Material
&
Methods
2. MATERIAL AND METHODS

A) MATERIALS

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

<table>
<thead>
<tr>
<th>S.no</th>
<th>Source/Company</th>
<th>Chemicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Genei Bangalore, India</td>
<td>Molecular weight markers – MWM,</td>
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<td>5.</td>
<td>Sigma Chemicalsco. USA</td>
<td>Bovine serum albumin, Bromelain,</td>
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<td></td>
<td></td>
<td>Chymotrypsin, Curcumin, Griess reagent, Ficin,</td>
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<td></td>
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<td>hydrogen peroxide, Ovalbumin, Papain,</td>
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<td></td>
<td></td>
<td>riboflavin, Sephadex G-75, Sodium nitrite,</td>
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<td></td>
<td>Soyabean trypsin inhibitor, Trypsin, 2-mercaptoethanol, sodium azide,</td>
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<td></td>
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<td>Blue dextran, Dopamine and serotonin</td>
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<td>6.</td>
<td>Qualiegens Fine Chemicals, India</td>
<td>ammonium sulphate, bromophenol blue,</td>
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<td>disodium hydrogen phosphate, ethyl alcohol,</td>
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<td>potassium iodide, sodium benzoate, sodium carbonate,</td>
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<td>sodium chloride, sodium hydroxide, sodium potassium tartarate, sulphuric</td>
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<td>acid, thiourea, trichloroaceticacid, TEMED, Tris (hydroxymethyl amninomethane).</td>
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<td>Sisco Research Lab (SRL), India</td>
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<td></td>
<td>casein, L-cysteine, glucose, commassie brilliant blue- R250, EDTA, Folin</td>
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<td>Ciocalteau’s phenol reagent, glycine, phenol and sucrose.</td>
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<td>Drugs</td>
<td>Amytriptyline (Merind manufactured by work hardt limited),Fluoxetine</td>
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<td></td>
<td></td>
<td>(Cadila pharmaceutical limited),Haloperidol (Torrent pharmaceutical ltd)</td>
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<td></td>
<td></td>
<td>Donepezil (Eisai pharmaceutical limited)</td>
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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°C 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°C 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°C 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°C. After 4hrs precipitate was removed by centrifugation at 11000rpm for 30 minutes at 4°C and the supernatant thus collected was made 60% saturated with ammonium sulphate. The solution was allowed to stand for 4hrs at 4°C, the precipitate thus obtained by centrifugation at 11000 rpm for 30 minutes at 4°C 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°C 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 deaerated 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 30 minutes 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 appropriate aliquots 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−1cm−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°C in 0.05 M sodium phosphate buffer, pH 7.5. The enzyme inhibitor complex was further incubated with casein for 30 minutes at 37°C 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 caseinolytic 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°C
for 10 minutes in the presence of 0.14M cysteine. Then the volume was made up to 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°C. 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 containing 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 into 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°C 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

Comassie 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

Stoichiometry 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°C. 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 at 37°C. 1 ml of 2% casein was added and again incubated for 30 minutes at 37°C. 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].
\[
\frac{I_0}{V_i} = k_i \left[ 1 + \frac{[S]_0}{k_m} \right] \frac{V_i}{V_0} + E
\]

Where \([I_0]\), \([E]\) and \([S]_0\) are initial concentrations, enzyme and substrate respectively. \(V_0\) is the velocity without inhibitor. The plot of \([I]\) against \(V_0/V_i\) is a straight line, the slope of which gives:

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

True \(K_i\) was obtained from a replot of \(K_i(\text{app})\) against \([S]_0\). Similar experiments for \(K_i\) determination were done for ficin and bromelain 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±20°C. 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+1°C in Shimadzu spectrofluorimeter model RF-540 equipped with a data recorder DR-3. The excitation wavelength was 250nm (\(\lambda_{\text{max}}\)) 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°C 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%. H₃PO₄. 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.
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 spectrofluorimeter model RF-540 equipped with data recorder DR-3 25±0.1°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 H2O2 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 ε=350/M/cm) [Marries
HOCI 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 HOCI**

BC (1μM) was incubated with HOCI (1-5μM) for 30 min or with 5μM HOCI 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, HOCI 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 HOCI Induced Modification of Cystatin**

BC (1μM) was incubated with 5μM of HOCI 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 H₂O₂ was estimated prior to experiment by the absorbance at 230 nM and ε = 0.081 mM⁻¹ cm⁻¹ [Andrease (1955)].

**Treatment of Brain Cystatin with H₂O₂**

BC (1μM) was incubated with (1mM - 250mM) H₂O₂ 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 H₂O₂ 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 H₂O₂ induced modifications of Cystatin**

1μM of BC was incubated with 250mM of H₂O₂ 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 presence 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 Alzheimer drug) and neurotransmitter (Dopamine and Serotonin) with the Cystatin.
2.10 SPECTROCOPI STUDES

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

\[
\frac{F_0}{F} = 1 + K_{sv} [Q]
\]

Where \(F_0\) and \(F\) are the fluorescence intensities in the absence and presence of quencher respectively, \(K_{sv}\) 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 independently 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)]

\[
\log \left( \frac{F_0 - F}{F} \right) = \log K + n \log [Q]
\]
Where $K$ and $n$ are the binding constant and number of binding sites respectively thus a plot of $\log \left( \frac{(F_0-F)}{F} \right)$ 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°C for 30 min before the activity was measured. Activity of untreated BC was taken as 100%.