inhibitory activity ***[Fig-25]*** .BC when incubated at constant temperature of 90 ^{0}C for varying time interval where it retained almost 65% of its activity upto 20 min and had 45% activity till 80 minutes ***[Fig-26]*** .These properties are in accordance with other reported cystatins like that from bovine Muscle ,

chicken cystatins , stefin A and stefin B. *[Barrett et al. (1984), Machleidt (1986))]* it is also in accordance with high molecular weight cystatins reported from goat kidney and human placenta *[Zehra (2005) , Sumbul (2006) , Rashid et al. (2006)]*. BC showed maximum emission at 340nm when excited at 280 nm. However on complexation with papain the fluorescence of BC was enhanced and maxima shifted to 350nm ***[Fig-28]***. These changes suggest that the environment of tryptophan residue is predominantly perturbed either by exposure of aromatic residue to the solvent or it may originate from local interactions affecting chromophoric groups of the two proteins. Difference spectrum of Cystatin papain complex showed trench at 245 nm and positive peak at 285 nm ***[Fig-27]*** These spectroscopic changes indicate that the environment of several aromatic amino acid residues in protein have been altered upon interaction of the inhibitor with papain, such changes are consistent with earlier reported results for interaction of low and high molecular weight Kininogen's *[Baba et al. (2005)]*, cystatin from human placental *[Rashid (2006)]* and rat cystatin with papain *[Takeda (1988)]* Studies were also done for inhibition of various cystiene proteinases by cystatin. BC was found to be potent inhibitors of thiol proteinases. ***[Fig-29]*** It is evident by their Ki values. It was readily apparent that the degree of inhibition varied with absolute concentration of the inhibitor as expected for tight binding reversible inhibition. The brain cystatins exhibited a Ki value for papain as 1nM ***[Fig-30]***, for ficin it was 1.85 nM ***[Fig-31]*** and for bromelain the value was found to be 2.25 nM respectively ***[Fig-32]***, as shown in ***Table-5***. A comparison of Ki (app) values indicates that this cystatin has highest affinity for papain, then ficin and least with bromelain. It has been reported that cystatin isolated from other sources generally do not inhibit bromelain but in our present study bromelain is found to be inhibited by cystatin as reported earlier for goat kidney *[Zehra et al. (2005)]*, human placental cystatin *[Warwas and Sawicki (1985) Rashid et al. (2006)]* and human spleen CPI *[Jarvinen and Rinnie (1982)]*.The values are in good comparison with other proteinase inhibitors. The inhibition constant (Ki) for chicken cystatin and is reported to be 5×10^{-12} M for both with papain. Ki values of nanomolar range has been given for cathepsin B, H and L with cystatin A, *Barrett et al. (1984)*, cystatin C, *Machleidt et al. (1986)*, chicken

cystatin and cystatin D, *Balbin et al. (1994).* The Ki values of various cystatins have been summarized in ***Table-5.***

The increasing value of Ki for BC with an increase in the substrate concentration suggests the inhibition to be competitive. This finding is supported by the apparent results of *Nicklin and Barrett (1984)* for the inhibition of human cathepsin B by chicken cystatin. Ki (app) values for chicken cystatin with papain and cathepsin B was reported as 0.05 and 0.39nM and 1.85 and 3.68nM respectively.

The results of the chapter gave comprehensive information about the purity molecular weight, shape, specificity and kinetics of inhibition of BC with papain, ficin and bromelain. The overall comparison showed that BC has compact in shape and inhibited papain more effectively as compared to other two proteinases.

The binding stoichiometry of BC with papain is found to be 1:1 Accurate K_i values were determined by working at lower enzymes concentrations and using equation derived by *[Krupa and Laidler (1959), Henderson (1972)].* IC_{50} is the concentration of inhibitor at which 50 % of the enzyme is inhibited IC_{50} value of BC for the three proteinases papain , ficin and bromelain were 0.09 µM , 0.12 µM and 0.15 µM respectively .Low IC_{50} value for papain suggests a greater affinity for the inhibitor towards the enzyme. Hence, the lowest value for papain further justifies the higher affinity of the purified cystatins for papain. These value indicate that the affinity of BC for these proteinases are in order of papain > ficin> bromelain. *Katunuma and Kominami (1985)* isolated Cystatin from rat liver have found the IC_{50} value 0.16µg for papain and 0.046µg for ficin and IC_{50} value of 4.2µg was obtained for cathepsin B and 0.14µg for cathepsin H.

Owing to the absence of carbohydrate content, disulphide linkages and all other results obtained for BC, it can be placed in type I cystatins, the Stefins class of cystatin superfamily. BC however resembles cystatin purified from goat kidney *[Zehra (2005)]* and other cystatins with respect to high molecular weigth hence, it is more appropriate to conclude that BC is a variant of class I and class II of cystatin superfamily. Furthermore, it can also be concluded that all tissue cystatin are not same because of the specific

physiological function they perform in the mammalian body. Owing to the importance and myriad of crucial functions cystatins perform in the mammalian body, the study is of great significance as it sheds light on the physicochemical properties of cystatins of one of the most important mammalian organ, the brain.

Results

Chapter-2

Effect of Free radical on Brain Cystatin

3.2 RESULT

3.2.1 NITRIC OXIDE (NO) INDUCED FUNTIONAL AND STRUCTURAL MODIFICATION OF BRAIN CYSTATIN

Functional inactivation of Cystatin by nitric oxide [NO]

When Cystatin was incubated with NO, generated by the reaction of SNP at varying concentrations there was 54% loss in antiproleolytic activity at 50μM of SNP for 30 min of incubation. This loss in antiproleolytic activity increased to 84% at 2mM concentration.However increase in length of exposure upto 2 hrs at 50 μM concentration did not show any significant decrease in antiproteolytic activity, 59% loss was noted after after 1 hrs of incubation which increase to 66% after 2½ hrs of incubation ***[Table-6 and 7].***

Structural modification of BC by nitric oxide [NO]

Nitric oxide when generated from 50μM of SNP resulted in the major loss of tryptophan fluorescence (38%), it was observed that when BC incubated with NO generating SNP, as the concentration increases fluorescence intensity decreases upto 97% at 2mM ***[Fig-33]*** this concentration when varied with time causes 48% loss in tryptophan fluorescence after 1 hrs of incubation, decrease in tryptophan fluorescence increased upto 59% at 2:30 hrs of incubation ***[Fig-34].***

Prevention of structural and functional modification of BC by curcumin

Curcumin (0-50 μM) was used to validate its scavenging capacity for NO and protection of Cystatin against the NO damage. The results indicated that when Cystatin was incubated with SNP, in the prescence of curcumin it was able to deplete SNP induced damage to BC, this damage is maximally reduced by 50μM of curcumin , the loss in tryptophan fluorescence when incubated with SNP for 30 min was 38%. This loss decrease to 27% when incubated along with 30μM of curcumin when this concentration increases

TABLE-6 EFFECT OF VARYING CONCENTRATION OF SNP ON ANTIPROTEOLYTIC ACTIVITY OF CYSTATIN

Cystatin (1μM) was incubated for 30 min with different SNP concentrations taking native Cystatin inhibitory activity as 100%. was measured by caseinolytic method of kunitz (1947)

S.NO	Different Concentrations of SNP incubated along with BC(1μM)	% Remaining *Inhibitory* Activity
1	BC alone	100
2	BC +0.05 Mm SNP	46 ± 0.59
3	BC + 0.1 mM SNP	40 ± 0.91
4	BC + 0.2 mM SNP	32 ± 0.92
5	BC + 0.5 mM SNP	25 ± 0.77
6	BC + 1 mM SNP	19 ± 0.91
7	BC + 2 mM SNP	16 ± 0.51

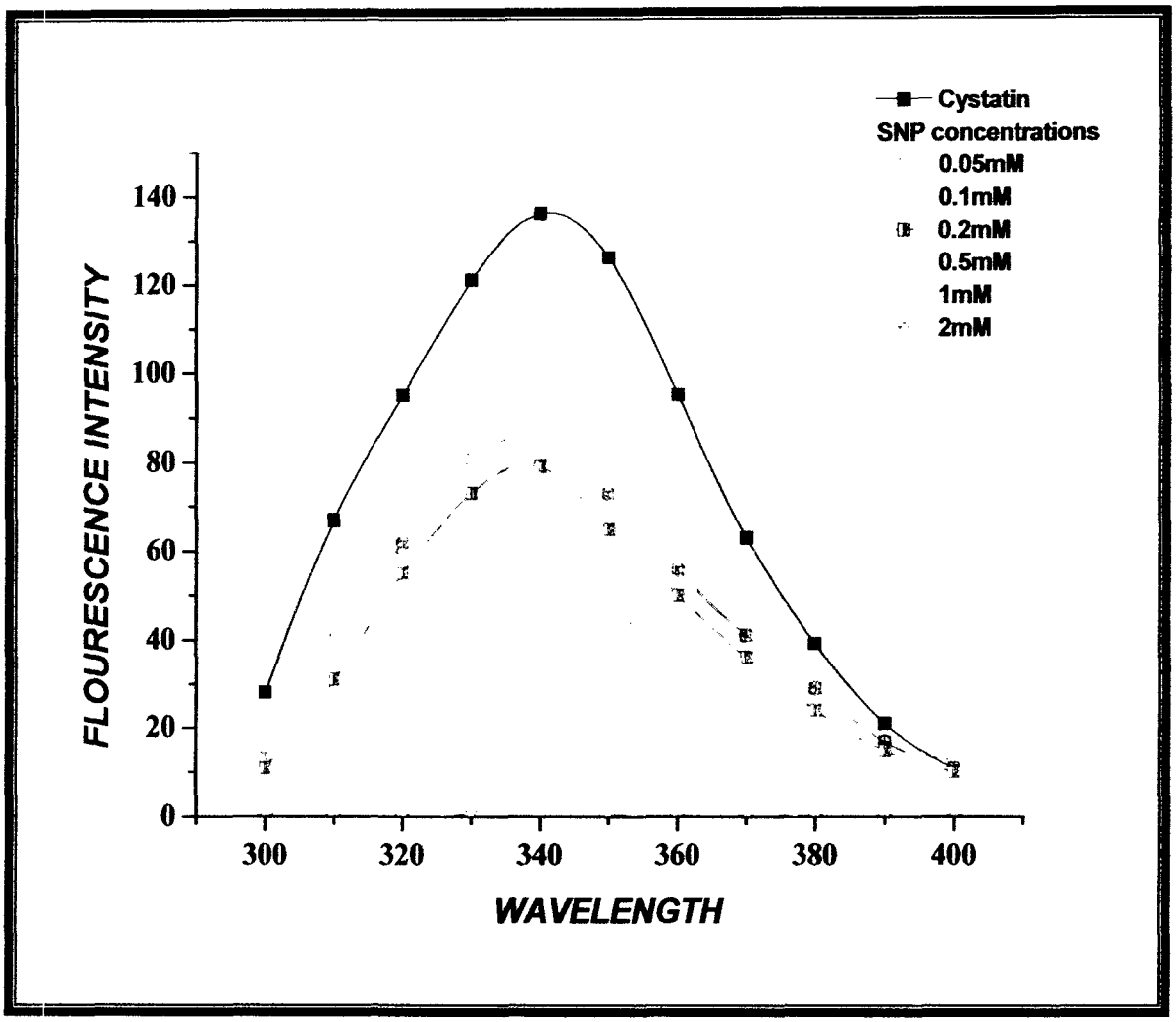
All data are expressed as mean ± S.E for four different sets of experiments statical significance was conducted employing ONE WAY ANOVA .a probability level of 0.05 was selected showing results are significant.

TABLE-7 EFFECT OF VARYING TIME INTERVALS ON ANTIPROTEOLYTIC ACTIVITY OF SNP TREATED CYSTATIN.

BC was incubated with SNP (0.05Mm) for varying time interval taking native inhibitory activity as 100%. Inhibitory activity was measured by caseinolytic method of kunitz (1947)

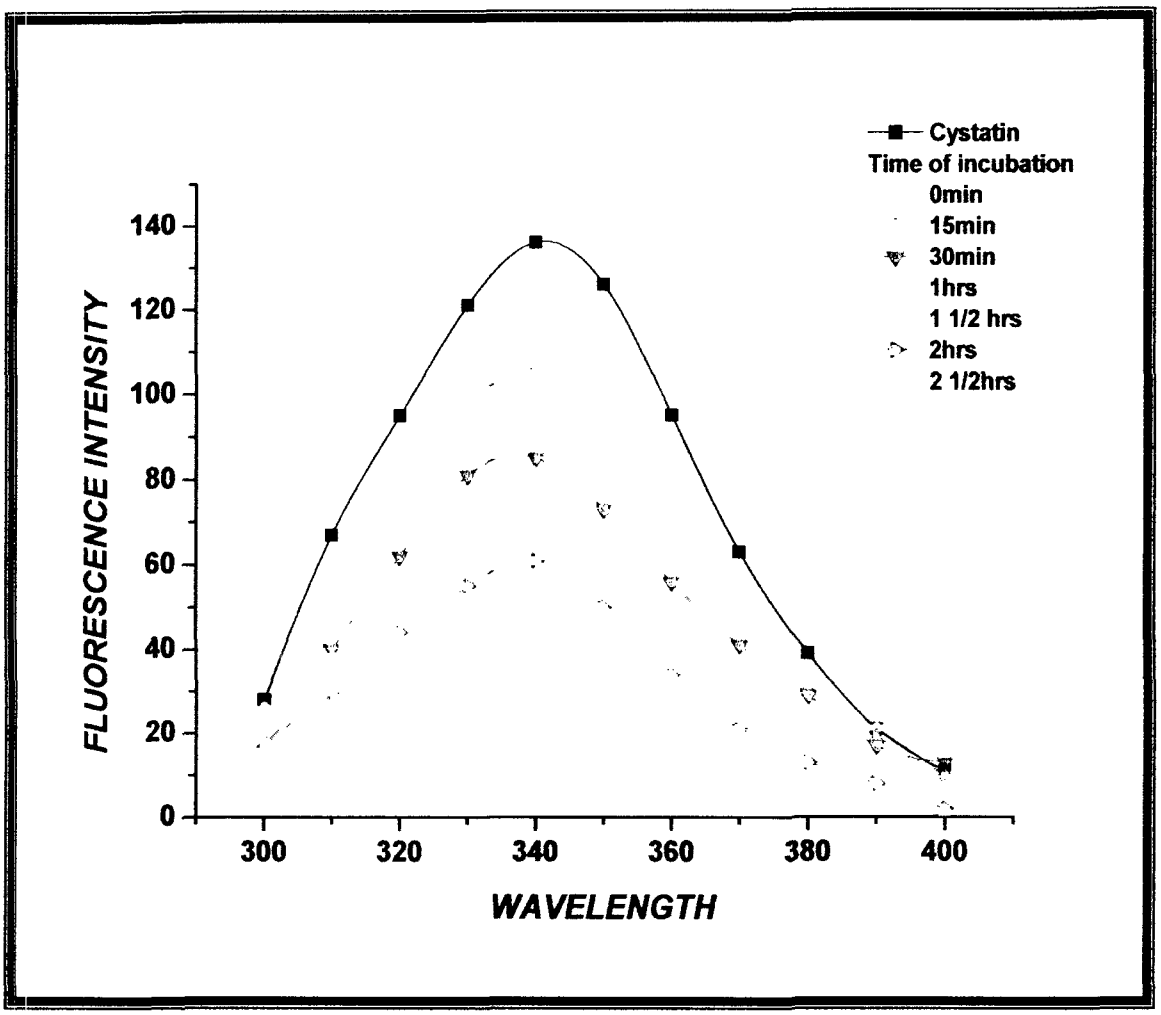
S.NO	Time of Incubation of SNP (0.05mM)with BC(1µM)	% remaining Inhibitory Activity
1	BC alone	100
2	0 min	51 ± 0.93
3	15 min	49 ± 0.87
4	30 min	46 ± 0.59
5	60 min	41 ± 0.64
6	90 min	39 ± 0.62
7	120 min	35 ± 0.91
8	150 min	34 ± 0.71

All data are expressed as mean ± S.E for four different sets of experiments statical significance was conducted employing ONE WAY ANOVA .a probability level of 0.05 was selected showing results are significant.



Percent decrease in tryptophan fluorescence in the presence of SNP

Cystatin alone fluorescence	SNP+BC (0.05mM)	SNP (0.1 mM)	SNP (0.2 mM)	SNP (0.5 mM)	SNP (1 mM)	SNP (2 mM)
100	38	40	42	64	81	97



Percent decrease in tryptophan fluorescence in the presence of SNP with different time of incubation BC concentration was 1 μM.

Cystatin alone fluorescence	0 min	15 min	30 min	1 hrs	1 ½ hrs	2 hrs	2 ½ hrs
100	23	33	38	48	51	55	59

further, loss in tryptophan fluorescence decreased to 8% at 50μM of curcumin concentration. ***[Fig-35]***. The similar results are obtained when caseinolytic activity was assessed, showing maximum protection at 50μM of curcumin ***[Table-8].***

Preventive effect of Querecitin on structural and functional inactivation of BC

With varying concentration of querecitin (50-250 μM) there is prevention of degradation of BC by NO. The functional damage is reduced to 20% at 150μM of querecitin while this damage was further condensed as the concentration of querecitin increase to 250μM , showing only 11% decrease in antiproteolytic activity. ***[Table -9]***. When SNP was incubated with Cystatin the decline in tryptophan fluorescence was 38% , this loss in tryptophan fluorescence reduced to 25% at 150μM of quercetin. ***[Fig-36]*** which was further reduced to 13% at 250μM of quercetin. Showing maximum protection by 250 μM of quercetin , the measurement of antiproteolytic activity and fluorescence along with quercetin shows protective effect by reducing the damage caused by SNP at 50μM

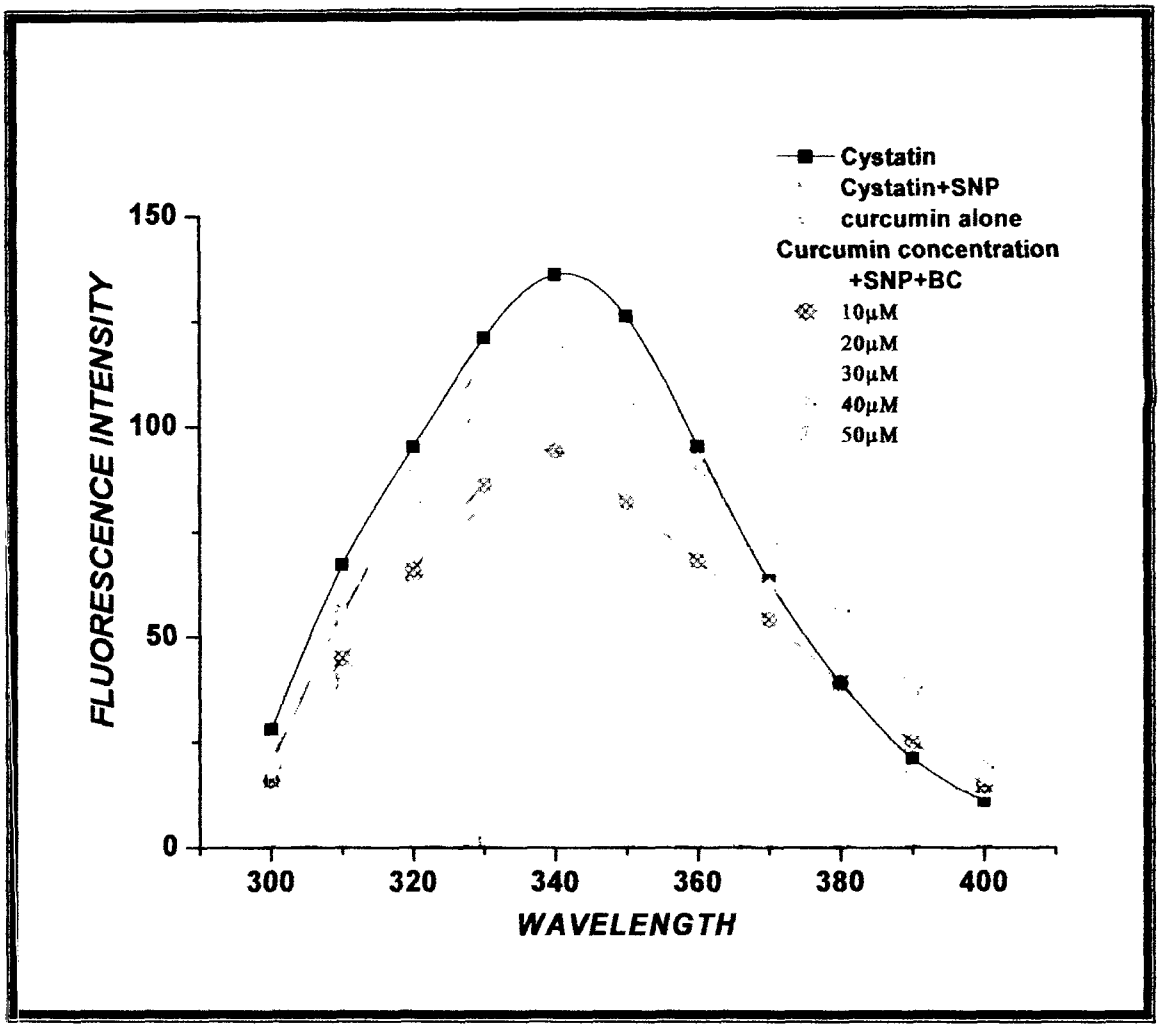
3.2.2 HYPOCHLOROUS ACID (HOCl) INDUCED STRUCTURAL AND FUNCTIONAL MODIFICATION OF CYSTATIN

Structural modication of Cystatin by hypochlorous acid (HOCl)

When 1μM of BC was incubated with increasing concentration of HOCl. The samples were analyzed for impact on structural integrity of BC by the technique of fluorescence ***[Fig-37]*** Significant loss in native protein was observed (73%) when Cystatin was incubated with 1μM of HOCl. very low fluorescence intensity was observed beyond 5μM of HOCl , while emission maximum remained unaffected at all concentration of HOCl.

Functional modification of BC in the prescence of HOCl

The exposure of 1μM of Cystatin to varying concentration of HOCl resulted in remarkable loss in antiproleolytic activity as determined by the method of *Kunitz (1947)* at concentrations as low as 1uM of HOCl ***[Table-10]***



Percent decrease in tryptophan fluorescence in the presence of curcumin

Cystatin alone fluorescence	BC+SNP	10 μM	20 μM	30 μM	40 μM	50 μM
100	38	31	26	27	13	8

TABLE-8 EFFECT OF VARYING CONCENTRATION OF CURCUMIN ON ANTIPROTEOLYTIC ACTIVITY OF NITRIC OXIDE TREATED BRAIN CYSTATIN.

Cystatin (1μM) was incubated with SNP (0.05Mm) in the presence and absence of curcumin taking native Inhibitory activity as 100%. It was measured by caseinolytic method of kunitz (1947).

S.NO	Different Concentrations of Curcumin incubated with 1 μM of BC along with SNP	% Remaining in Inhibitory Activity
1	BC (native with out SNP)	100
2	SNP + Brain Cystatin	46 ± 0.59
3	BC+ SNP+10 μM Curcumin	72 ± 0.93
4	BC+ SNP+20 μM Curcumin	80 ± 0.77
5	BC+ SNP+30 μM Curcumin	85 ± 0.90
6	BC+ SNP+40 μM Curcumin	88 ± 0.77
7	BC+ SNP+50 μM Curcumin	90 ± 0.63

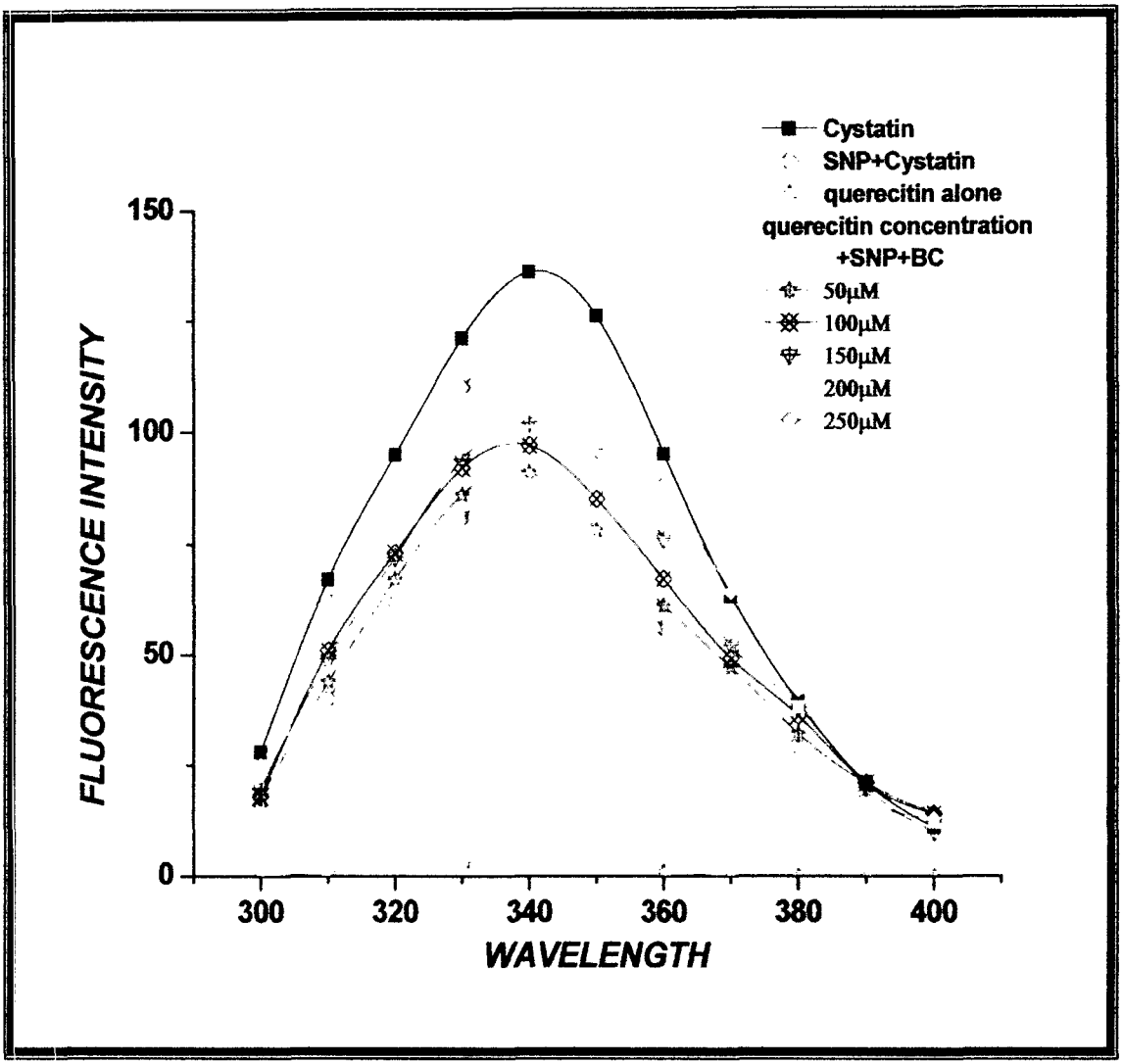
All data are expressed as mean ± S.E for three different sets of experiments statical significance was conducted employing ONE WAY ANOVA .a probability level of 0.05 was selected showing results are significant.

TABLE-9 EFFECT OF DIFFERENT CONCENTRATIONS OF QUERECITIN ON ANTIPROTEOLYTIC ACTIVITY OF NO TREATED CYSTATIN.

BC (1μM) was incubated with SNP (0.05Mm) for 30 min in the presence and absence of querecitin taking native as 100%. Inhibitory activity was measured by caseinolytic method of *Kunitz (1947)*

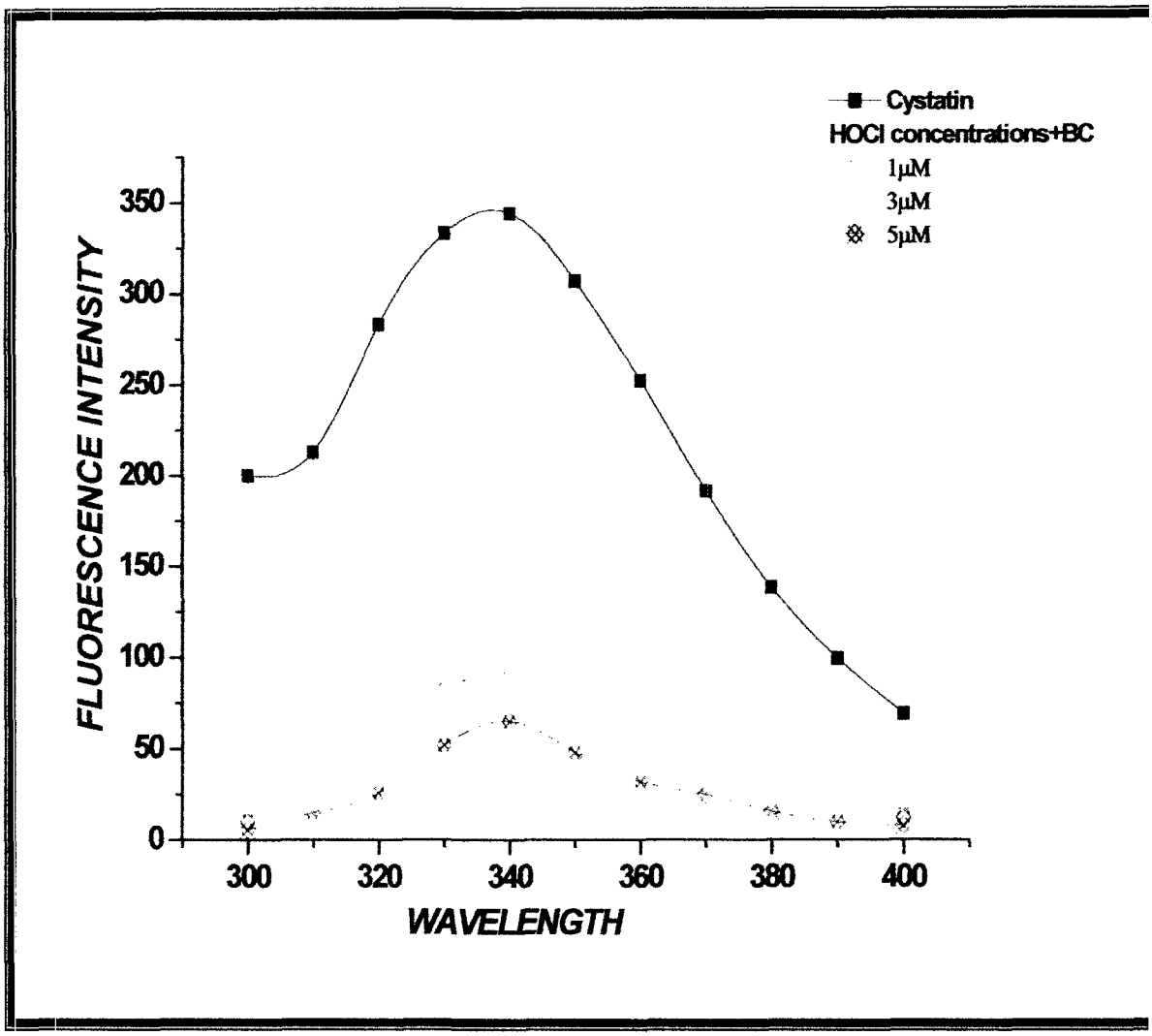
S.NO	BC with different concentration of Querecitin along SNP	% Remaining Inhibitory Activity
1	BC alone	100
2	SNP+Cystatin	46 ± 0.59
3	BC+ SNP +50 μM Querecitin	48 ±0.93
4	BC+ SNP +100 μM Querecitin	77 ± 0.51
5	BC+ SNP +150 μM Querecitin	80 ± 0.77
6	BC+ SNP +200 μM Querecitin	83 ± 0.75
7	BC+ SNP +250 μM Querecitin	89 ± 0.92

All data are expressed as mean ± S.E for three different sets of experiments statical significance was conducted employing ONE WAY ANOVA .a probability level of 0.05 was selected showing results are significant



Percent Decrease in Tryptophan Fluorescence in the presence of Querecitin

Cystatin alone fluorescence	Cystatin+SNP	50 µM	100 µM	150 µM	200 µM	250 µM
100	38	33	29	25	19	13



Percent Decrease in Tryptophan Fluorescence in the presence of HOCl

Cystatin alone fluorescence	HOCl (1μM)	HOCl (3μM)	HOCl (5μM)
100	73	78	82

TABLE- 10 EFFECT OF VARYING CONCENTRATIONS OF HOCL ON ANTIPROTEOLYTIC ACTIVITY OF CYSTATIN.

Cystatin (1μM) was incubated with different concentrations of HOCl. For 30 min Inhibitory activity was measured by the caseinolytic method of *kunitz (1947).*

S.NO	Different Concentrations of HOCl incubated along with BC(1μM)	% Remaining Inhibitory Activity
1	BC alone	100
2	BC +1 μM HOCl	68 ±0.88
3	BC +3 μM HOCl	50 ±0.71
4	BC +5 μM HOCl	18 ±0.77

All data are expressed as mean ± S.E for three different sets of experiments statical significance was conducted employing ONE WAY ANOVA .a probability level of 0.05 was selected showing results are significant

which lead to 32% loss in activity of BC. The inhibitor was only 18% active at 5μM of HOCl showing 82% loss in activity. Beyond this HOCl concentration no papain activity was detected.

Protective effect of different scavengers on the damage of BC by HOCl

Flavonoids have been exclusively studied for their anti oxidant properties against free radicals damage ***[Fig-38]*** however their effect on hypochlorous acid mediated damage is less known.

Thus present work was also aimed for exploring the potential of these compounds curcumin, quercetin, Glucose, sodium benzoate and Mannitol for their effect on BC diminished the extent of loss of antiproteolytic activity and fluorescence intensity, causing the inhibitory potential as well as fluorescence of cystatin close to native.

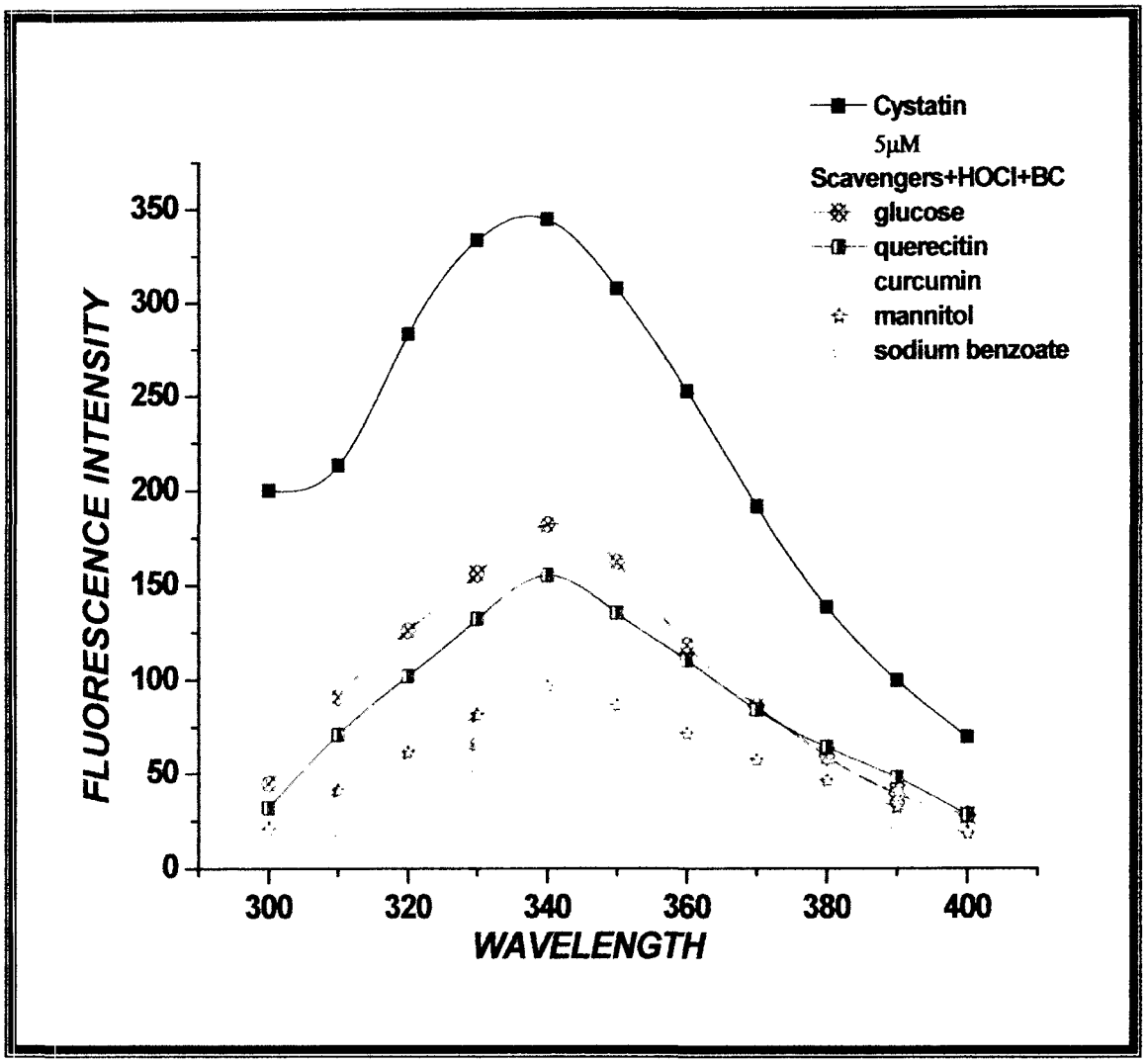
Glucose was found to be most effective as it caused only 47% decrease in fluorescence of BC in the presence of HOCl as compared to 55-77 % quecching in the prescence of other scavengers These results are supported by the inhibitory activity determinations in the presence of scavengers listed in ***Table-11*** ,which shows these coumpunds are effective for protection in the order of glucose > querecitin > curcumin > mannitol > sodium benzoate

3.2.3 HYDROGEN PEROXIDE INDUCED FUNTIONAL AND STRUCTURAL MODIFICATION OF CYSTATIN

Hydroperoxidase such as H_2O_2 and lipid hydroperoxidase have been implicated as mediator of cellular injuries in a variety of clinical conditions including cancer etc *[Pryor et al 2006].*

Thus deleterious effects of H_2O_2 and protective effect of polyphenols and other compounds were assessed on BC.

To directly quantitate the effects of exposure of oxidant [H_2O_2] on BC, the antiproteolytic activity of BC was measured in the presence of the component by the method of Kunitz (1947) using casein as substrate as described in the methods section.



Percent Decrease in Tryptophan Fluorescence in the presence of Scavengers

Cystatin alone fluorescence	BC+HOCl (5μM)	Glucose	Querecitin	Curcumin	Mannitol	S.Benzoate
100	73	47	55	65	72	77

TABLE-11 EFFECT OF DIFFERENT SCAVENGERS ON ANTIPROTEOLYTIC ACTIVITY OF HOCl TREATED CYSTATIN.

BC (1Mμ) was incubated with different scavengers (25mM) in the presence of HOCl (5μM) for 30 min .Inhibitory activity was measured by caseinolytic method of kunitz (1947).

S.NO	BC (1μM)incubated with HOCl in the prescence of each scavengers 25mM	% Remaining Inhibitory Activity
1	BC alone	100
2	BC+HOCl (5 μM)	18 ± 0.77
3	BC+HOCl (5 μM)+Curcumin	68 ± 0.93
4	BC+HOCl (5 μM)+Querecitin	66 ± 0.60
5	BC+HOCl (5 μM)+Sodium benzoate	55 ± 0.90
6	BC+HOCl (5 μM)+Mannitol	35 ± 0.75
7	BC+HOCl (5 μM)+Glucose	47 ± 0.51

All data are expressed as mean ± S.E for four different sets of experiments statical significance was conducted employing ONE WAY ANOVA .a probability level of 0.05 was selected showing results are significant

Functional inactivation of BC by H_2O_2

H_2O_2 caused only modest inactivation of cystatin. Even at high concentration of H_2O_2 (250mM) 40% of antiproleolytic activity of BC was retained ***[Table-12]*** .To study the impact of H_2O_2 on activity of BC as a function of time 1µM of BC was incubated with 250mM H_2O_2 in dark for varying time interval [0-60 min] and activity of Cystatin was determined by the method of *[Kunitz (1947)].* A gradual decline in activity of BC was observed showing 60% loss after 30 min of incubation with H_2O_2 (250 mM) .

Structural modification of BC in the prescence H_2O_2

1µM of Cystatin was exposed to (1-250µM) H_2O_2 in dark for 30 min and the samples were analyzed by fluorescence spectra, significant changes were observed ***[Fig-39]***, the results obtained showed emission maxima at 340nm with H_2O_2 incubation, showing no shift in λ_{max}. At 1 mM of H_2O_2 showing no significant decline in fluorescence intensity while at higher concentration of H_2O_2 (250µM) the fluorescence intensity decreased to 82% as compared to native .

The fluorescence result of BC under native conditions at 1mM of H_2O_2 not showed significant decline in fluorescence intensity. When Cystatin was incubated with H_2O_2 at 250µM and studied as a function of time, with in 10 min the antiproteolytic activity decreased 77 % with fluorescence intensity decrease to 82%. ***[Table -13 and Fig-.40]***

PROTECTIVE EFFECT OF DIFFERENT SCAVENGER'S ON STRUCTURAL AND FUNCTIONAL DAMAGE OF CYSTATIN CAUSED BY H_2O_2

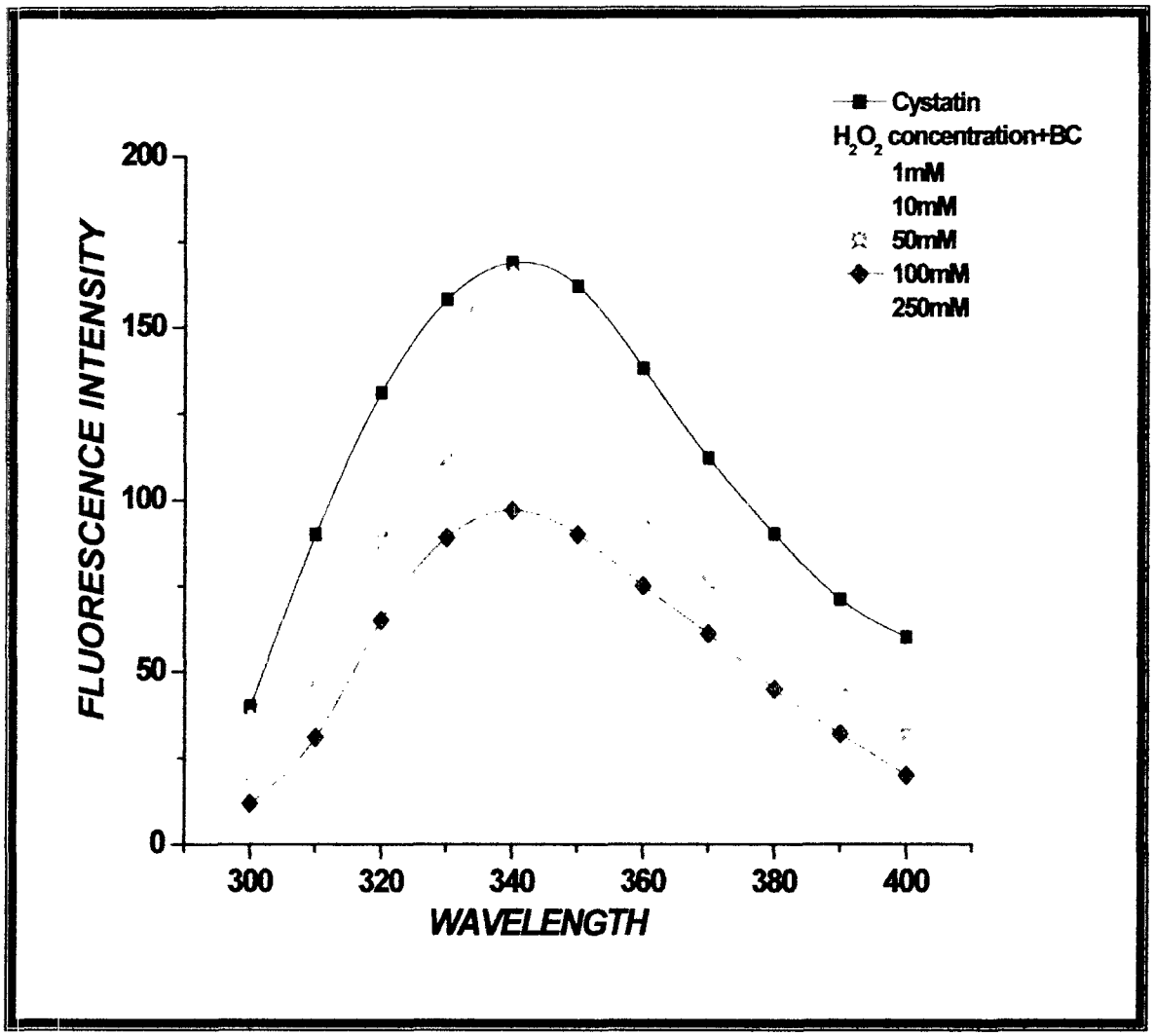
The effect of various scavengers on H_2O_2 induced Cystatin inactivation was studied using sodium benzoate, querecitin, curcumin and mannitol. Mannitol did not show protection infact offered 27% enhancement in fluorescence intensity of treated BC since mannitol and sodium benzoate are specific hydroxyl scavengers.This suggest only partial involvement of these radicals in H_2O_2 mediated BC damage. Among antioxidants and polyphenols curcumin (120µM) and Quercetin offered significant (80%) protection 250mM

TABLE-12 EFFECT OF VARYING CONCENTRATIONS OF H_2O_2 ON ANTIPROTEOLYTIC ACTIVITY OF CYSTATIN

BC(1μM) was incubated with different concentration of H_2O_2. I nhibitory activity was measured by caseinolytic method of kunitz (1947) taking native as 100%.

S.NO	Different Concentrations of H_2O_2 incubated along with BC(1μM)	% Remaining Inhibitory Activity
1	BC alone	100
2	BC + 1 mM H_2O_2	88 ± 1.2
3	BC + 10 mM H_2O_2	76 ± 2.1
4	BC + 50 mM H_2O_2	52 ± 0.98
5	BC + 100 mM H_2O_2	45 ± 1.1
6	BC + 250 mM H_2O_2	40 ± 0.98

All data are expressed as mean ± S.E for three different sets of experiments statical significance was conducted employing ONE WAY ANOVA .a probability level of 0.05 was selected showing results are significant.



Percent Decrease in Tryptophan Fluorescence in the presence of H_2O_2

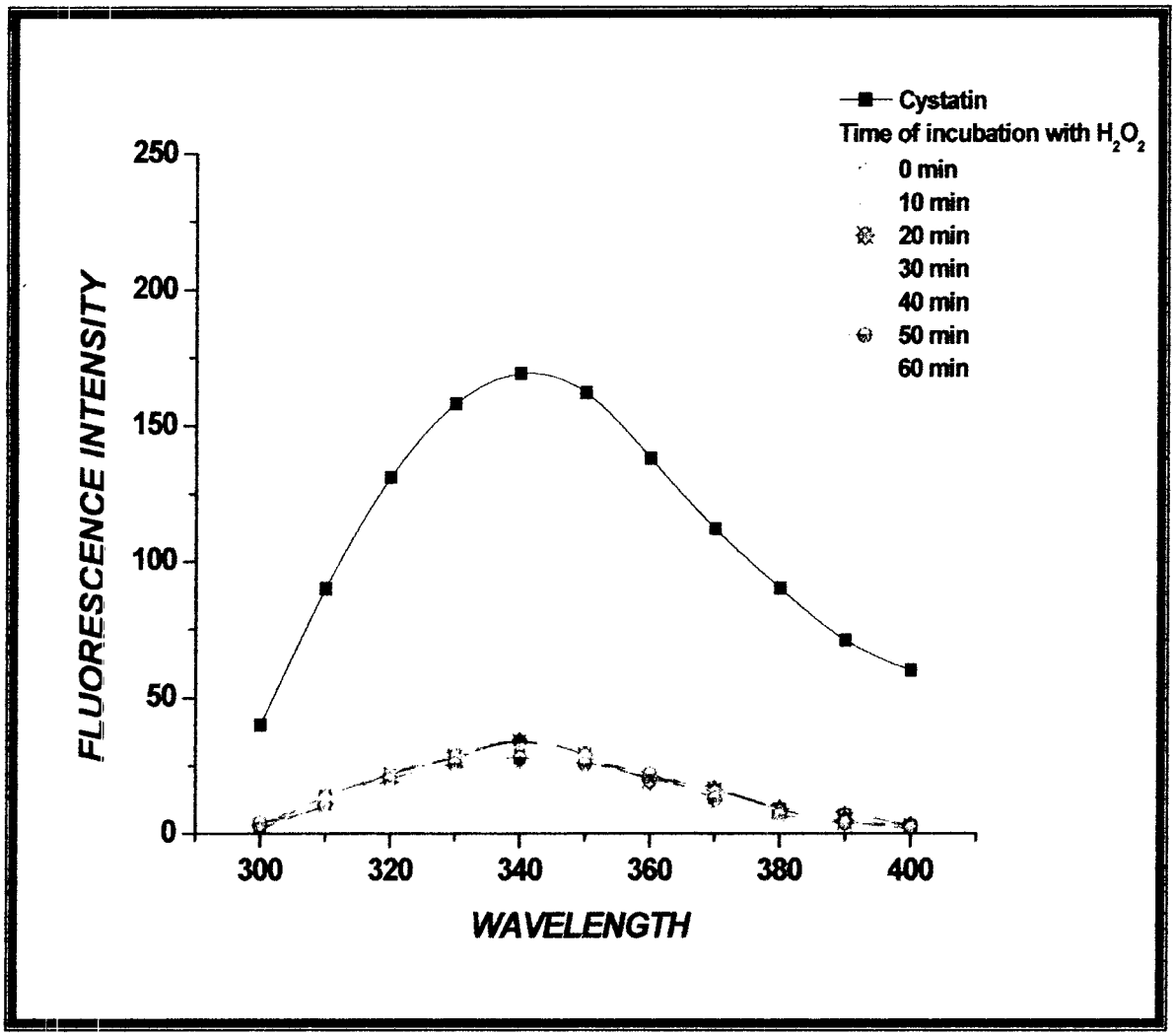
Cystatin alone fluorescence	H_2O_2 (1mM)	H_2O_2 (10mM)	H_2O_2 (50mM)	H_2O_2 (100mM)	H_2O_2 (250mM)
100	2	21	31	43	82

TABLE-13 EFFECT OF H_2O_2 ON ANTIPROTEOLYTIC ACTIVITY OF CYSTATIN INCUBATED FOR VARYING TIME INTERVAL.

BC (1μM) was incubated with H_2O_2 (250mM) for varying time interval.The Inhibitory activity was measured by caseinolytic method of kunitz (1947) taking native as 100%

S.NO	Time of Incubation of H_2O_2(250mM)with BC(1μM)	% Remainng Inhibitory Activity
1	BC alone	100
2	0 min	82 ± 0.93
3	10 min	77 ± 1.22
4	20 min	56 ± 0.60
5	30 min	40 ± 0.98
6	40 min	12 ± 0.93
7	50 min	8.5 ± 0.62
8	60 min	6 ± 0.62

All data expressed as mean ± S.E for four different sets of experiments statical significance was conducted employing ONE WAY ANOVA .a probability level of 0.05 was selected showing results are significant.



Percent decrease in tryptophan fluorescence in the presence of H_2O_2 with different time of incubation

Cystatin alone fluorescence	0 min	10 min	20 min	30 min	40 min	50 min	60 min
100	80	80	80	82	82	83	84

of H_2O_2 incubated with 1μM of Cystatin The samples when analyzed for caseinolytic activity of papain *[Kunitz (1947)]* In the prescence of H_2O_2 , BC showed mediated decline in activity ***[Table-14].*** However at 120μM of curcumin and Quercetin prompted restoration of lost activity near to native These natural antioxidant also neutralize the loss in tryptophan fluorescence and retrieved the native like fluorescence pattern in cystatin, with curcumin and querecitin showing maximum protection. ***[Fig-41]***

3.2.4 RIBOFLAVIN INDUCED STRUCTURAL AND FUNCTIONAL MODIFICATION OF BRAIN CYSTATIN

Riboflavin upon mediation with fluorescent light generates reactive oxygen species like superoxide anion (O_2) and triplet oxygen ($^3O_2$), flavin radicals and substantial amount of hydroxyl radical *[Husain et al 2005].*

Functional inactivation of brain Cystatin by Riboflavin

Effect of riboflavin on BC function was assessed by monitoring changes in its antiproleolytic activity by caseinolytic assay of papain *[Kunitz 1947]* 1μM of BC was incubated with increasing concentration of photoilluminated riboflavin (5-50μM) or with 50μM of riboflavin for varying time intervals. The results obtained are summarized in ***[Table-15]*** Exposure of BC to increasing concentration of riboflavin resulted in rapid decline of antiproteolytic activity at 50 μM concentration 76 % activity was lost with more than half of its inactivation taking place at concentration as low as 20μM similarly increase in length of exposure (0-60 min) ***[Table-16]*** of BC with riboflavin causes loss of inhibitory activity towards papain, with more than 50% inhibition taking place after 10 min of incubation.

Structural modification of Cystatin by riboflavin

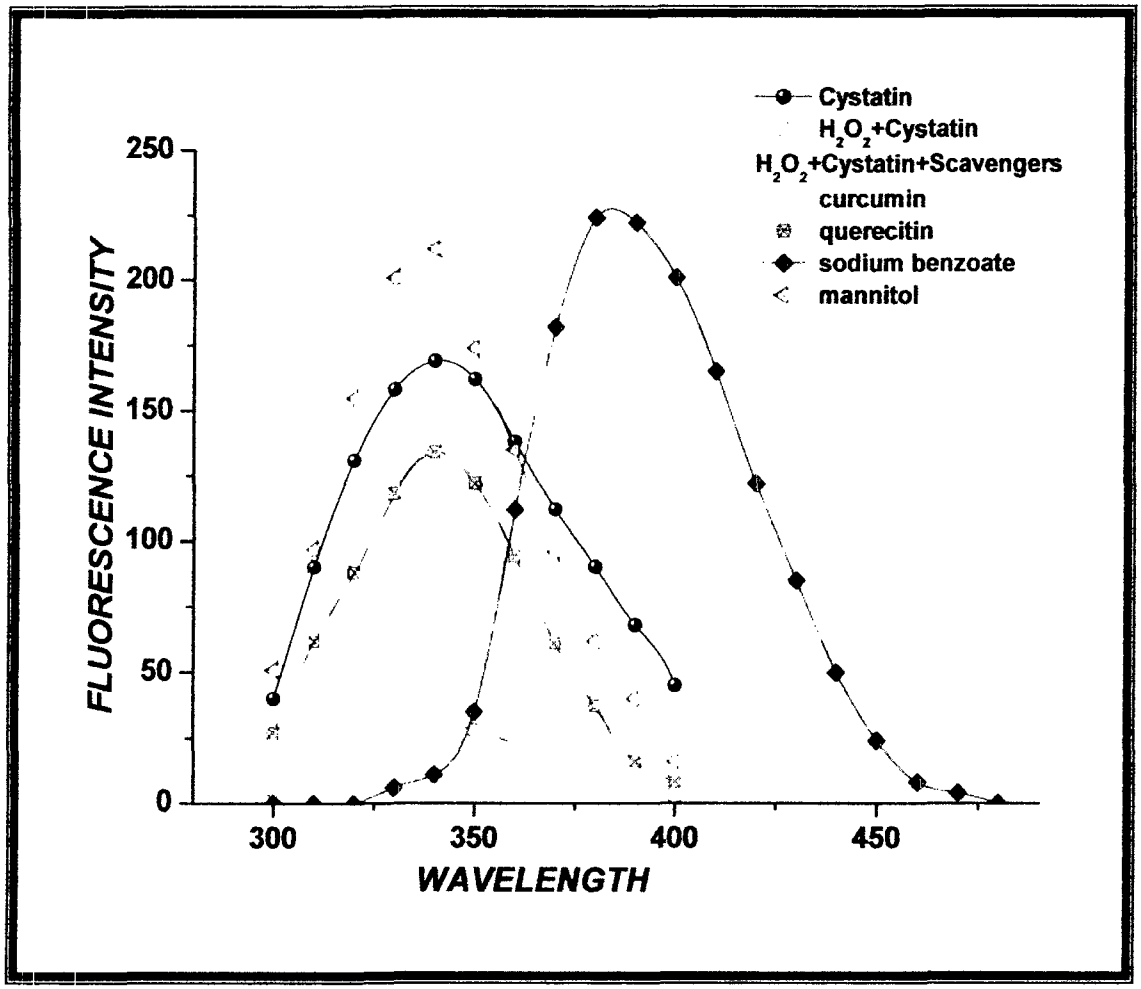
Treatment of Brain Cystatin with photo illuminated riboflavin drastically altered its intrinsic fluorescence properties. The fluorescence emission spectra of native BC gave an emission maximum at 340nm when excited at 280nm. BC (1μM) was incubated with increasing concentration of photo illuminated riboflavin [5-50μM] and was analyzed by fluorescence spectroscopy ***[Fig-42]*** to assess the effect on the conformation of protein.

TABLE-14 EFFECT OF DIFFERENT SCAVENGERS ON ANTIPROTEOLYTIC ACTIVITY OF H_2O_2 TREATED BRAIN CYSTATIN.

BC (1 μM) was incubated with H_2O_2 (250mM) in the presence of different scavengers (25mM).Inhibitory activity was measured by caseinolytic method of kunitz(1947).

S.NO	BC incubated with H_2O_2 along different scavengers	% Remainng Inhibitory Activity
1	BC alone	100
2	BC+ H_2O_2 (250mM)	40 ± 0.98
3	BC+ H_2O_2 (250mM) + Querecitin (120μM)	83 ± 0.94
4	BC+ H_2O_2 (250mM) + Curcumin (120μM)	82 ± 0.92
5	BC+ H_2O_2 (250mM) + Sodium benzoate	72 ± 0.92
6	BC+ H_2O_2 (250mM) + Mannitol	75 ± 0.75

All data are expressed as mean ± S.E for three different sets of experiments statical significance was conducted employing ONE WAY ANOVA .a probability level of 0.05 was selected showing results are significant.



Percent Decrease in Tryptophan Fluorescence in the presence of Scavenger

Cystatin alone fluorescence	H_2O_2 +BC	curcumin	querecitin	S.benzoate	mannitol
100	82	20	21	93	-

TABLE-15 EFFECT OF VARYING CONCENTRATIONS OF PHOTOILLUMINATED RIBOFLAVIN ON ANTIPROTEOLYTICACTIVITY OF BRAIN CYSTATIN

Cystatin (1 µM) was incubated for 30 min with different Riboflavin concentration. Inhibitory activity was measured by caseinolytic method of Kunitz (1947) taking native as 100%.

S.NO	Different Concentrations of Riboflavin incubated along with BC(1µM)	Percent Inhibitory Activity
1	BC alone	100
2	BC + 5 µM Riboflavin	62 ± 0.77
3	BC + 10 µM Riboflavin	57 ± 0.88
4	BC + 20 µM Riboflavin	40 ± 1.08
5	BC + 30 µM Riboflavin	38 ± 0.92
6	BC + 40 µM Riboflavin	30 ± 0.77
7	BC + 50 µM Riboflavin	24 ± 0.55

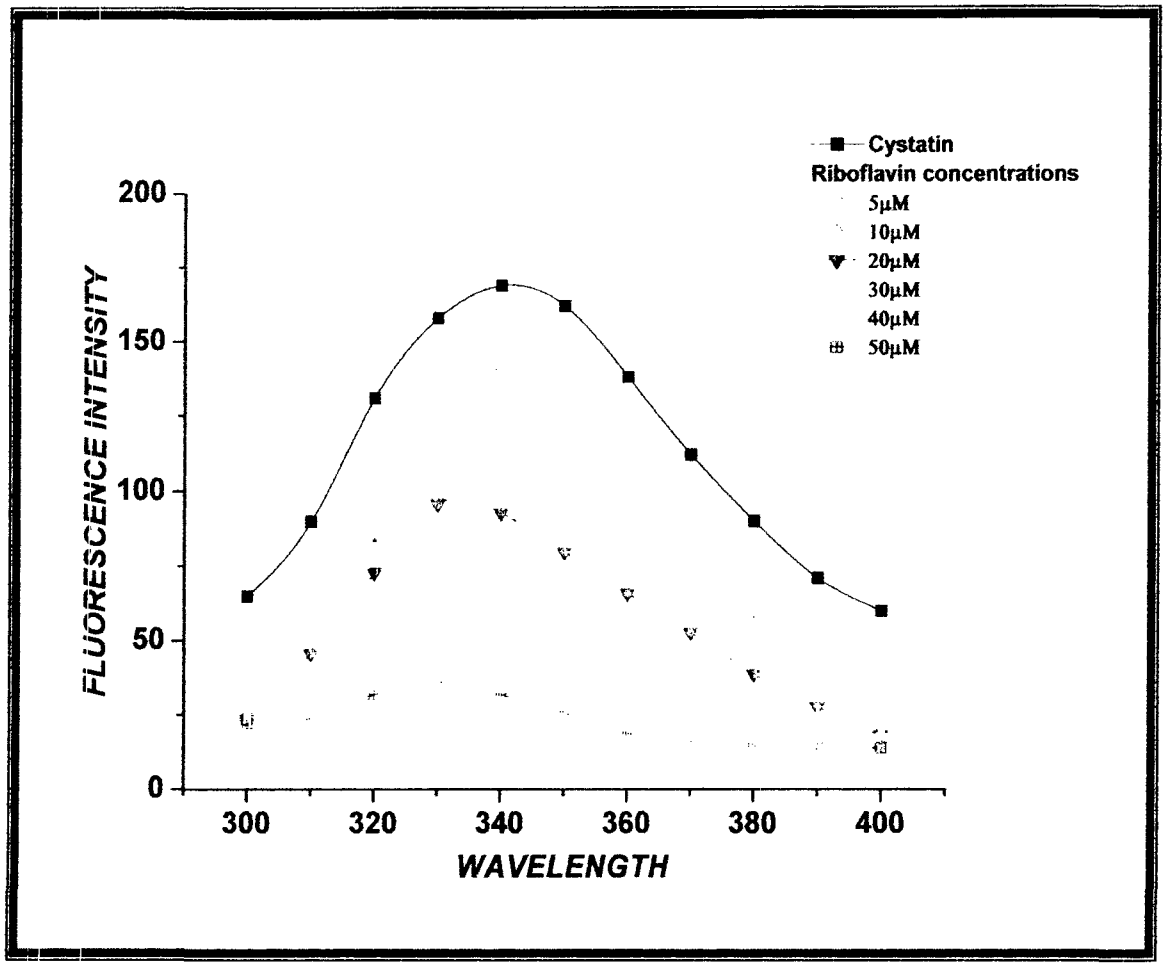
All data are expressed as mean ± S.E for three different sets of experiments statical significance was conducted employing ONE WAY ANOVA .a probability level of 0.05 was selected showing results are significant.

TABLE-16 EFFECT OF RIBOFLAVIN ON ANTIPROTEOLYTIC ACTIVITY OF CYSTATIN INCUBATED FOR VARYING TIME INTERVAL.

BC (1μM) was incubated with (50μM) for varying time interval. Inhibitory activity was measured by caseinolytic method of Kunitz (1947) taking native as 100%.

S.NO	Time of Incubation Riboflavin(50μM)with BBC(1μM)	% Remaining Inhibitory Activity
1	BBC	100
2	0	45 ± 0.62
3	10 min	38 ± 0.93
4	20 min	29 ± 0.92
5	30 min	24 ± 0.55
6	40 min	18 ± 0.78
7	50 min	12 ± 0.77
8	60 min	9 ± 0.88

All data are expressed as mean ± S.E for three different sets of experiments statical significance was conducted employing ONE WAY ANOVA .a probability level of 0.05 was selected showing results are significant



Percent Decrease in Tryptophan Fluorescence in the presence of riboflavin

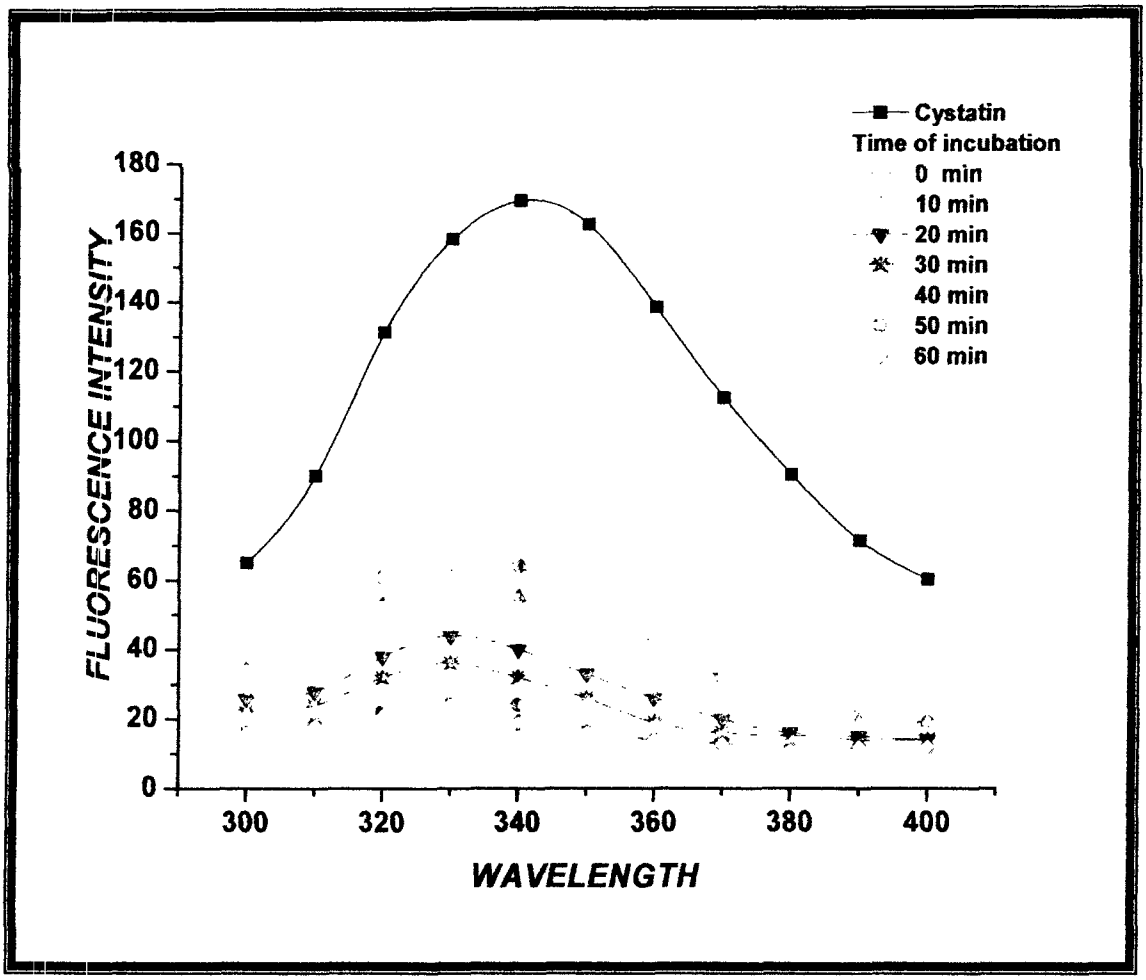
Cystatin alone fluorescence	5 μM	10 μM	20 μM	30 μM	40 μM	50 μM
100 %	16	29	45	51	63	81

Samples were excited at 280nm and emission range was 300-400nm. At 10μM of riboflavin no change in emission λ_{max} however 29% decline in fluorescence intensity was observed. Beyond this concentration (20μM) 10nm of blue shift in fluorescence intensity for native was observed with profound decline in tryptophan fluorescence (45%). Effect of 50μM of riboflavin on intrinsic fluorescence with 1μM of BC was also studied for various time periods of (0-60 min) .A time dependent gradual loss in fluorescence intensity with similar blue shift of 10nm was observed after 10 min of incubation ***[Fig-43]***.

PROTECTIVE EFFECT OF SCAVENGERS AND ANTIOXIDANTS

To detect the type of ROS involved in Cystatin inactivation various free radical scavengers were used. The results obtained showed that maximum suppression of BC inactivation was caused by potassium iodide (40%) closely followed by sodium azide, sodium azide is a scavenger of singlet oxygen and potassium iodide is a scavenger of flavin triplet state sodium benzoate, mannitol and thio urea eliminates hydroxyl radicals *[Khan and Khan (2004), Martinez Cayucla (1995)]*. These hydroxyl radical scavenger also offered some protection showing moderate role of this radicals in riboflavin mediated BC inactivation Biological antioxidant Glucose was analyzed for suppression of cystatin inactivation by riboflavin and it was found to be the most effective among natural antioxidants (57% protection) ***[Table-.17]***

To ascertain the effect of various scavengers to defeat the unfavorable consequences of photodynamic action of riboflavin on structure of BC, fluorescence spectra of treated cystatin in the presence of scavengers was obtained and percent retention of tryptophan fluorescence was determined. ***[Fig-44]*** Treated BC retained only 32% of native tryptophan fluorescence in. reaffirming the involvement of flavin triplet state and singlet oxygen in photodynamic modification of BC by riboflavin in the prescence of different scavengers, maximum retention of tryptophan fluorescence was shown by potassium iodide and sodium azide showed only 10 and 49 % decrease respectively Mannitol, Thio Urea and sodium benzoate exhibited only 10-15 % retention implicating only faint involvement of hydroxyl radical among biological antioxidant. Glucose again showed good protection.



Percent decrease in tryptophan fluorescence in the presence of Riboflavin with different time of incubation

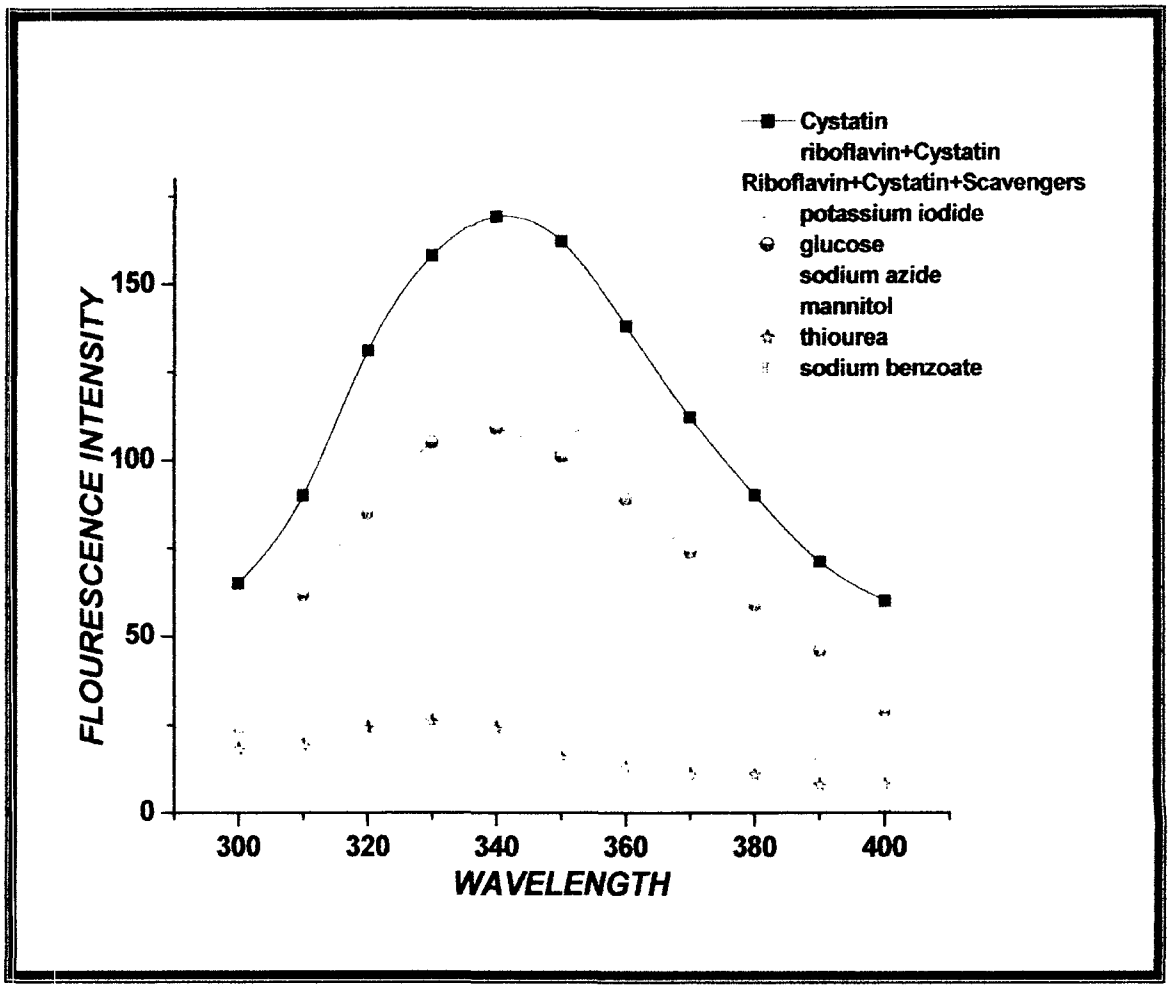
Cystatin alone fluorescence	0 min	10 min	20 min	30 min	40 min	50 min	60 min
100	62	67	76	81	83	86	89

TABLE-17 EFFECT OF DIFFERENT SCAVENGERS ON ANTIPROTEOLYTICACTIVITYOF RIBOFLAVIN TREATED CYSTATIN.

BC (1 µM) was incubated with Riboflavin (50 µM) for 30 min in the presence of different scavengers (25mM).Inhibitory activity was measured by caseinolytic method of *Kunitz (1947)*.

S.NO	BC (1 µM) incubated with Riboflavin in the prescence of different scavengers	% Remaining Inhibitory Activity of BC
1	BC alone	100
2	BC + 50 µM Riboflavin	24 ± 0.55
3	BC + 50 µM Riboflavin + Glucose	57 ± 0.77
4	BC + 50 µM Riboflavin + Potassium iodide	40 ± 0.88
5	BC + 50 µM Riboflavin + Sodium azide	37 ± 0.91
6	BC + 50 µM Riboflavin Thiourea	27 ± 0.93
6	BC + 50 µM Riboflavin + Mannitol	20 ± 0.76
7	BC + 50 µM Riboflavin + Sodium benzoate	10 ± 0.68

All data are expressed as mean ± S.E for three different sets of experiments statical significance was conducted employing ONE WAY ANOVA .a probability level of 0.05 was selected showing results are significant.



Percent Decrease in Tryptophan Fluorescence in the presence of Scavenger

Cystatin alone fluorescence	Riboflavin +BC	P.Iodide	Glucose	S.azide	Mannitol	Thiourea	S.Benzo
100	32	10	36	49	83	86	89

Discussion

Chapter-2

DISCUSSION

Reactive oxygen and nitrogen species (ROS/RNS) like superoxide anions, hydroxyl radicals, hydrogen peroxide, and nitric oxide are the intermediates of regular pathway of aerobic metabolism and processes. These reactive species generated from normal metabolism or exogenous insults lead to peroxidation of membrane lipids and damages to cellular macromolecules like DNA and proteins. Proteins are major targets for ROS as a result of their abundance in biological systems. Oxidants react rapidly with protein backbone and side chain residues causing various deleterious consequences like protein damage and degradation *[Davies et al. (1987a)]*, modification of amino acids *[Davies et al. (1987b)]*, and changes in secondary and tertiary structures of proteins.

NITRIC OXIDE (NO) is a pleitropic molecule that is needed for physiological function. It induces vasodilatation and inhibits apoptosis and plays a significant role in memory processes, making it a putatively valuable therapeutic agent in ageing associated diseases. However, NO can be harmful under oxidative stress conditions, due to oxidation of functional protein especially in the brain. Thus, when NO is produced in excessive amounts in the brain. It changes from a physiological neuromodulator to a neurotoxic factor. NO also act as a highly reactive free radical gas. It can participate as a cytotoxic effector molecule and pathogenic mediator when produced at high rates by inflammatory stimuli induced nitric oxide synthase or over stimulation of constitutive forms of enzyme *[Radi (2004)]*. In contrast to the protective action of low levels of NO, its excess has injurious outcomes due to worse oxidative damage *[Pryor (2006)]*.

Free radical NO leads to extensive loss of BC function. ***[Fig-6 and 7]*** There are number of reports for protein inactivation by NO through amino acid modification e.g. goat lung cystatin *[Khan et al. (2009)]*, and catalase *[Sigfrid et al. (2003)]*.The results showed that nitric oxide free radical leads to functional and structural damage of BC. The functional inactivation of BC may be due to oxidation of tryptophan residues as shown by the intrinsic fluorescence studies.

The damage of cystatins by NO was further investigated, in the presence of antioxidants .Use of curcumin and quercetin showed that these compounds can prevents. This damage to a very significant level ***[Table-8 and Fig-35].*** It was found that 50μM concentration of curcumin and 250 μM of qerecitin exhibited remarkable protection of BC against the functional ***[Table - 9]*** and structural damage ***[Fig-36]*** by NO.

Oxidative stress plays a major role in the pathogensis of various diseases including cerebral ischemia- reperfusion injury, hemorrahage neuronal cell injury, hypoxia and cancer *[valko (2007)].* It was reported that curcumin exhibits strong anti oxidant properties comparable to vitamin E and C *[Toda et al. (1985)]* Dietary supplemantation of curcumin was also found to be beneficial in neurodegenative diseases such as Alzheimer, s diseases *[Calabrese (2003) , Yang (2005)].*

The present study is of great significance showing that purified Cystatin could be damage by reactive oxygen species which are generated during various neural diseases. As cystatins play significant role in controlling the unwanted proteolysis in mammalian system, thus because of their important functions they are involved in various pathophysiological conditions including *[Berntein et al. (1996)],* cerebral amyloid aniopathy *[Jensson (1990)]* and other neurodegenerative diseases *[Shannon (2002)],* their inactivation needs to be prevented.to maintain protease – antiprotease imbalance.

Hypochlorous Acid (HOCl) The reactive species like HOCl and H_2O_2 have been recognized as characteristic components causing inflammation *[Martinez-Cayuela 1995].* Macrophages and neutrophils reduce molecular oxygen to superoxide anion, as a part of host defence system to neutralize the invading pathogens *[Marnett et al. (2003)].* The superoxide produced is rapidly dismutated to hydrogen peroxide. Activated neutrophils release the enzyme myeloperoxidase that reacts with H_2O_2 and chloride ions present to form HOCl *[Marnett et al. (2003)],* a reactive oxygen metabolite, that can modify amino acid residues, induce conformational changes in proteins and inactivate enzyme and enzyme inhibitors *[Wasil ei al. ; Dean et al. (1997)].*

HOCl, a strong oxidant, is known to oxidize many other important biomolecules such as DNA, enzymes and antiproteinases *[Jerlich et al. (2000), Whiteman et al. (2003), Szuchman-Sapir et al (2008)].* The results

reveal that BC, in the presence of HOCl lost its antiproteolytic activity rapidly (within 30 min, 82% loss occurred at 5μM HOCl). ***[Table-10]***

Inactivation of proteins like α_2 M, by HOCl has been reported earlier. *Whiteman et al. (2003)* reported a 90% loss in activity of isolated α_1 and α_2 macroglobulin in the presence of 7μM HOCl. HOCl usually causes aggregation of proteins as has been shown for fibronection *[Vissers and Winterboum (1991)]*, caprine α_2 M *[Khan and Khan (2004)]*, ovalbumin *[Olszowski et al. (1996)]*, apohaemoglobin and apomyoglobin *[Chapman et al. (2003)]*. HOCl reacts with amide groups of protein backbone and also with free amino groups of lysine residues of proteins yielding chloramines. These chloramines can than effectuate protein fragmentation.

At low HOCl concentrations preferably lysines are modified limiting the rapid backbone fragmentation *[Hwakins and Davies (1998)]*. The fluorescence profile of HOCl-treated BC reveals complete loss of intensity at concentrations above 5μM without any change in λ_{max} of emission. ***[Fig-37]*** Also, HOCl is prejudiced to modify tyrosine, phenylalanine (by chlorination and by sulphoxidation and oxy-acid formation) that to oxidize tryptophan *[Stadtman and Levine (2003)]*.This might also explain the unchanged λ_{max} of BC mainly attributable to tryptophan. Curcumin (120 μM) was able to deplete HOCl induced damage to some extent ***[Fig-38]***, followed closely by querecitin. Diminution of hypochlorite induced damage on human serum albumin by flavonoids has been reported by *Firuzi et al. (2004)*.

Hydrogen peroxidase (H_2O_2).It was observed that H_2O_2 lead to inactivation of BC towards papain ***[Table-12]*** owing to destruction of active site tryptophan residues. Major loss of tryptophan fluorescence intensity was observed in BC on incubation with 250 mM H_2O_2. ***[Fig-38]***

Quenching of intrinsic fluorescence of BC with increasing H_2O_2 concentration occurs probably due to oxidation of tryptophan residues leading to conformational change as reported earlier *[Khan and Khan (2004)]*. H_2O_2 was found to be a mild inactivator of BC relative to HOCl (and other radicals studied). The treated inhibitor lost only 40% of its antiproteolytic activity in presence of 250mM H_2O_2 .Use of scavengers showed prevention of H_2O_2 mediated damage. ***[Table-14].***

The utilization of scavengers, quenchers and enhancers is the method used to investigate the role of ROS involved in the photosensitization reaction *[Rywkin et al. (1992)]* and radical mediated damage. In order to investigate the type of ROS involved in inactivation of BC various scavengers like sodium benzoate mannitol, bioflavonoids, curcumin and querecitin were used. It was found that mannitol known to eliminate hydroxyl radicals have shown no preventive effect on H_2O_2 induced damage of BC. ***[Fig-41]*** While In case of sodium benzoate H_2O_2 showed marked red shift of 35nM, indicating changes in the microenvironment of tryptophan residues and the loss of native folded state of BC.

Riboflavin on photoillumination generates singlet oxygen, hydroxyl radicals and flavin triplet state *[Dahl and Richardson (1980)]* .The results reveal the loss BC functional activity on exposure to photosensitized riboflavin. ***[Fig-42]*** This bears similarity to sheep plasma high molecular weight kininogen (HMWK) *[Baba et al. (2004)],* membrane proteins *[Ali et al. (1991)],* BSA, invertase, lysozyme and trypsin *[Hasan et al. (2006)]* damage caused by photosensitized riboflavin.

So free radical scavengers were used for detection of the type of ROS involved. ***[Table-17]*** shows the scavenging effect of sodium azide, potassium iodide, mannitol, and thiourea. Sodium azide is a scavenger of singlet oxygen, potassium iodide scavenges triplet state and thiourea and mannitol scavenge hydroxyl radicals *[Martinez and Caycula (1995)].* Photoactivated riboflavin damage of BC showed the involvement of flavin triplet state in the damage. Potassium iodide protects 40% of inhibitory activity of BC caused by riboflavin inactivation. Retention of inhibitory activity in treated BC was also found in the presence of glucose, which is a known biological antioxidant *[Arad et al. (1980)].* These findings indicate that BC inactivation and degradation proceeds via hydroxyl pathway.

Thus from the data obtained for riboflavin induced damage on BC, it can be conclude that hydroxyl radicals leads to extensive structural and functional modification of purified Cystatin. ***[Fig- 44 &Table-17].*** It was also found that potassium iodide also inhibits BC inactivation by riboflavin indicating involvement of flavin triplet state. Among biological antioxidants only glucose was found to have significant effect on BC inactivation. Thus results obtained

with specific ROS scavengers suggested that singlet oxygen and flavin triplet state were predominantly responsible for BC damage. Same results are reported for sheep HMWK and catalase *[Baba et al. (2004); Cardoso et al. (2006)].* and goat brain cystatin *[Sumbul and Bano (2008)].*Damaging effects of riboflavin have been documented in various other proteins also . *[Ali et al. (1991) , Hasan et al (2006)].* Among the various forms of ROS generated, singlet oxygen is of particular physiological significance because of its selectively long life in aqueous solution, its ability to cross cell membrane barrier and high reactivity towards biomolecules *[Joshi (1998)].*

Curcumin (Cur) and Querctin (QE) These bioflavonoid are known for exerting pleiotropic health benefit through their antioxidant and anti-inflammatory, anti-inflammatory, antimicrobial anticancer and antidiabetic activities *[Aggarwal et al. (2007) , Takahama et al. (2009) , Sreejayan and Rao (1997)].*

In the light of this work and the previously reported results it can be said that curcumin and querecitin exhibits very beneficial effects against the damage caused by nitrogen free radicals and ROS .Efficacy of these natural antioxidants against the detrimental effects of NO, HOCl, H_2O_2 and photosensitized riboflavin studied on BC showed concentration dependent protective effect for all the four reactive species *[Huang et al. (1988)].*

The protective effects of curcumin may be attributed mainly to its antioxidants properties. The study of the effects of curcumin and querecitin on inflammatory and other oxidative stress conditions may help to develop some novel drugs.

Photodynamic effects of various sensitizers have long been applied to delineate structural and functional properties of large number of enzymes and other biologically active proteins *[Hopkins and Spikes (1969)].* In the present study, it has been shown that BC is susceptible to reactive oxygen species generated by various oxidants ,which mimics biological exposure to oxygen radicals *[Spike (1977)].*

Results

Chapter-3

Interaction of Cystatin with Drug

1-Amytriptyline

2-Fluoxetine

3-Haloperidol

4-Donepezil

3.3 RESULTS

ANTIDEPRESSANTS

Interaction of four drugs Amytriptyline, Fluoxetine, Haloperidol and Donepezil with cystatin

3.3.1 INTERACTION OF AMYTRIPTYLINE WITH CYSTATIN

For macromolecules, the fluorescence measurements can give some information about the binding of small molecules with proteins, such as the binding constants, binding sites and binding affinity. Fluorescence intensity of a compound can be decreased or quenched by interactions of protein with drugs.

Fluorescence Spectra of cystatin with Amytriptyline

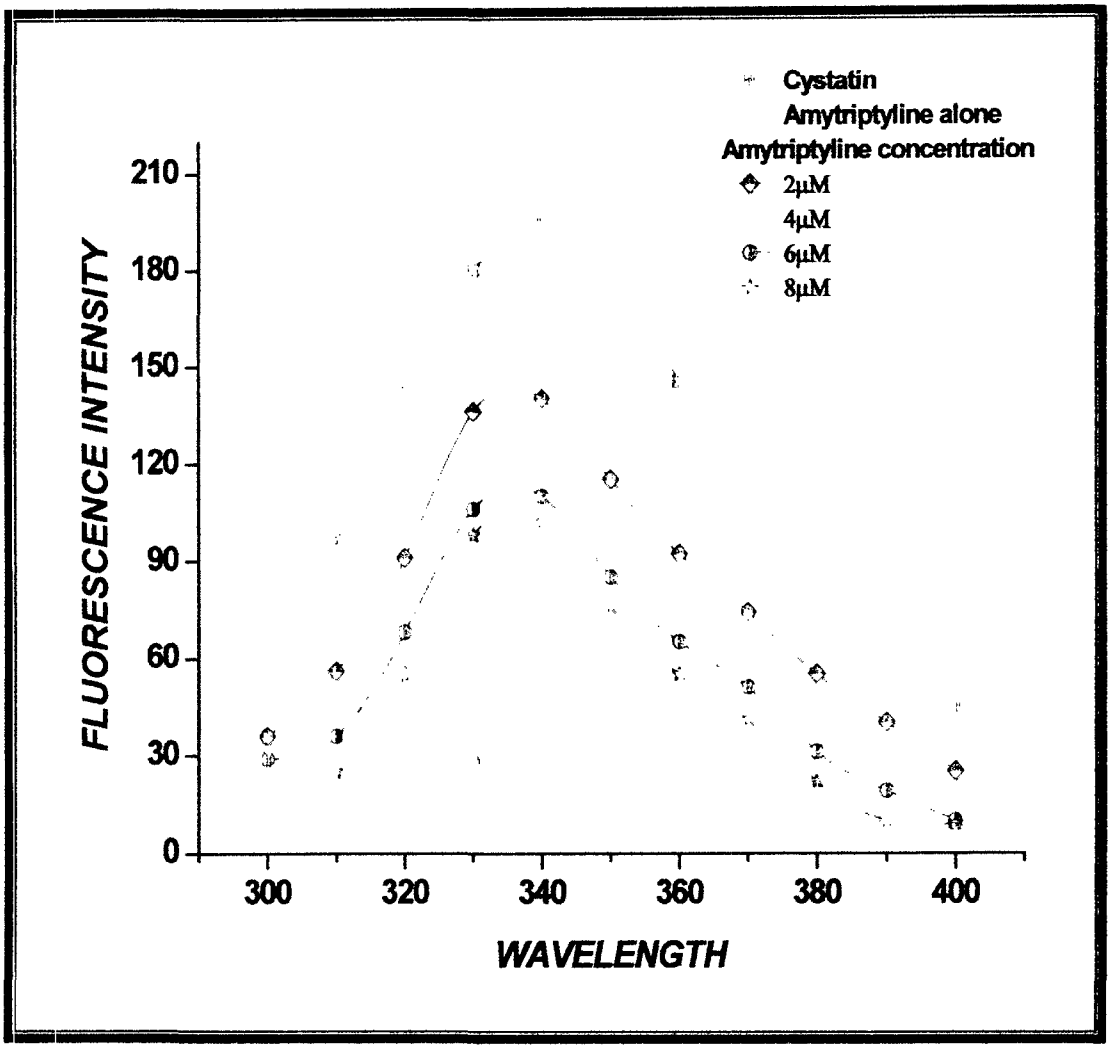
The fluorescence spectra of cystatin (1μM) in the presence of different concentrations of amytriptyline were recorded in the range of 300-400 nm upon excitation at 280 nm. Amytriptyline caused quenching of the intrinsic fluorescence of cystatin ***[Fig-45]*** with no shift in wavelength, however with increase in Amytriptyline concentration, fluorescence intensity was further decreased.

The maximum decrease in fluorescence intensity (48%) occurred at 8μM concentration . These results indicated that there were interactions between amytriptyline and brain cystatin and the binding reactions resulted in non-fluorescent complex.

Fluorescence quenching data was analysed by the Stern-Volmer equation.

$$\frac{F_o}{F} = 1 + K_{sv}[Q]$$

Where F_0 and F are the steady-state fluorescence intensities in the absence and presence of quencher, respectively, **Ksv** the Stern-Volmer quenching constant and **[Q]** is the concentration of quencher. ***[Table-24]***



Percent decrease in tryptophan fluorescence of BC in the presence of Amytriptyline

Cystatin alone fluorescence	Amytriptyline alone	2 μM	4 μM	6 μM	8 μM
100	32	29	38	44	48

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 *[Feng et al (1998) Gao et al (2004)]*

$$\frac{\log[F_0 - F]}{F} = \log K + n \log[Q]$$

Where **K** and **n** are the binding constant and the number of binding sites, respectively. Thus, a plot of **Log $(F_0-F)/F$** *versus* **[Q]** has been used to determine **K** as well as **n.** The value of K was found to be 3.01×10^6 Mol^{-1} and the no of binding sites were determined as 1 for amytriptyline. ***[Table- 24]***

Determination of ΔG^0 of interaction between Amytriptyline and Cystatin

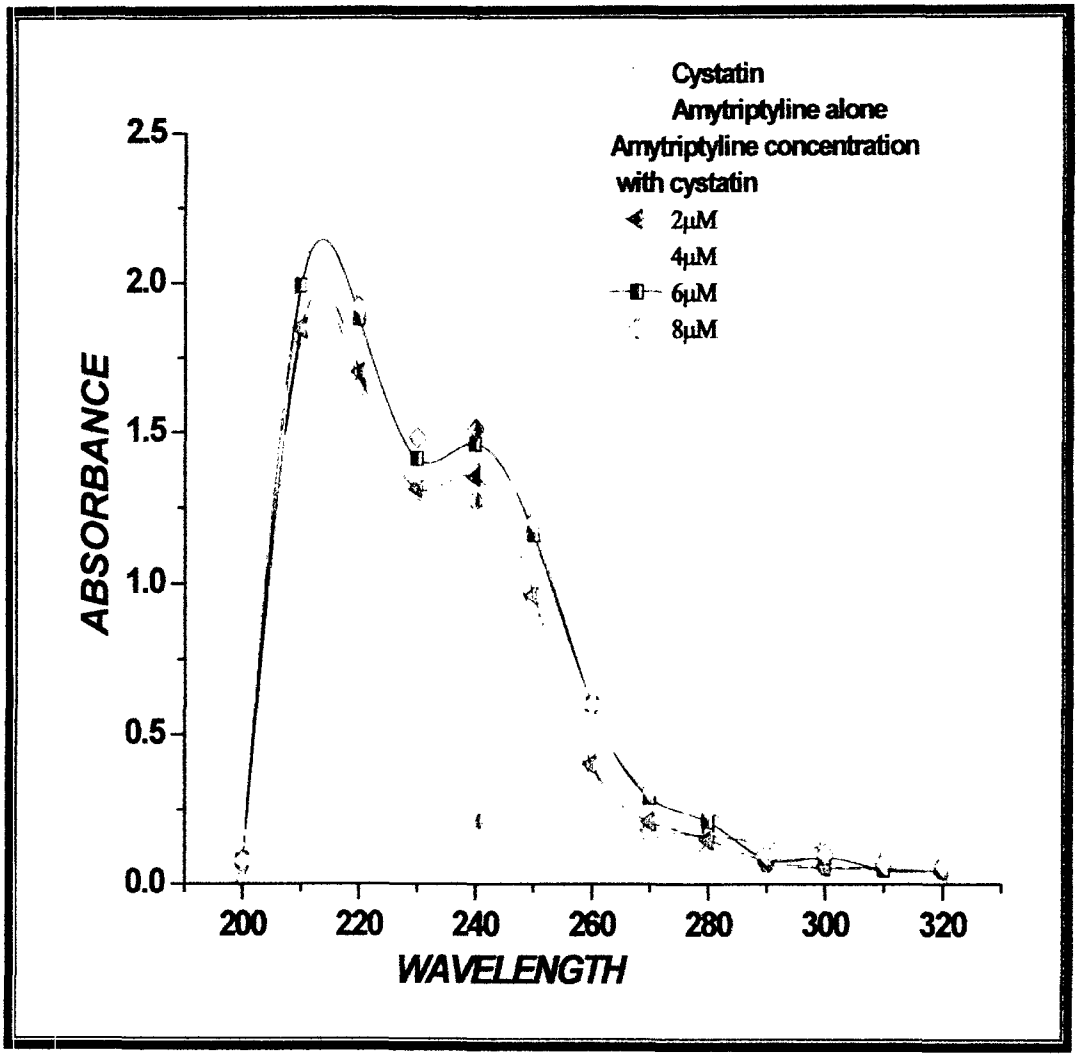
The forces of interaction between drugs and biomolecules include hydrophobic force, electrostatic interactions, van der Waals interactions and hydrogen bonds. In order to identify the interacting forces between amytriptyline with cystatin, the thermodynamic parameters i.e. free change (ΔG^0) of the interactions were calculated from the following equations: ***(Table-24)***

$$\Delta G^\circ = -RT\,Ink\,(KJ/mol)$$

The value obtained for cystatin – amytriptyline interaction was -36.97 kJ/mol indicating the reaction to be spontaneous

UV-vis absorption spectra of Amytriptyline cystatin complex

The interaction between Amytriptyline-cystatin was studied from UV-VIS absorption spectral data ***[Fig-46]***. Cystatin concentrations were fixed at 1µM while the Amtriptyline concentration was varied from 2µM-8µM. Absorption spectra of native Cystatin in the presence and absence of Amytriptyline were recorded in the range of 200-300 nm.


Cystatin
Amytriptyline alone
Amytriptyline concentration
with cystatin
2μM
4μM
6μM
8μM
ABSORBANCE
2.5
2.0
1.5
1.0
0.5
0.0
200
220
240
260
280
300
320
WAVELENGTH

The UV-vis absorption spectra were computed at all the antidepressant concentrations. However, no profound changes were noted at 2-8 μM Amytriptyline .The spectra obtained for BC at 2μM Amytriptyline shows two distinct positive peaks at 210 nm and 240 nm, the gross conformation of Cystatin at all concentrations of Amytriptyline was found to be unaffected. The UV absorption intensity of Cystatin increased with the variation of Amytriptyline concentration. The Addition of drug did not result in the shift of λ_{max} towards longer wavelength. However there was quenching in fluorescence. This evidence clearly indicated the interaction and some complex formation between drug and Brain Cystatin *[Cui et al (2004) Hu et al (2004)]*

Inhibitory activity of Cystatin in the presence of Amytriptyline

Effect of Amytriptyline on Cystatin function was assessed by monitoring changes in its antiproleolytic activity by caseinolytic assay of papain [Kunitz 1947] 1μM of Cystatin was incubated with increasing concentration of Amytriptyline (2-8μM). The results obtained are summarized in ***[Table-18]*** When Cystatin incubated with increasing concentration of Amytriptyline, it resulted in rapid decline of antiproteolytic activity showing 58% loss at 6μM amytriptyline with half of its inactivation taking place at a concentration of 8μM.The data obtained also indicated that inactivation of Cystatin by Amytriptyline is concentration dependent.

3.3.2 INTERACTION OF FLOUXETINE WITH BRAIN CYSTATIN

Studies on the binding mechanism between protein and small molecules provide useful information. For example, a detailed characterization of drug-protein binding properties was essential for understanding the function of drugs hence, interest in drug – protein interaction has attracted much attention.

Fluorescence spectra of fluoxetine with brain cystatin

In this study fluorescence spectra of Cystatin (1μM) in the presence of different concentrations of fluoxetine were recorded in the range of 300-400 nm upon excitation at 280 nm.

TABLE-18 Inhibitory activity of Cystatin in the presence Amytriptyline

Changes in the inhibitory activity of cystatin (1μM) after incubation with increasing concentration of Amytriptyline (2 - 8μM) for 30min in the final reaction volume of 1 ml in 0.05M sodium phosphate buffer pH 7.5.

S.NO	Amytriptyline concentration with cystatin	%Inhibitory Activity Retained
1	Cystatin alone	100
2	Cystatin + 2 μM Amytriptyline	71 ± 0.922
3	Cystatin +4 μM Amytriptyline	57 ± 0.755
4	Cystatin +6 μM Amytriptyline	42 ± 0.623
5	Cystatin +8 μM Amytriptyline	38 ± 0.98

All data are expressed as mean ± S.E for four different sets of experiments statical significance was conducted employing ONE WAY ANOVA .a probability level of 0.05 was selected showing results are significant.

The Drug caused quenching of the intrinsic fluorescence of Cystatin *[Fig-47]* with 10 nm of blue shift in wavelength. As the concentration of fluoxetine increases, fluorescence intensity decreases, maximum decrease in fluorescence intensity occurred at 2μM of drug concentration leading to quenching upto 51% These results indicated that there were interactions between fluoxetine and cystatin (BC) moreover the binding reactions resulted in non-fluorescent complex.

The fluorescence quenching data was analysed by the Stern-Volmer equation as described earlier for amytriptyline. K_{sv} the stern volmer quenching constant value indicates the affinity of binding obtained at 298K was is shown in ***Table-24.***

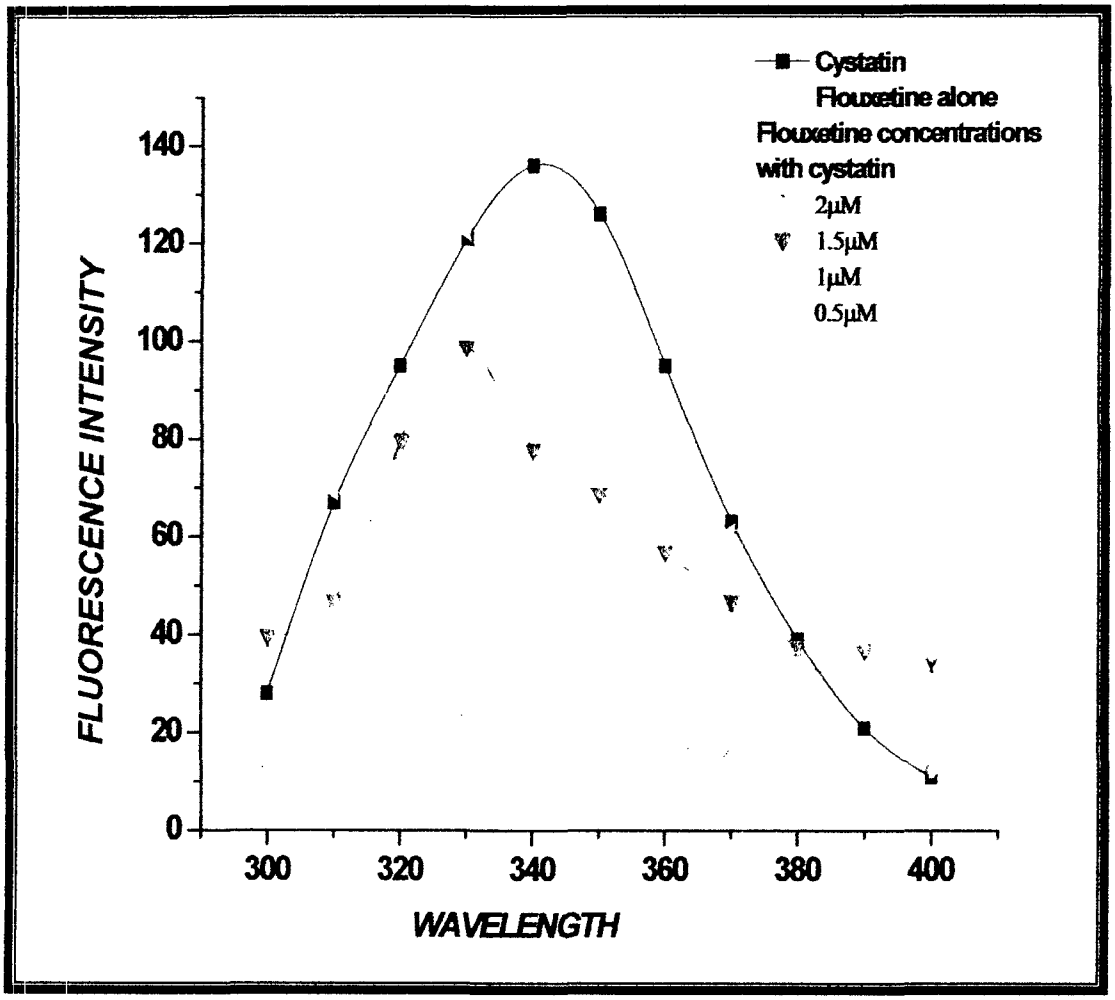
Determination of binding constant (K) and number of binding sites (n) these value were calculated as described earlier in amytriptyline section. The value of binding constant K was found to be 5.03 $x10^6$ Mol^{-1} and the no of binding sites was equal to 1 for fluoxetine ***[Table-24]***

ΔG^0 of interaction between Fluoxetine and Cystatin

Free energy change (ΔG^0) of the interactions was calculated (as described earlier in amytriptyline section).The value was found to be -38.2KJ/mol showing the reaction to be spontaneous. ***[Table-24]***

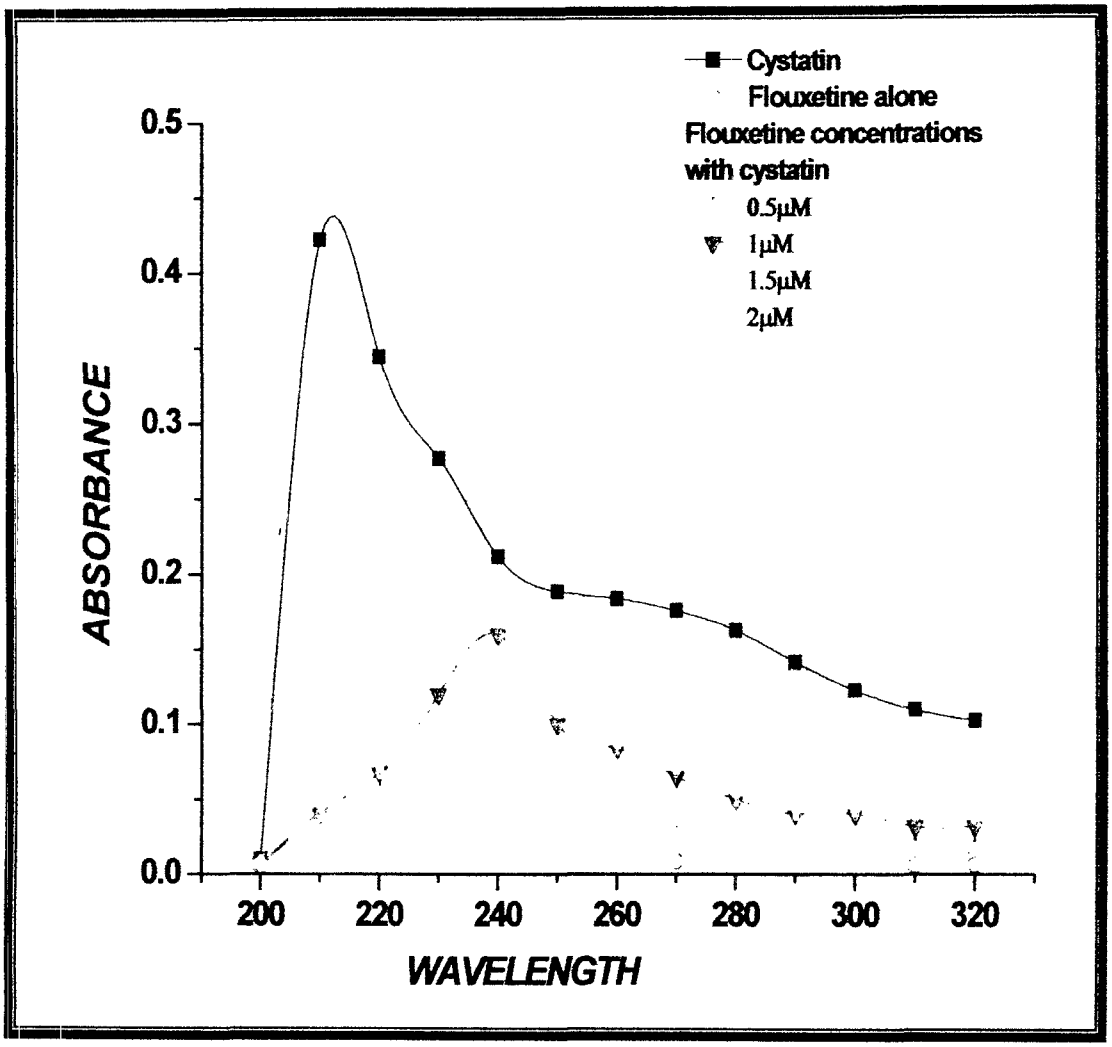
UV-vis absorption studies of Fluoxetine Cystatin complex

The interaction between Fluoxetine with cystatin was also studied from UV-vis absorption spectral data. Cystatin concentrations were fixed at 1μM while the Fluoxetine concentration was varied from 0.5μM-2μM. Absorption spectra of cystatin in the presence of Fluoxetine were recorded in the range of 200-300 nm.The UV absorption intensity of cystatin increased with the variation of Fluoxetine concentration. UV absorbance spectra of Cystatin, flouxetine and their complexes are shown in ***[Fig.48]*** Cystatin showed peak in the region 200-210 nm, while on complexation with fluoxetine profound changes were introduced and their was peak shift of 30nm (red shift) with enhanced absorbance as compared to fluoxetine.



Percent decrease in tryptophan fluorescence in the presence of flouxetine

Cystatin alone fluorescence	Fluoxetine alone	0.5 μM	1.0 μM	1.5 μM	2 μM
100	85	35	38	43	51


Cystatin
Flouxetine alone
Flouxetine concentrations with cystatin
0.5μM
1μM
1.5μM
2μM
ABSORBANCE
0.0
0.1
0.2
0.3
0.4
0.5
200
220
240
260
280
300
320
WAVELENGTH

The UV-vis absorption spectra were computed at all the fluoxetine concentrations. However, little change was noted between 0.5-2 µM fluoxetine. The spectra obtained for Cystatin interaction with 2 µM, fluoxetine showed peaks at 240 nm, the gross conformation of BC at all concentrations of fluoxetine was not effected significantly.

Inhibitory activity of Brain Cystatin in the presence of fluoxetine

The results obtained indicate that inactivation of Brain cystatin by fluoxetine is concentration dependent. 1µM Cystatin was incubated with increasing concentrations of fluoxetine(0.5-2 µM) in 50 mM sodium phosphate buffer pH 7.5 at room temperature for 30 min, its inhibitory activity was determined by caseinolytic assay of papain *[Kunitz, 1947]*. The activity of native cystatin was taken as 100%. On interaction with 0.5 µM fluoxetine, 48% loss of cystatin activity was noticed ***[Table-19]***. However At 1µM drug concentration 52% of inhibitor activity was compromised, a drastic decline (90%) was noticed at 2 µM fluoxetine drug concentration and the inhibitor retained only 10% of its original papain inhibition potential.

3.3.3 INTERACTION OF HALOPERIDOL WITH CYSTATIN

Haloperidol is an older antipsychotic drug used in the treatment of schizophrenia and, more acutely, in the treatment of acute psychotic states and delirium.

Fluorescence spectra of Haloperidol with brain cystatin

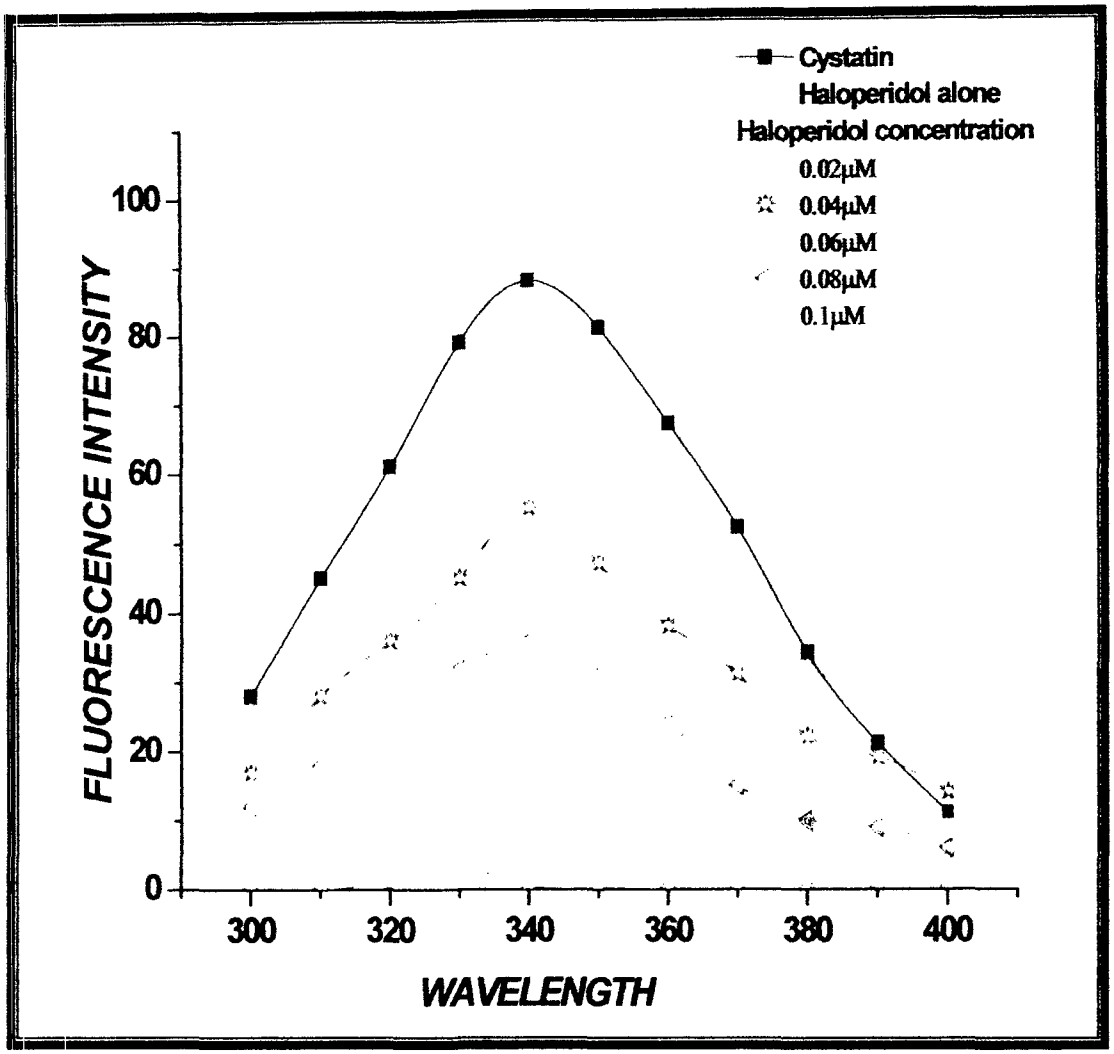
When 1µM of BC was incubated with increasing concentration of Haloperidol. 1µM of BC treated with increasing concentration of Haloperidol (0.02-0.1µM) and was subjected to fluorescence spectroscopy to assess the effect on conformation.the protein samples were excited at 280nm and emission range was 300-400nm. Haloperidol at 0.02µM did not induce any change in emission λ_{max} however 68% decline in fluorescence intensity was observed. Beyond this concentration (0.08µM) profound decline in fluorescence intensity (75%) was observed. Significant quenching in parent protein was observed (95%) when cystatin was incubated with 0.1µM of Haloperidol. ***[Fig-49]***.

TABLE-19 INHIBITORY ACTIVITY OF BRAIN CYSTATIN IN THE PRESENCE OF FLUOXETINE

Table shows changes in the inhibitory activity of Brain cystatin after incubation for 30 min with increasing concentration of Fluoxetine. BC (1μM) treated with varying concentration of Fluoxetine (0.5μM-2μM) for 30 min in the final reaction volume of 1 ml in 0.05 M sodium phosphate buffer pH 7.5.

S.NO	BC with Fluoxetine Concentration	% Inhibitory Activity Remaining
1	Cystatin alone	100
2	Cystatin + 0.5 μM Fluoxetine	52 ± 0.394
3	Cystatin + 1 μM Fluoxetine	48 ± 0.770
4	Cystatin + 1.5 μM Fluoxetine	25 ± 0.911
5	Cystatin + 2 μM Fluoxetine	10 ± 0.518

All data are expressed as mean ± S.E for four different sets of experiments statical significance was conducted employing ONE WAY ANOVA .a probability level of 0.05 was selected showing results are significant.



Percent decrease in tryptophan fluorescence in the presence of Haloperidol with cystatin

BC alone fluorescence	Haloperidol alone	0.02 μM	0.04 μM	0.06 μM	0.08 μM	0.1 μM
100	98	68	77	69	75	95

Stern–Volmer constant

For fluorescence quenching, the decrease in intensity is usually described by the well-known Stern–Volmer equation as described earlier for amytriptyline antidepressant. The value of Ksv was found to be 15 x10 ^{6} mol $^{-1}$ for Haloperidol ***[Table-24].***

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

(n) the value was determined as described earlier in amytriptyline section. The binding constant and the number of binding sites was found to be 7.988 x10 ^{6} mol $^{-1}$ and 1.1 respectively ***[Table-24]***

ΔG^0 of interaction between Haloperidol with Cystatin

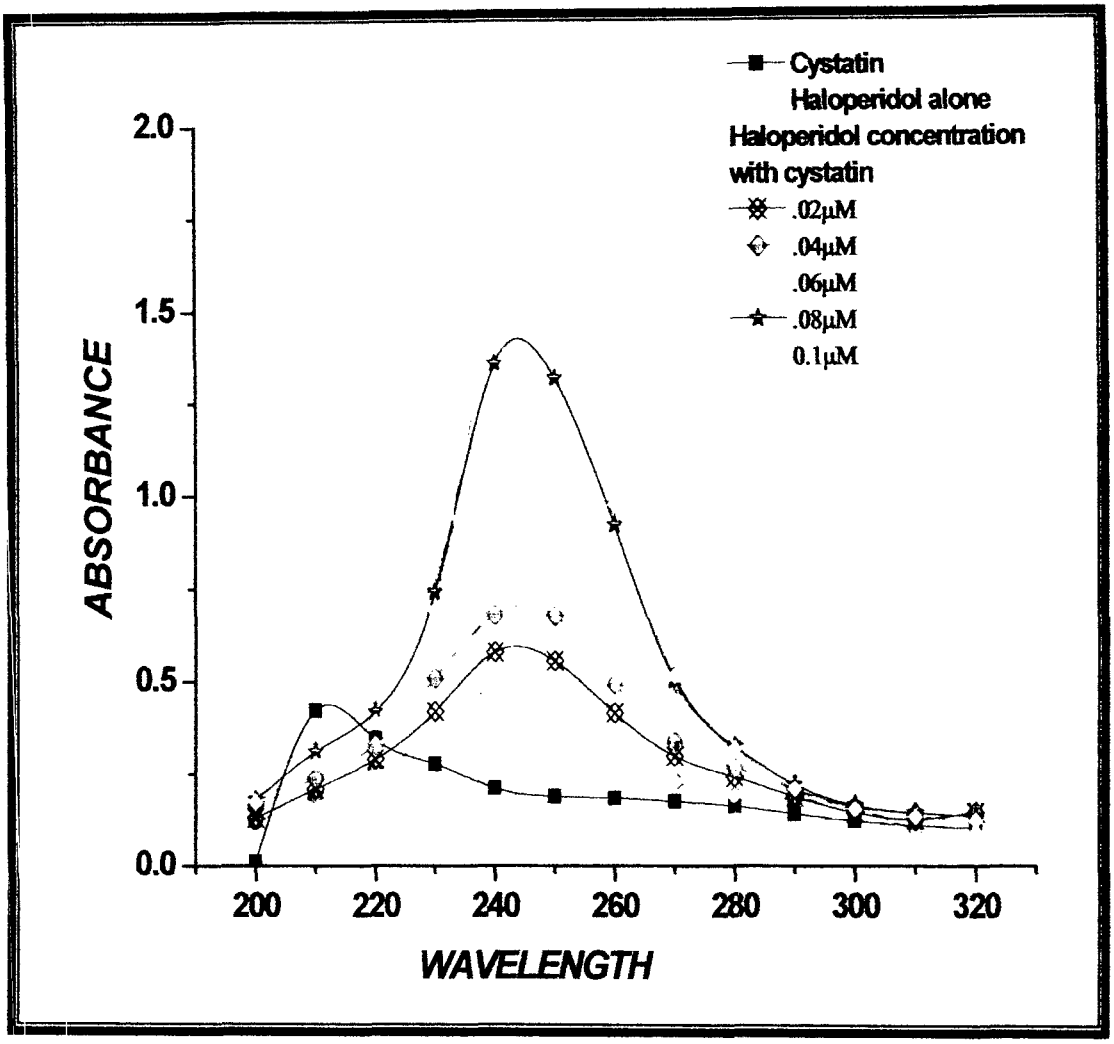
Free energy change (ΔG^0), of the interactions was calculated from the equations was -39.37 KJ/ mol as described earlier in amytriptyline section. ***[Table-24]***

Absorption spectra of cystatin in the presence and absence of Haloperidol

The interaction between Haloperidol with cystatin was studied from UV-VIS absorption spectral data cystatin concentrations was fixed at 1µM while the haloperidol concentration was varied from 0.02µM-0.1µM. Absorption spectra of native cystatin and in presence of Haloperidol were recorded in the range of 200-300 nm. The UV absorption intensity of cystatin increased with the variation of Haloperidol concentration. UV absorbance spectra of cystatin, Haloperidol and their complexes are shown in ***[Fig-50]*** BC showed peak in the region 200-210 nm while on complexation with Haloperidol profound changes were introduced and their was peak shift of 30nm (red shift). in the range of 200-300 nm, with deep enhancement in absorbance.

Inhibitory activity of Cystatin in the presence of Haloperidol

The obtained data indicates that the inactivation of Brain cystatin by Haloperidol is concentration dependent. 1µM cystatin was incubated with increasing concentrations of the drug (0.02-0.1 µM) in 50 mM sodium phosphate buffer pH 7.5 at room temperature for 30 min and its inhibitory


Cystatin
Haloperidol alone
Haloperidol concentration
with cystatin
.02μM
.04μM
.06μM
.08μM
0.1μM
ABSORBANCE
2.0
1.5
1.0
0.5
0.0
200
220
240
260
280
300
320
WAVELENGTH

activity was determined by caseinolytic assay of papain *[Kunitz, 1947]*. The activity of native cystatin was taken as 100%. The exposure of 1μM of cystatin to varying concentrations of Haloperidol resulted in remarkable loss in its antiproleolytic activity. At concentration as low as 0.06μM haloperidol showed 42% loss in activity of cystatin .On interaction with 0.02 μM haloperidol ,29% loss of BC activity was noticed ***[Table-20]***. At 0.1μM drug concentration the inhibitor retained only 36% of its original papain inhibition potential.

3.3.4 INTERACTION OF DONEPEZIL WITH BRAIN CYSTATIN

Alzheimer Disease (AD) is a progressive brain disorder that gradually destroys a person's memory and ability to learn reason, make judgments, communicate and carry out daily activities. The greatly reduced concentration of acetylcholine in the cerebral cortex is a significant factor in Alzheimer Disease *[German (2003)]*. The inhibition of acetyl cholinesterase (AChE) activity may be one of the most realistic approaches to the symptomatic treatment of Alzheimer Disease. AChE is responsible for degradation of the neurotransmitter acetylcholine (ACh) in the synaptic cleft of neuromuscular junctions and of neuronal contacts in the central nervous system *[Kasa et al. (2000); Tabet (2006)]*. Many medicinal agents, as donepezil, is used for treatment of Alzheimer Disease, belong to the important class of acetyl cholinesterase inhibitors (AChEIs) *[Kaur and Zhang (2008)]*. The results of the interaction of donepezil with cystatin is given below.

Intrinsic fluorescence studies of Cystatin in the presence of Donepezil

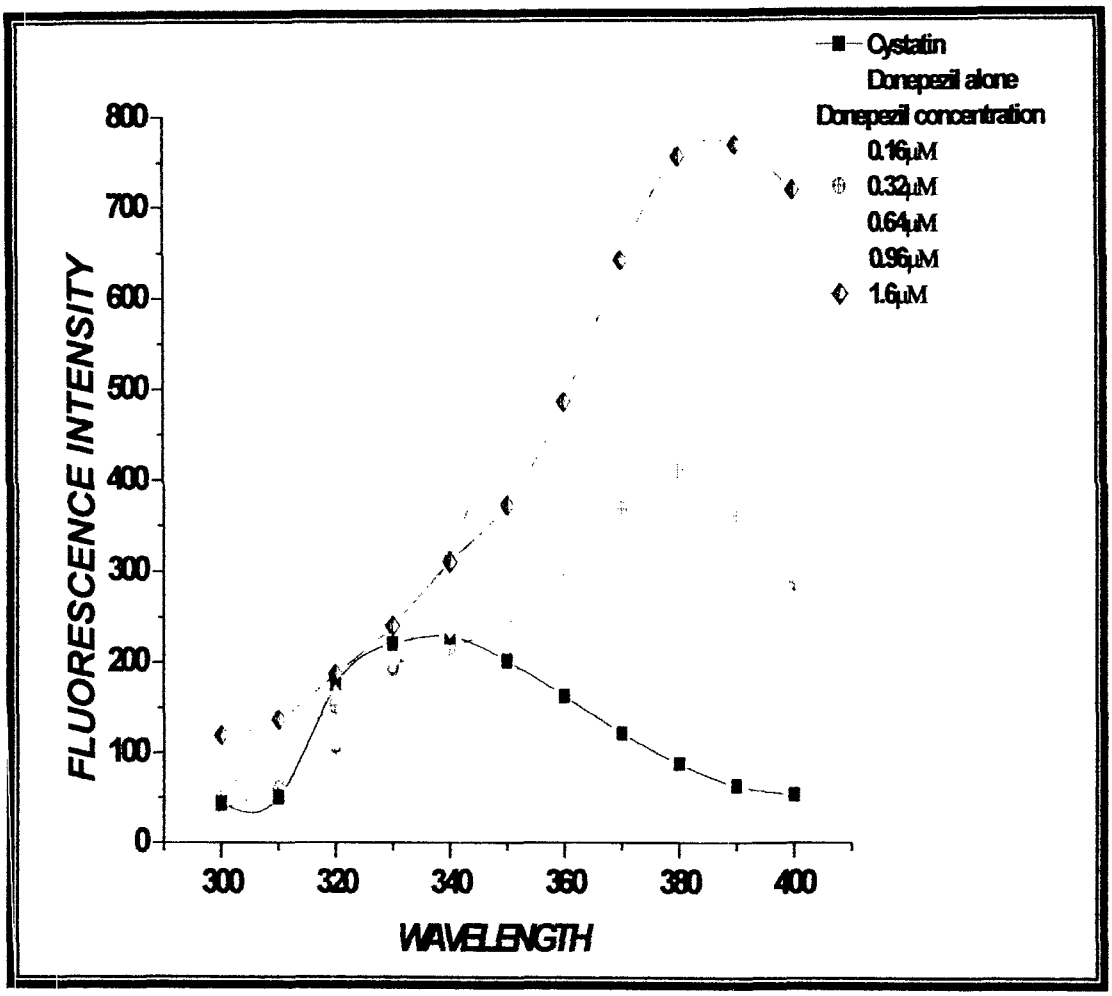
Cystatin (1μM) was incubated with various concentration of Donepezil varying from 2 to 10 μM for30 min. The fluorescence was recorded in the wavelength region of 300-400 nm after exciting the protein solution at 280 nm for total protein fluorescence. Donepezil caused unfolding of the Cystatin as indicated by enhancement in fluorescence intensity accompanied by the red shift of 40nm as compared to λ_{max} of native Cystatin (330nm) while the drug (native) shows λ_{max} at 370 nm however when it forms complex with Cystatin there was shift in λ_{max} of 10nm with significant enhancement in fluorescence intensity at 1.6 μM ***[Fig-51]***.

TABLE-20 INHIBITORY ACTIVITY OF CYSTATIN IN THE PRESENCE OF HALOPERIDOL

Changes in the inhibitory activity of cystatin after incubation for 30 min with increasing concentration of Haloperidol. Cystatin (1μM) was treated with varying concentration of Haloperidol(0.02 μM-0.1μM) for 30min in the final reaction volume of 1 ml in 0.05M sodium phosphate buffer pH 7.5.

S.NO	Haloperidol concentrations with cystatin	% Remaining Inhibitory Activity
1	Cystatin alone	100
2	Cystatin + 0.02 mM Haloperidol	71 ± 0.938
3	Cystatin +0.04 μM Haloperidol	65 ± 0.915
4	Cystatin +0.06 μM Haloperidol	58 ± 0.920
5	Cystatin +0.08 μM Haloperidol	43 ± 0.770
6	Cystatin +0.1 μM Haloperidol	36 ± 0.911

All data are expressed as mean ± S.E for four different sets of experiments statical significance was conducted employing ONE WAY ANOVA .a probability level of 0.05 was selected showing results are significant.


Cystatin
Donepezil alone
Donepezil concentration
0.16μM
0.32μM
0.64μM
0.96μM
1.6μM
FLUORESCENCE INTENSITY
WAVELENGTH
0
100
200
300
400
500
600
700
800
300
320
340
360
380
400

UV-vis spectra of Cystatin in the presence and absence of Donepezil

Cystatin concentrations were fixed at 1μM while the Donepezil concentrations varied from 0.16μM-1.6μM. Absorption spectra of native cystatin and in the presence and absence of Donepezil were recorded in the range of 200-300 nm. The UV absorption intensity of Cystatin increased with increasing concentration of Donepezil concentration, however the absorption intensity decrease slighty may be due to disruption or perturbation of absorbing groups ***[Fig-52]***.

Inhibitory activity of Cystatin in the presence of Donepezil

Changes in the inhibitory activity of cystatin with increasing concentration of Donepezil is shown in ***[Table-21]***. Effect of Donepezil on cystatin function was assessed by monitoring its changes in antiproleolytic activity by caseinolytic assay of papain *[Kunitz 1947]* 1μM of cystatin was incubated with increasing concentration of Donepezil (0.16-1.6μM) . Exposure of cystatin to increasing concentration of donepezil resulted in rapid decline of antiproteolytic activity , 85% decline in the activity was seen at 1.6 μM of donepezil with more than half of the inactivation of cystatin was taking place at concentration as low as 0.32μM.

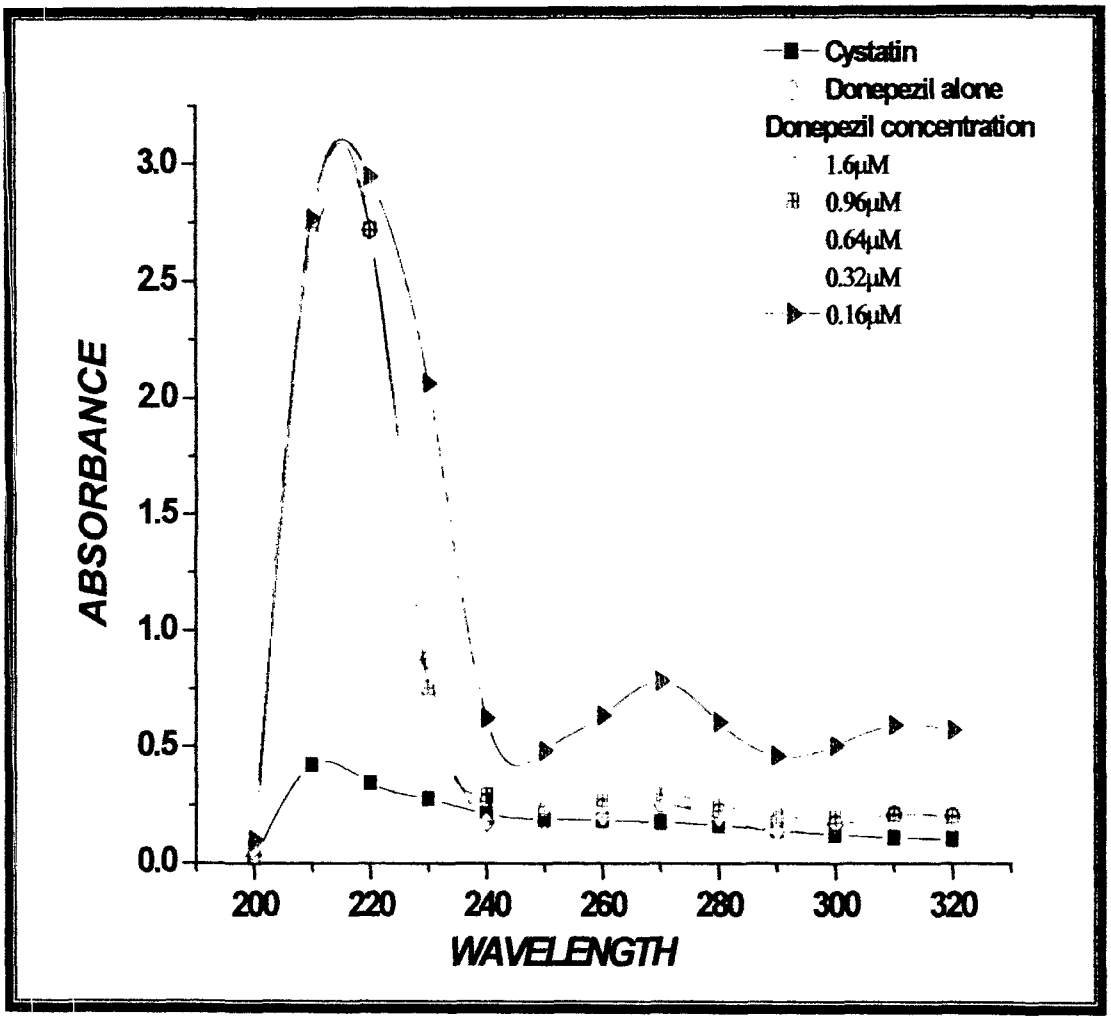

Cystatin
Donepezil alone
Donepezil concentration
1.6μM
0.96μM
0.64μM
0.32μM
0.16μM
ABSORBANCE
3.0
2.5
2.0
1.5
1.0
0.5
0.0
200
220
240
260
280
300
320
WAVELENGTH

TABLE-21 INHIBITORY ACTIVITY OF CYSTATIN IN THE PRESENCE OF DONEPEZIL

Table shows Changes in the inhibitory activity of Brain cystatin after its incubation for with increasing concentrations of Donepezil. Cystatin (1µM) treated with varying concentration of Donepezil (0.16µM-1.6µM) for 30min in the final reaction volume of 1 ml in 0.05M sodium phosphate buffer pH 7.5.

S.NO	Drug Concentration	% Remaining inhibitory activity of cystatin
1	Cystatin alone	100
2	Cystatin + 0.16 µM Donepezil	57 ± 0.623
3	Cystatin +0.32 µM Donepezil	40 ± 0.938
4	Cystatin +0.64 µM Donepezil	38 ± 0.772
5	Cystatin +0.96 µM Donepezil	24 ± 0.932
6	Cystatin +1.6 µM Donepezil	15 ± 0.680

All data are expressed as mean ± S.E for three different sets of experiments statical significance was conducted employing ONE WAY ANOVA .a probability level of 0.05 was selected showing results are significant.

Discussion

Chapter-3

DISCUSSION

Antipsychotic and antidepressant drugs are imperative in the treatment of schizophrenia and other brain disorders. These drugs exert their therapeutic effects at least in part through perturbation of the dopamine, noradrenalin and serotonin neurotransmitter systems in the brain, but additional molecular mechanisms of action are likely to contribute to their clinical effects.

Antidepressants such as SSRIs (Amytriptyline and Fluoxetine) and SNRIs (Haloperidol) produce their effects on the central nervous system via their actions on synaptic transmission. In general, these antidepressants work on a very limited number of transmitters, including catecholamines and acetylcholine. Antidepressant drugs increase noradrenergic and serotonergic transmission in the brain and may affect the secretion of anterior pituitary hormones by regulating the hypothalamic secretions *[Schüle (2006)]* the aim of the present study was to investigate the effect of antidepressants on cystatin structural and functional modification.

Binding studies are used to determine the ability of an antidpressants to attach to a particular site of a protien. Drug binding is evaluated at various drug concentrations and Information derived from binding studies have been used to determine and compare the affinity of different antidepressants for cystatin. However, binding parameters are only the first step in understanding overall pharmacologic effects. .In the present study some binding reaction Parameters, including the binding constants and the numbers of the binding sites were determined.

Amitriptyline is a medication used to treat various forms of depression, pain associated with the nerves (neuropathic pain), and to prevent migraine headaches. It is often used to manage nerve pain resulting from cancer treatment. Such injury to nerves causes burning, tingling sensation. Amitriptyline acts to block reabsorption of chemicals that transmit nerve messages in the brain.

Intrinsic fluorescence of proteins provide considerable information about protein structure, quenching, enhancement of intensity and spectral shift which are used for elucidation of related structure-dynamics in proteins

[Lakowicz (2006)]. In the present study, fluorescence spectroscopic measurements were undertaken to gain insight into the interaction of Brain cystatin with amytriptyline. It was found that in the presence of drug fluorescence intensity decreased indicating a conformational change in protein ***[Fig-45]***.

When 1μM of Cystatin was incubated with 2 μM of amytriptyline for 30 min and the samples were analyzed by fluorescence, complex changes were observed . Fluorescence emission spectrum showed no shift in λ_{max} , with not much decline in fluorescence intensity as compared to native . The negative value of ΔG° reveals that the interaction process is spontaneous. Our finding is similar to the binding of Congo red with human serum albumin .The value for the number of binding sites (n) equal to 0.89 showing that there is one independent binding site of interaction. The K_{sv} value reported for amytriptyline - cystatin complex was 1.15 x 10^5 / M indicating the quenching in fluorescence to be static.

Absorption spectral measurements of Cystatin in the presence of amytriptyline provided information related to changes in their structure ***[Fig-46]*** The two positive peaks at 210 and 240 nm observed for amytriptyline - cystatin complexes at all concentrations studied , shows contribution from phenylalanine and histidine residues *[Donovan (1969)].* When cystatin incubated along with amytriptyline for varying concentration it caused decrease in antiproleolytic activity, there was 58% loss in antiproleolytic activity at 6μM of Amytriptyline concentration. This loss in antiproleolytic activity increased to 62% at 8μM. ***[Table-18].***showing a concentration dependent effect

Fluoxetine, is a selective serotonin uptake inhibitor is clinically useful in treating depression and may be useful for management of a variety of other psychiatric and metabolic derangements. Fluoxetine antagonizes the neurotoxic effects of p-chloroamphetamine, a compound that depletes serotonin *[Wong et al. (1975); Wona and Bvmaster (1976)].* Fluoxetine is effective in the treatment of depression *[Stark and Hardison (1985)]* and obesity *[Carruba et al. (1985)].*

Fluorescence technique has been widely used for drug–protein studies *[Ahmad et al. (2004) ; Yang et al. (2008)]* .In this study the addition of increasing concentrations of fluoxetine caused a progressive reduction of the fluorescence intensity of cystatin fluoxetine complex, ***[Fig-47]*** with 10nm of blue shift in the wavelength λ_{max} (emission maximum). Thus, the fluorescence was strongly quenched, whereas λ_{max} was decreased from 340 to 330 nm by the addition of 2μM of fluoxetinea , shift that reasonably can be attributed to the increased hydrophobicity (or a decreased polarity) of the region surrounding the tryptophan site *[Kragh-Hansen (2001)]*. Similar spectral features were observed for the interaction of compound [Zn(L_2)(phen)] with BSA.

Stern–Volmer equation is used to study the interaction of Cystatin with fluoxetine. The interaction forces between proteins and ligands may comprise hydrophobic, hydrogen bonds, van der Waals, and electrostatic interactions *[Timaseff and Peeters (1972)]*. The free energy change (ΔG^0) is estimated for the interaction of fluoxetine with Cystatin is shown in ***[Table-24]***. The negative values of the free energy (ΔG^0), support the assertion that the binding process is spontaneous.

The UV-vis absorption difference spectra were computed at all the drug concentrations. However, profound changes were noted only for those obtained at 0.5-2 μM fluoxetine ***[Fig-49]***. The spectra obtained for fluoxetine interacted with 1 μM Cystatin, shows peaks at 240 nm with shift of 30 nm.

When 1 μM Cystatin was incubated with increasing concentrations of the fluoxetine (0.5-2 μM) in 50 mM sodium phosphate buffer pH 7.5 at room temperature for 30 min, its inhibitory activity dereased in concentration dependent manner *[Kunitz (1947)]*. On interaction with 0.5 μM fluoxetine 48 % loss of Cystatin activity was noticed ***[Table-19]***. At 1 μM drug concentration 52% of inhibitor's activity was compromised. A drastic decline (90%) was noticed at 2 μM drug concentration.

Although fluoxetine is a competitive inhibitor of serotonin uptake and interacts with the same portion of the carrier protein responsible for the transport of serotonin, the structural features responsible for substrate-carrier protein recognition may be different from those responsible for inhibitor-carrier

protein recognition. Additional studies are required to determine whether the structural overlap between fluoxetine and serotonin is biochemically and pharmacologically meaningful.

Haloperidol It is classified as a highly potent neuroleptic compound possessing a strong activity against delusions and hallucinations, most likely due to an effective dopaminergic receptor blockage in the mesocortex and the limbic system of the brain. The drug is rapidly absorbed. Plasma-levels reach their maximum within 20 minutes after injection. Concentrations 4-25 micrograms per liter are required for therapeutic action.

The Haloperidol drug- cystatin was chosen as an example for this kind of analytical study because of its importance as a common antidepressant. *Sulkowska et al.2007* investigated the binding interaction of methotexate to BSA by monitoring the changes in the fluorescence emission spectra of the protein in the presence of Methotexate .The results are very similar to our results when binding of haloperidol with Cystatin was studied. The binding constant between haloperidol and Cystatin was found to be $K= 7.988 \times 10^6$. showing strong binding affinity .Haloperidol- cystatin interaction gave one binding site with negative value of ΔG and one binding sites showing the reaction to be spontaneous ***[Table-24]***. The interactions between gemcitabine hydrochloride (GEM) and BSA showed similar results *[Kandagal et al. (2006)]*,The values of n for GEM- BSA interaction varied from 1.16 to 1.09.

The purified inhibitor was incubated with increasing concentrations of Haloperidol and its antiproteolytic potential was determined. Haloperidol diminished the activity of cystatin with maximum inactivation of the inhibitor at higher concentration within a short span of time ***[Table-20].*** At lower concentration inhibitor inactivation was less significant. Considerable activity (~58%) was retained by cystatin at 0.06 µM of drug concentration. Even at maximum concentration of the drug (0.1 µM) brain cystatin retained 36% activity. Drug induced inactivation of proteins has been reported earlier for other proteins also like horse liver alcohol dehydrogenase activity which was affected differentially by various drugs. Few like barbital, caffeine and diazepam exerted no effect however chlorpromazine, sulpiride, morphine etc. reduced the activity and phenytoin enhanced ADH activity *[Roig et al. (1991)]*.

Absence of any drug induced conformational change in cystatin suggest that inactivation of the inhibitor may be related to subtle changes in the conformation at the active site region induced by the drugs. Similar ligand binding functional changes have been shown for isocitrate dehydrogenase *[Serry and Farrell (1990)]*.

Donepezil is a cholinesterase inhibitor that is specific for acetyl cholinesterase.Donepezil has a relative bioavailability of 100% following oral administration.Alzheimer's disease is the most common cause of dementia and is a primary degenerative disease of the brain of unknown cause. Onset is usually late in life with increasing impairment of memory, developing gradually into a global impairment of cognition, orientation, linguistic ability and judgement. Acetylcholine is an important neurotransmitter associated with memory, and abnormalities in cholinergic neurones are among the many neurological and neurochemical abnormalities that develop in Alzheimer Disease. One approach to lessening the impact of these abnormalities is to inhibit the breakdown of acetylcholine by blocking the relevant enzyme. Cholinesterase inhibitors are the 'first-line' agents in the treatment of Alzheimer's disease. Cholinesterase inhibitors are rapidly absorbed through the gastrointestinal tract.

Drug induced changes in enzyme activities leading to adverse effects is the area of continual scientific investigation *[Priyamvada et al. (2008)]* .When drugs bind to a protein, the intramolecular structures can be altered, resulting in conformational change of the protein *[Salvi et al. (2001)] ; Guo et al (2004a); Cheema et al. (2008); Ahmed-Ouameur et al. (2006)]*. In the presence of donepezil, fluorescence intensity increased profoundly.Such a kind of change has also been documented earlier , after Interaction of ligands (phytohormones, cytokinins, abscisic and gibberellic acids) with wheat germ agglutinin resulting in 60% increase in fluorescence intensity of native protein *[Bogoeva et al. (2004)]*.

Donepezil-Cystatin complexation showed 40 nm red shift in λ_{max} indicating exposure of aromatic residues to the solvent caused by conformational rearrangement of the two proteins *[Monsellier and Bedouelle (2005); Vivian and Callis (2001)]*. Absorption spectral measurements of

cystatin in the presence of drugs showed a peak noticeable at 275 nm and 210 nm in spectra obtained at 0.16 μM Donepezil suggesting changes around tyrosine residues and mainly due to tryptophan *[Donovan, (1973)]*. There was a gradual decline in cystatin activity with increasing drug concentration resulting in 62% loss at 0.64 μM Donepezil ***[Table-21]***. Further magnitude of decline was relatively smaller with increasing drug concentration at 1.6 μM , Donepezil the inhibitor retained 15% of its antiproteolytic potential.

Thus, the results indicate that the UV absorption and fluorescence emission changes on donepezil mediated interaction are due to minor conformational changes in Cystatin mainly arising from local interactions affecting the chromophoric groups of the protein

Results

Chapter-4

Interaction of Neurotransmitters Dopamine and Serotonin with Cystatin

3.4. RESULTS

3.4.1 INTERACTION OF NEUROTRANSMITTER (DOPAMINE) WITH BRAIN CYSTATIN

SPECTRAL STUDIES

Fluorescence analysis of brain cystatin in the presence of Dopamine

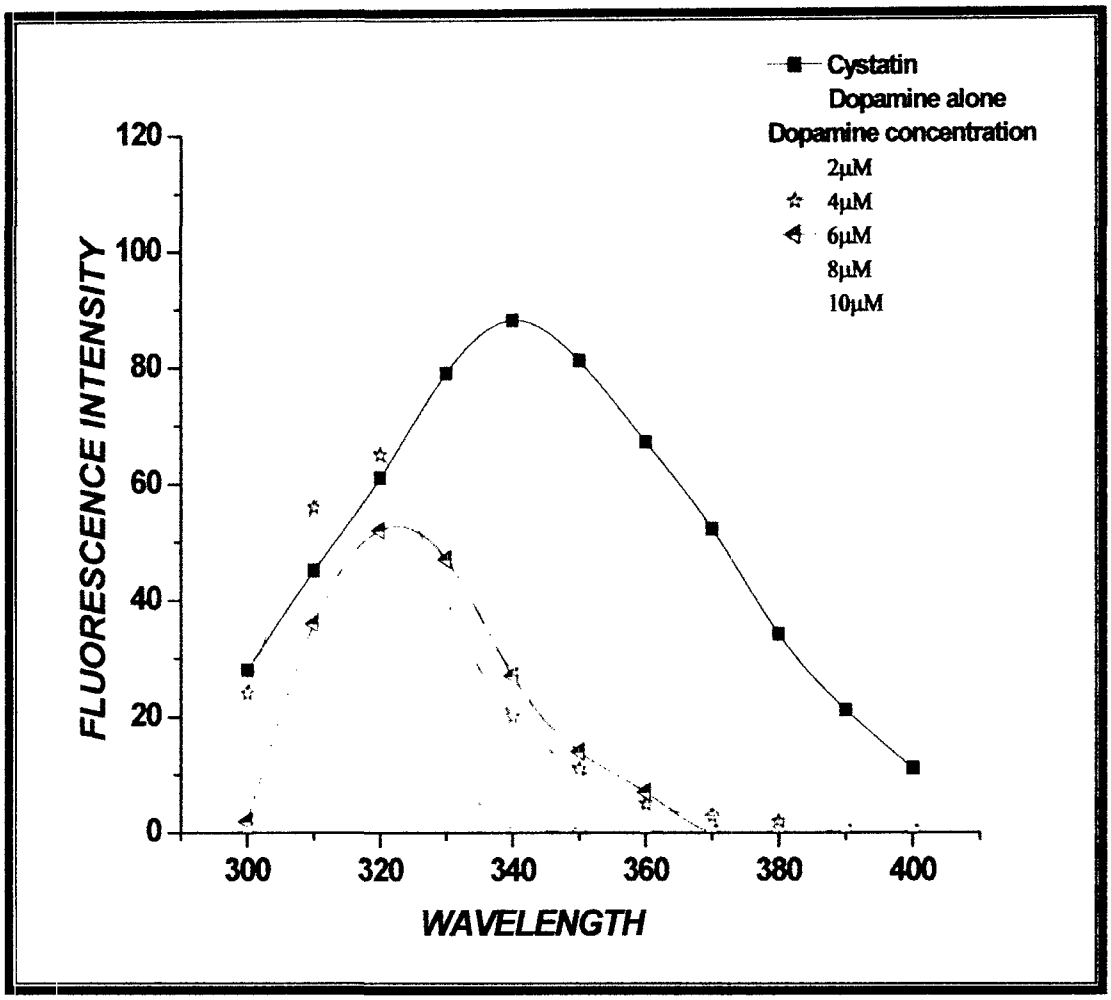
The fluorescence spectra of BC (1μM) was recorded in the presence of different concentrations (2-10 μM) of Dopamine after 30 min of incubation intensities were recorded in the range of 300-400 nm upon excitation at 280 nm. Dopamine caused quenching of the intrinsic fluorescence of BC accompanied by a blue shift (20 nm) ***[Fig-53]*** in wavelength at 2μM concentration. These results indicated that there were 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. (As discussed earlier for Amytriptyline) 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-24.***

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 *[Feng et al. (1998) , Gao et al (2004)] [As discussed earlier for Amytriptyline chapter-3]* 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-24]***

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 Amytriptyline with Cystatin the thermodynamic parameter, that is. Free energy



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	68	77	69	75	95

change (ΔG^0), of the interactions were calculated from the equations, it was found to be -36.58 KJ/mol *[As discussed earlier for Amytriptyline chapter-3]* ***[Table-24]***

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-54]***. 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)]*.

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-22*** .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 losed half of its inhibitory activity at 6 μM concentration indicating changes on complex formation.

3.4.2 EFFECT OF SEROTONIN ON CYSTATIN

Intrinsic fluorescence studies of Cystatin in the presence of Serotonin

The fluorescence spectra of Cystatin in the presence of different concentrations of Serotonin were recorded in the range of 300-400 nm upon excitation at 280 nm for fluorescence. Cystatin (1μM) was incubated with various concentration of Serotonin varying from 10 nM to 50 nM for 30min. The slits were set at 10 nm for excitation and emission. The path length of the sample was 1 cm.Serotonin causes unfolding of the BC as indicated by enhancement in fluorescence intensity ***[Fig-55].***

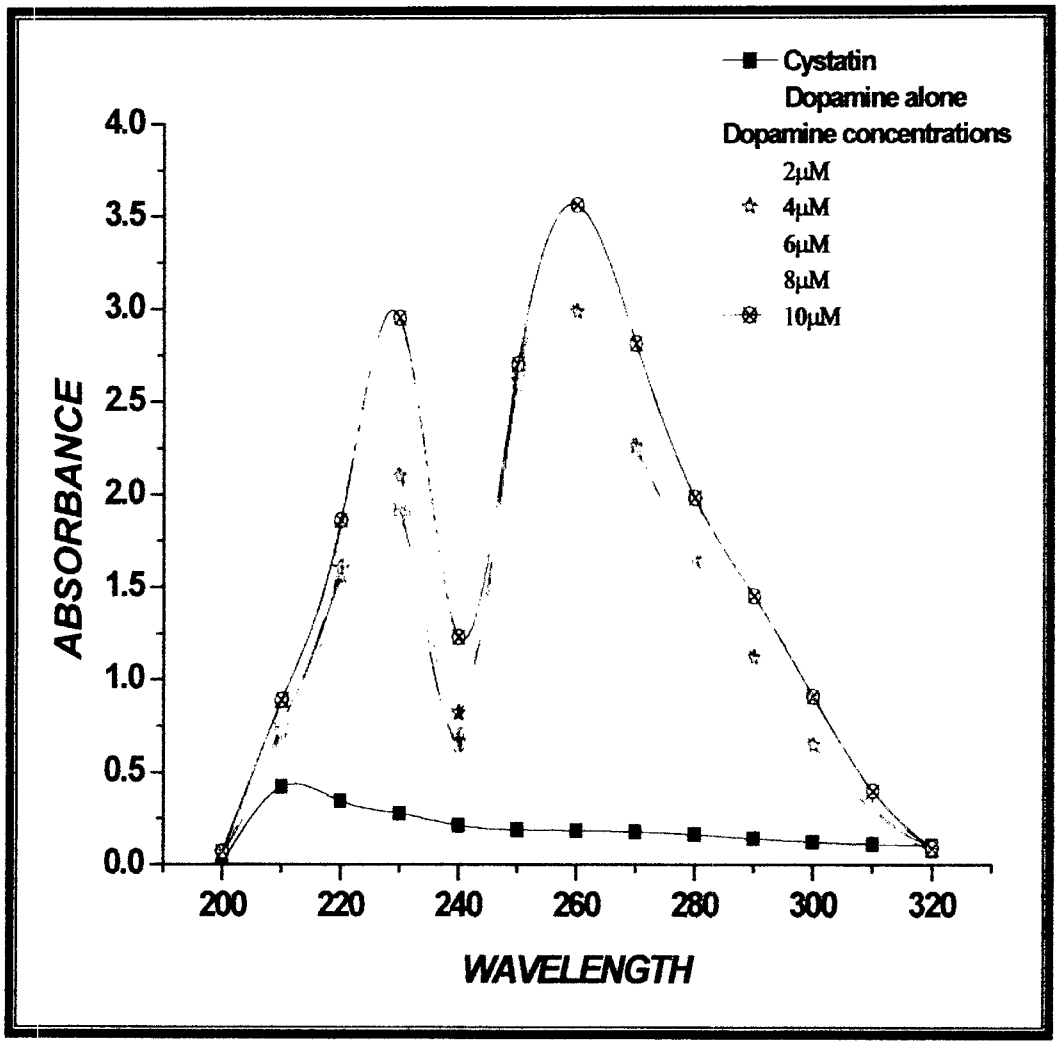
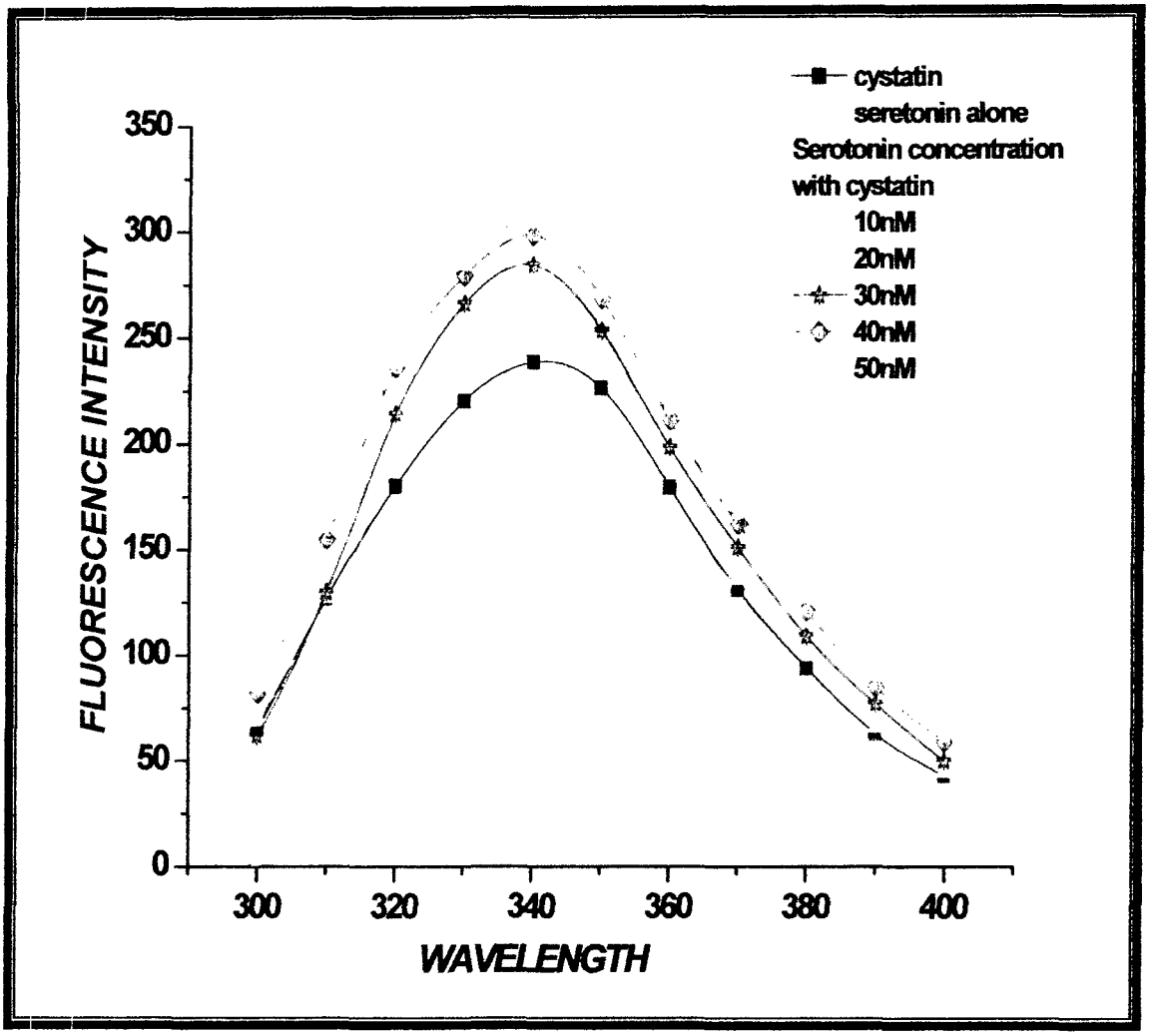

Cystatin
Dopamine alone
Dopamine concentrations
2μM
4μM
6μM
8μM
10μM
ABSORBANCE
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
200
220
240
260
280
300
320
WAVELENGTH

TABLE-22 INHIBITORY ACTIVITY OF CYSTATIN IN THE PRESENCE OF DOPAMINE

Changes in the inhibitory activity of Brain cystatin after incubation with increasing concentration of Dopamine are shown in the table 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.

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

All data are expressed as mean ± S.E for four different sets of experiments statical significance was conducted employing ONE WAY ANOVA .a probability level of 0.05 was selected showing results are significant.


cystatin
seretonin alone
Serotonin concentration
with cystatin
10nM
20nM
30nM
40nM
50nM
FLUORESCENCE INTENSITY
WAVELENGTH
0
50
100
150
200
250
300
350
300
320
340
360
380
400

UV-vis absorption studies of serotonin complex with cystatin

Cystatin concentration was fixed at 1μM while the serotonin concentration was varied from 10-50 μM. Absorption spectra of native cystatin in the presence and absence of serotonin were recorded in the range of 200-300 nm .The UV absorption intensity of BC decreased with increasing concentration of serotonin ***[Fig-56].*** Decrease in absorption intensity may arise due to perturbation of absorbing groups.

Inhibitory activity of BC in the presence of Serotonin

Changes in the inhibitory activity of cystatin with increasing concentration of serotonin is shown in ***[Table-23]***. The result showed that cystatin lost significant amount of inhibitory activity at 10 nM concentration of serotonin. Profound loss was seen at 30 nM of serotonin concentration this loss further increases and at 50nM major loss seen in inhibitory activity (85%) of cystatin.

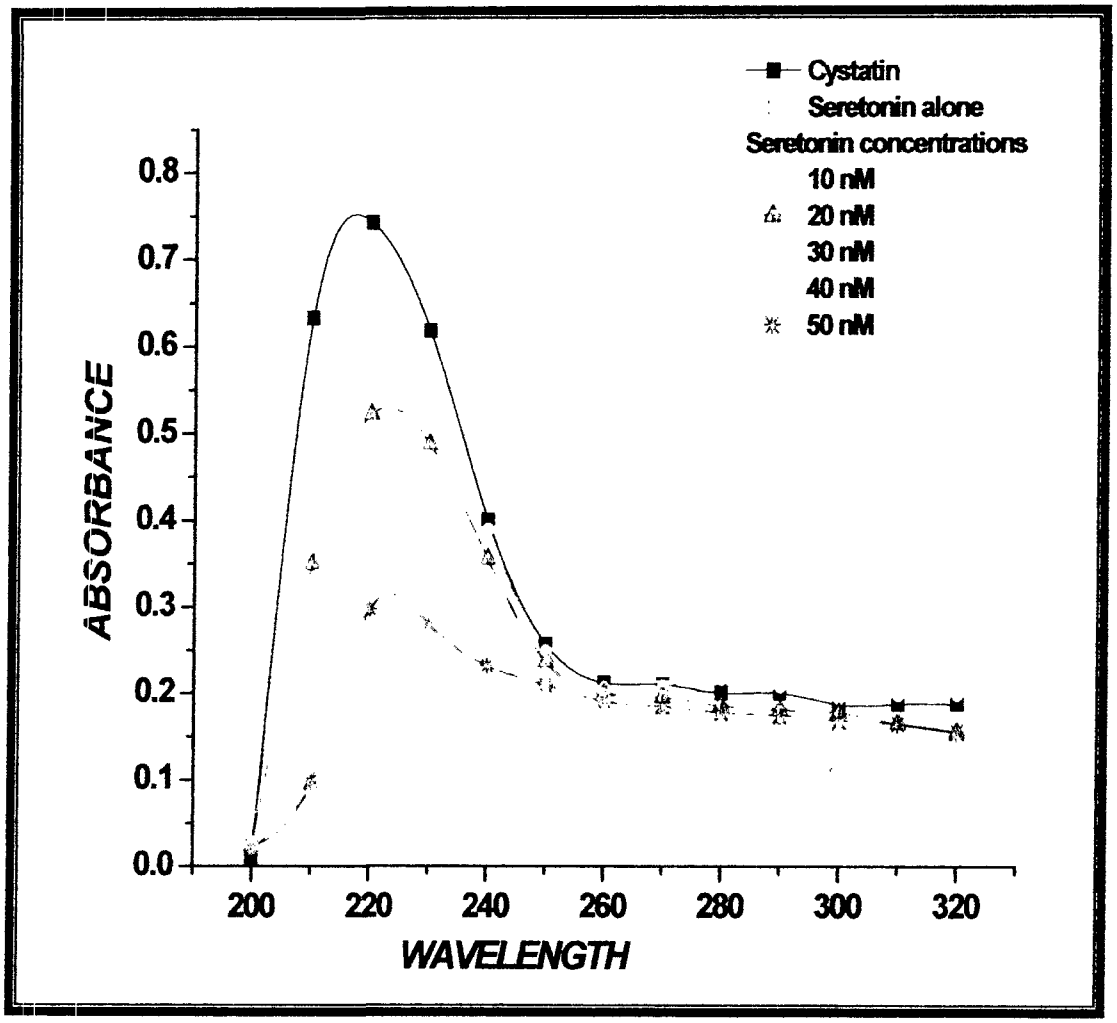

Cystatin
Seretonin alone
Seretonin concentrations
10 nM
20 nM
30 nM
40 nM
50 nM
ABSORBANCE
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
200
220
240
260
280
300
320
WAVELENGTH

TABLE-23 INHIBITORY ACTIVITY OF CYSTATIN IN THE PRESENCE OF SEROTONIN

Changes in the inhibitory activity of Brain cystatin after incubation with increasing concentration of Serotonin. Cystatin (1µM) was incubated with serotonin (10nM-50nM)for 30min in the final reaction volume of 1 ml in 0.05M sodium phosphate buffer pH 7.5.

S.NO	Concentration of Serotonin	% Remaining inhibitory activity of cystatin
1	Cystatin alone	100
2	Cystatin+10 nM Serotonin	46 ± 0.761
3	Cystatin+20 nM Serotonin	32 ± 0.745
4	Cystatin+30 nM Serotonin	25 ± 1.084
5	Cystatin+40 nM Serotonin	20 ± 0.926
6	Cystatin+50 nM Serotonin	15 ± 0.789

All data are expressed as mean ± S.E for four different sets of experiments statical significance was conducted employing ONE WAY ANOVA .a probability level of 0.05 was selected showing results are significant.

TABLE-24 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
AMYTRIPTYLINE	0.115×10^6	3.018×10^6	0.89	-36.966
FLOUXETINE	0.5×10^6	5.0317×10^6	0.79	-38.232
HALOPERIDOL	15×10^6	7.988×10^6	1.1	-39.377
DOPAMINE	0.5×10^6	2.592×10^6	0.798	-36.589

Discussion

Chapter-4

DISCUSSION

Dopamine It is a member of the catecholamine family, it is a precursor of norepinephrine and epinephrine. Dopamine is crucial for human movement 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 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) ; Huang B. et al. (2002)]* In addition, the effectiveness of drugs depends on their binding ability to target proteins. It has been shown that the distribution, free concentration and 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) ; Channu et al. (1999)]* Therefore, study of the interactions between proteins and drug molecules help to provide basic information on the pharmacological actions and bio-distribution.

When cystatin was interacted with dopamine it shows decrease in fluorescence .The quenching of cystatin is accompanied by the blue-shift in fluorescence ***[Fig-53]***. This indicates the increase of polarity of the fluorophore environment in the cystatin, probably due to the hydrogen bonds 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- 24]*** showed that complex formation between dopamine and Cystatin occur spontaneously, Our finding is similar to the binding of methotexate and berberine drug with human serum albumin *[Khan et al. (2007) Yan-Jun Hu et al. (2008)]* 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 relative to serotonin. The treated inhibitor lost only 44% of its

antiproteolytic activity in the presence of 4μM dopamine ***[Table-22].*** 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 *[Shahper et al. (2007)].*

Interaction between dopamine- cystatin complex 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 both the proteins is altered on complexation. ***[Fig-54]***. At higher ratios, shift of spectra towards longer wavelengths was observed. These evidences clearly indicated that there is interaction and complex formation between dopamine and cystatin results are supported from literature. *[Cui et al. (2004) ; Hu et al. (2004)].*

Serotonin is a neurotransmitter, a type of chemical that helps relay signals from one area of the brain to another. Although serotonin is manufactured in the brain, where it performs its primary function, some 90% of the serotonin supply is found in or from the digestive tract and in blood platelets. Because of its widespread distribution in cells, it is believed to influence a variety of psychological and other body functions. Out of approximately 40 million brain cells, most are influenced either directly or indirectly by serotonin.

Imbalance in serotonin levels may influence mood in a way that leads to depression. Possible problems include low production of serotonin in brain cell, a lack of receptor sites able to receive the serotonin that is made, inability of serotonin to reach the receptor sites, or a shortage in tryptophan(the chemical from which serotonin is made). If any of these biochemical glitches occur, researchers believe it can lead to depression, as well as obsessive-compulsive disorder, anxiety, panic, and even excess anger.

Drugs that enhance serotonergic neurotransmission are useful or potentially useful in treating a variety of major psychiatric and metabolic derangements, including depression, eating disorders, alcoholism, pain, anxiety, and obsessive-

compulsive behavior.*[Fuller (1986)]* Serotonin released at synapses is actively removed from the synaptic cleft via a presynaptic serotonin transport carrier proteins. Inhibitors of presynaptic reuptake augment physiological signals mediated by serotonin by increasing its availability in the synaptic cleft, thereby increasing postsynaptic receptor activation. The availability of a variety of compounds that selectively inhibit neuronal uptake of serotonin, without an effect on uptake of the catecholamine, Dopamine, has been invaluable in elucidating the central role of serotonin in several physiological systems and pathophysiological states*[Lemberger (1985)]*.

The interaction between serotonin and cystatin leads to unfolding of the protein.Intrinsic fluorescence studies of cystatin in the presence of Serotonin shows enhancement in fluorescence intensity with no shift in maximum emission (λ_{max}) which indicates perturbation in the environment of aromatic residues and unfolding of cystatin in the presence of serotonin ***[Fig-55].*** 1µM cystatin treated with increasing concentrations of the serotonin (10-50 nM) was analyzed spectroscopically by fluorescence and U.V measurements in the emission range from 300-400 nm and 200-300nm respectively. The results are shown in ***[Fig-55]***, at low concentration only mild changes were induced in cystatin in the presence of 10 nM serotonin, the fluorescence intensit increased slightly .However, at all the drug concentrations, wavelength of maximum emission (λ_{max}) remained unaltered.The U.V spectra obtained for cystatin -serotonin complex showed decrease in absorbance with increase in serotonin concentration***[Fig -56].***

The concentration of serotonin depicts the effect on cystatin activity. 1µM cystatin was incubated with increasing concentrations of serotonin (10-50 nM) in 0.05 M sodium phosphate buffer pH 7.5 at room temperature for 30 min. The activity of native cystatin was taken to be 100%. As shown in the ***Table-23,*** 75% loss in cystatin activity occurred at 30 nM concentration and 80% loss precipitated at 40 nM serotonin , at 50 nM only 15% activity was left.

All these results taken together show that serotonin causes unfolding in the cystatin as compared to binding taking place in case of dopamine. The two neurotransmitter dopamine and serotonin react differently with cystatin therefore cystatin might be playing some role in the mechanisim of action of this neurotransmitters.thus interaction of cystatin with dopamine and serotonin need to be further investigated to work out the mechanisim of these neurotransmitters.

Summary

SUMMARY

Cysteine proteinase inhibitors of the cystatin superfamily are ubiquitous in mammalian system. 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. Cystatin are widely distributed in mammalian system these proteins are non covalent tight binding inhibitors and they have been found to be evolutionary, structurally and functionally related proteins

On the basis of sub cellular localization, disulphide bonds, number of amino acid residues and sequence homology, cysteine proteinase inhibitors have been classified into three families. Family I called as **Stefins** which include members containing about 100 amino acid residues (MW 11,000), they lack disulphide bridges and carbohydrate content. These inhibitors are present in cells and tissues, Family II known as **Cystatin family** includes inhibitors of about 115 amino acid residues (MW 13000) and two disulphide loops towards the carboxyl terminal. They are found both in the cells and body fluids. **Kininogens** or family III cystatins are large precursor molecules of the vasoactive kinins. They are single chain high molecular weight glycoprotein, which serve a variety of physiological functions such as kinin delivery and induction of endogenous blood coagulation cascade. They are found only in blood plasma. These cystatins all together comprise of the cystatin superfamily.

Cystatins have been purified from several mammalian sources like muscle, heart , kidney, spleen, liver, skin and placenta. In the present study a cystatin, of molecular mass (44,000) has been purified from buffalo brain. The purification has been achieved by a simple three step procedure involving alkaline treatment (pH 11) ammonium sulphate fractionation and gel filtration chromatography on Sephadex G-75. Two protein peaks (peak-I and peak - II) with significant inhibitory activity in peak -I was obtained it was named as BC (Brain Cystatin) the pooled fractions of this peak on PAGE showed homogeneity on the basis of charge, BC was obtained with 64.13% yield and 384.72 fold purification peak - II was not taken into consideration because of

its insignificant inhibitory activity and low protein content. Purified protein consist of two polypeptide chains as determined by SDS-PAGE in the presence and absence of β-mercaptoethanol, The molecular weight of the inhibitor was found to be 31.62 KDa and 12.58KDa ,however by gel filtration the molecular weight was 43.6 KDa .The stoke's radius of BC was revealed as 27 A^0. Purified Cystatin was lacking in both carbohydrate and disulphide bonds and the protein was found to be maximally active in the temperature range of 25 - 60 ^{0}C The pH stability was in between pH 6.0- 10.0.

BC strongly inhibited activities of proteases like papain, bromelain and ficin, with no inhibition of serine proteases namely trypsin and chymotrypsin. The inhibition kinetics revealed that the cystatin is a tight binding inhibitor. The respective Ki values of BC obtained for the three proteinases in the order of papain, ficin and bromelain were 1.0 nm 1.85 nm and 2.25nm. The IC_{50} values for BC were 0.09μM, 0.12μM and 0.15μM for papain, ficin and bromelain, respectively. kinetic parameters taken together imply that the cystatin binds more effectively to papain, then bromelain and least with ficin. The results of the study show that purified cystatin may be considered as a variant of type I cystatin , the stefins class of cystatin superfamily.

The effect of nitric oxide on functional and structural modifications of BC was also investigated. It was found that nitric oxide quenched tryptophan fluorescence. Protective effect of Curcumin and querecitin (widely used as scavengers of NO) were analyzed on cystatin dysfunction by NO . Both the scavengers were found to have very significant preventive effect against NO induced functional and structural damage to BC, they were able to protect 90% of the antiproteolytic activity of cystatin damage by NO.

To investigate the effect of ROS on cystatin, HOCl and H_2O_2 were used as oxidants. The results reveal that BC, in the presence of HOCl lost its antiproteolytic activity rapidly. Another oxidant hydrogen peroxide (H_2O_2) showed significant functional and structural damage at 250mM concentration. Effect of riboflavin studied with BC showed that oxidant inactivated the inhibitor, leading to 76% loss of inhibitory activity at 50 μM riboflavin concentration . Various antioxidants namely Quercetins ,curcumin glucose and scavengers Mannitol ,Sodium azide, Thiourea ,Potassium iodide and

Sodium benzoate used in the study indicated the involment of hydroxyl radicals and flavin triplet in the damage.

To investigate the effect of drugs on cystatin four drugs (amytriptyline, fluoxetine , haloperidol , donepezil) and two neurotransmitter (dopamine and serotonin) were used in this study . Amytriptyline with its increasing concentration it caused quenching of the intrinsic fluorescence of cystatin, with no shift in wavelength.

Fluoxetine is approved for the treatment of major depression. This Drug also causes quenching of the intrinsic fluorescence of Cystatin with 10 nm of blue shift in wavelength. Fluorescence intensity decreased, with a maximum decrease at 2μM concentration of the drug. On interaction of cystatin with 0.5 μM fluoxetine, loss of cystatin inhibitory activity was also noticed. Haloperidol is a typical antipsychotic drug it also gave a decline in fluorescence intensity and inhibitory activity. All these results indicated binding of Amytriptyline Fluoxetine and Haloperidol with cystatin.

Donepezil, a reversible acetylcholinesterase inhibitor. It caused unfolding of the cystatin as indicated by enhancement in fluorescence intensity. The inhibitor was further analyzed for its interaction with neurotransmitters dopamine and serotonin. Dopamine is essential for the normal functioning of the central nervous system. It caused quenching of the intrinsic fluorescence with a blue shift. However Serotonin caused unfolding of the BC as indicated by enhancement in fluorescence intensity. The result showed that cystatin lost significant amount of inhibitory activity at 10 nM concentration of serotonin.

Results of fluorescence on binding of the drugs were further analyzed by stern volmer equation for the no binding site , binding affinity and free energy change of the reaction .Results indicated one binding site for each drug amtriptyline (0.89) Fluoxetine (0.79) haloperidol (1.1) and dopamine (0.798). The binding affinity value was found to be in the range of 3.018×10^6 , 5.0317×10^6 , 7.988×10^6 and 2.592×10^6 for amtriptyline , Fluoxetine, haloperidol dopamine respectively showing highest binding affinity for haloperidol . The free energy change (ΔG^0) value was in the order of -39.377 > -38.232 > -36.966 >-36.589 for Haloperidol, Fluoxetine, Amytriptyline and Dopamine respectively, showing that the reaction of these drugs with cystatin

was spontaneous. Investigations in this work not only gives a detailed physiochemical characterization of the cystatin purified from buffalo brain but also gives a comprehensive information about drug-protein interaction. Understanding the conformational changes that result in a protein by various drug treatments may provide a powerful tool for drug design.

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Biography

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