Materials & Methods
2. MATERIALS

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

1. Gene I, Bangalore, India

Molecular weight marker-PMW-M, Goat anti-rabbit IgG alkaline phosphatase conjugate.

2. Hi-Media, India

Agar agar, agarose, Freund's complete adjuvant, Freund's incomplete adjuvant, nutrient agar, nutrient broth, sodium azide.

3. Merck, India

Benzoyl-DL-arginine-p-nitroanilide hydrochloride (DL-BAPA), Papain.

4. Pharmacia Fine Chemicals, Sweden

Blue dextran, Sephadex G-75.

5. Qualigens Fine Chemicals, India

Acetone, ammonium persulfate, ammonium sulphate, bromophenol blue, cupric sulphate, cupric chloride, DMSO, disodium hydrogen phosphate, ethyl alcohol, glacial acetic acid, hydrochloric acid, isopropanol, mannitol, methanol, monosodium dihydrogen phosphate, potassium iodide, silver nitrate, sodium bicarbonate, sodium carbonate, sodium chloride, sodium formate, sodium hydroxide, sodium lauryl sulphate, sodium potassium tartrate, sodium thiosulfate, sulphuric acid, thiourea, trichloroacetic acid, TEMED, tris (hydroxymethyl amino methane), Tween-20.

6. Sigma Chemicals Co. USA

Bromelain, ficin, pepsin, ovalbumin, PVDF membrane and CAPS buffer.
7. Sisco Research Lab (SRL), India

Acrylamide, bis-acrylamide, bovine serum albumin, casein, chymotrypsin, L-cysteine, comassie brilliant blue-R250, cytochrome C, DEAE-cellulose, DTNB, EDTA, Folin's phenol reagent, GdnHCl, glycine, p-nitrophenyl phosphate, phenol, ribonuclease, riboflavin, sucrose and urea.
3. METHODS

PURIFICATION OF GOAT KIDNEY CYSTATIN

Fresh goat kidneys (100g) were collected from the abbatoir immediately after slaughter. The kidneys were homogenised in 1% (w/v) NaCl, 2% (v/v) butanol and 0.2M EDTA at 4°C. The supernatant was collected by centrifugation at 3000rpm at 4°C in a Beckman J-21 cooling centrifuge.

Alkaline Treatment

The supernatant obtained was subjected to alkaline pH of 10.0 by 3N NaOH and incubated at 37°C for 10min. After the incubation, the pH of the supernatant was again brought to pH 7.5 using 2N HCl. The precipitated proteins were removed by centrifugation at 3000rpm at 4°C in a Beckman J-21 cooling centrifuge.

Ammonium Sulphate Fractionation

The supernatant was fractionated with 50% saturation of ammonium sulphate with gentle stirring at 4°C. After 4-6h, the precipitate was removed by centrifugation at 3000rpm for 15min and the supernatent thus collected was made 80% saturated with ammonium sulphate. The solution was allowed to stand at 4°C for 10h, the precipitate recovered by centrifugation at 3000rpm for 20min and dissolved in 15ml of 50mM sodium phosphate buffer, pH 7.5. The fraction was extensively dialysed against several changes of the same buffer at 4°C to remove ammonium sulphate.

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 3h in a boiling water bath. The gel fines were removed by suspending the gel in
two to four fold excess 0.05M sodium phosphate buffer, pH 7.5 and allowing 90-95% of the gel to settle down. The remaining gel in supernatant was rapidly removed by suction. 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 glass rod into the 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 (0.05M 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 0.05M 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 millilitre fractions were collected and assayed for protein and cystatin activity. Homogeneity of the preparation was analysed by 10% PAGE.

**Ion-Exchange Chromatography**

Appropriate amount of DEAE-cellulose was suspended with 15 volumes of 0.5N hydrochloric acid for half an hour with constant stirring and washed in a Buchner funnel. The washing was continued till the pH of the filtrate was approximately equal to 7.0. The exchanger was then treated with 15 volumes of 0.5N sodium hydroxide for half an hour and washing continued till the pH of the filtrate approached neutrality. The DEAE-cellulose was then resuspended in operating buffer (0.05M sodium phosphate buffer, pH 8.5) to get a homogenous slurry. This slurry was poured on a vertically mounted glass column plugged with glass wool.
with the help of a glass rod. The column was left standing for one hour. Flow rate was subsequently adjusted with the help of stop cock until constant rate of flow higher than required for final elution reached. Finally the required flow rate was adjusted. The column was then equilibrated with excess of the operating buffer till the pH of the effluent was identical to that of the eluting buffer. Elution was performed with a linear gradient (0-0.3M) of sodium chloride in 0.05M sodium phosphate buffer, pH 7.5. Pooled fractions after gel filtration having protein and activity were loaded. Three millilitre fractions were collected and assayed for inhibitory activity and protein concentration.

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 tubes and final volume was made upto 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 10min of incubation at room temperature with 0.5ml of 1.0N Folin-Ciocalteu's phenol reagent. The tube were instantly vortexed. The colour developed was read at 660nm after 30min against a reagent blank. A standard curve was prepared using BSA.

Protein in the column fractions was monitored at 280nm in Spectronic-D21 spectrophotometer.

Carbohydrate Estimation

The procedure described by Dubois (1956) was followed. Two millilitre aliquots containing 10-70µg of carbohydrate was pipetted into a test tube and 0.05ml
80% phenol added. This was followed by the addition of 0.5 ml of concentrated sulphuric acid, the stream of acid being directed against the liquid surface rather than against the side of the test tube in order to obtain good mixing. The tubes were allowed to stand for 10 min thoroughly mixed and again incubated for 20 min at 30°C. The colour intensity was measured at 490 nm for the quantitation of hexose content. Glucose was used as the standard.

**Thiol Group Estimation**

The procedure described by Ellman (1959) was followed for estimating the thiol groups of cystatin. SDS and β-mercaptoethanol induced appearance of free thiol group in the cystatin was followed by titration with DTNB reagent. Appropriate aliquots of 0.2 ml native, SDS and β-mercaptoethanol treated cystatin were mixed with 0.1 ml of DTNB reagent (prepared by dissolving 40 mg in 100 ml of 0.05 M Tris-EDTA buffer, pH 8.0) in a total volume of 3.1 ml. The absorbance was read after 15 min at 412 nm in Spectronic-D21 spectrophotometer. Free thiol concentration was calculated from the absorbance using molar extinction coefficient of 13,600 M⁻¹ cm⁻¹ for the thionitrobenzoic acid group released. A standard plot was prepared using cysteine.

**Assay of Cystatin Inhibitory Activity**

**Caseinolytic assay for papain**

Inhibitory assay of cystatin was performed as described by Kunitz (1947). Kidney cystatin was examined for its ability to prevent thiol proteinases from digesting casein. For determination of inhibitory activity, papain was activated in the presence of 0.2 M EDTA and 0.5 M cysteine for 10 min prior to incubation of papain cystatin complex for 30 min at 37°C in 0.05 M sodium phosphate buffer, pH 7.5. The enzyme inhibitor complex was further incubated with casein for 30 min at
37°C and reaction stopped by the addition of 10% TCA. Acid insoluble material was removed by centrifugation at 2500rpm for 15min. The supernatant was analyzed for acid soluble peptides with Folin's phenol reagent as described by Lowry et al (1951). Ficin inhibition was also assayed by similar method.

**Amidase Assay for Papain**

Papain amidase activity was also measured as described by Ganrot (1966) by using chromogenic synthetic substrate DL-BAPA. Papain (0.1mg/ml) was activated in 20mM EDTA and 50mM cysteine at 37°C for 10min. After incubation at 37°C, 2.0ml freshly prepared BAPA solution (prepared by dissolving 5mg in 1.0ml DMSO and then the solution was brought to 11ml with phosphate buffer) was added and the volume was made upto 3.0ml with 50mM sodium phosphate buffer, pH 7.5. The reaction was stopped by the addition of 0.5ml of 30% acetic acid and the absorbance of the yellow coloured p-nitroanilide formed was determined at 410nm.

**Assay of Caseinolytic Activity of Bromelain and Ficin**

The proteolytic activity of bromelain was measured according to the method of Murachi and Neurath (1960). The enzyme was activated at 37°C for 10min in the presence of 20mM cysteine. Then the volume was made upto 1ml by sodium phosphate buffer, pH 6.0. One millilitre of 0.5% casein was added and incubated for 30min at 37°C. The reaction was stopped by 1.0ml 5% TCA. Acid insoluble material was removed by centrifugation at 2500rpm for 15min. The supernatant was analyzed for acid soluble peptides which were hydrolyzed by the enzyme by Folin's phenol reagent as described by Lowry et al (1951).
SLAB GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis (PAGE)

PAGE was performed by the tris-glycine system of Laemmli (1970) using the apparatus manufactured by Biotech, India. Concentrated stock solution of 30% acrylamide containing 0.8% bisacrylamide and 1.5M Tris, pH 8.8 were mixed in appropriate proportions to the required final concentration. It was then poured into the mould formed by the glass plates (8.5-10cm) separated by 1.5mm thick spacers. Bubbles and leaks were avoided. A comb providing template for 7 sample wells was inserted into the stacking gel solution before polymerisation began. The polymerisation was complete in about 30min after which the comb was removed and wells overlaid with the running buffer. Routinely, a 10% acrylamide gel was used. Samples containing 20-40μg of protein were prepared to give a final concentration of 62.5mM Tris-HCl pH 6.8, 10% (v/v) glycerol and 0.001% bromophenol blue as tracking dye.

SDS Polyacrylamide Gel Electrophoresis (SDS PAGE)

SDS PAGE was essentially performed by the method of Laemmli (1970). Concentrated stock solutions were mixed in appropriate order to give a final concentration of 10%. Protein samples were prepared in solution containing 62.5mM tris-HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol and 0.001% (w/v) bromophenol blue. The samples were incubated at 100°C for 5min. Electrophoresis was performed at 100V till the tracking dye reached the bottom of the gel. Running buffer used during electrophoresis contained 1% SDS in addition to 192mM glycine and 25mM Tris-HCl pH 6.8.
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 in 50% methanol and 10% acetic acid for at least 4h. For destaining, the gels were incubated with shaking in 5% methanol and 7.5% acetic acid at room temperature.

Silver Staining of the Gel

The gel after electrophoresis was silver stained by modified Blum's silver stain method (Nesterenko, 1994). The staining method was initiated by fixation for 10mins in 50% acetone, 1% trichloroacetic acid and 0.015% formaldehyde with subsequent washings. After a second fixation in 50% acetone only the gel was pretreated with 10% sodium thiosulfate and then impregnated with 20% silver nitrate and 37% formaldehyde. The protein bands were developed by immersing the gel in 2% sodium carbonate, 37% formaldehyde and 10% sodium thiosulfate for 30s to 1 min. The reaction was stopped by 1% glacial acetic acid and incubated for 15min in 1% glycerol after rinsing in distilled water.

MOLECULAR WEIGHT DETERMINATION

The molecular weight of goat cystatin was determined under native and denaturing conditions by gel filtration chromatography and SDS PAGE, respectively.

The molecular weight of native goat cystatin was computed from its elution volume on a Sephadex G-75 column (2x70cm). The column was calibrated by determining the elution volume of some marker proteins-bovine serum albumin (68KDa), ovalbumin (45KDa), ribonuclease (12KDa) and cytochrome C
(11.7KDa). This data was analyzed according to the theoretical treatment of Andrews (1964) method. The linear plot between $V_e/V_o$ and $\log M$ was used in calculating the molecular weight of goat cystatin where $V_e$ is the elution volume of the protein and $V_o$ is the void volume of the column determined using blue dextran.

Molecular weight of cystatin 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 was plotted against the logarithms of molecular weight. The standard proteins used were phosphorylase b (97KDa), bovine serum albumin (68KDa), ovalbumin (45KDa) and carbonic anhydrase (29KDa) and soyabean trypsin inhibitor (20KDa). The analysis of data indicated a linear relationship between $\log M$ and relative mobility ($R_m$) and the plot was used in calculating the molecular weight of cystatin.

**IMMUNOLOGICAL PROCEDURES**

**Immunization of Rabbits**

Antibodies against goat cystatin were raised in healthy male albino rabbits. 300μg of electrophoretically pure cystatin in 0.5ml of normal saline emulsified with equal volume of Freund's complete adjuvant was injected simultaneously. Two weeks later 150μg of cystatin was injected along with Freund's incomplete adjuvant. A similar second and third booster dose were given in the third and fourth week respectively and the animal was bled after an additional one week. The blood collected was allowed to coagulate at 22°C for 3h. The antisera was decomplemented at 57°C for 30min and stored at -20°C in small aliquots.
**Immunodiffusion**

Immunodiffusion was performed essentially by the method of Ouchterlony (1962). One percent molten agarose in normal saline containing 0.2% sodium azide was poured in on glass plate and allowed to solidify at room temperature. Required number of wells were cut and the slides were stored at 4°C. Fifteen to twenty microlitres of suitably diluted antisera and required amount of antigen prepared in normal saline were added in different wells. The reaction was allowed to proceed for 12-24h in a moist chamber at room temperature. The petriplates were immersed in normal saline to remove non-specific precipitin lines.

**Enzyme Linked Immunosorbent Assay (ELISA)**

**Direct Binding ELISA**

*Buffers and Substrate:* Tris buffer saline (TBS), pH 7.4. 20mM tris, 150mM sodium chloride. Tris buffer saline Tween-20 (TBS-T), pH 7.4. 20mM tris, 14.3mM sodium chloride, 200mg potassium chloride and 5ml Tween 20 was dissolved in 1 litre distilled water and pH adjusted to 7.4 with 1N hydrochloric acid. Bicarbonate buffer, pH 9.5, 50mM containing 0.02% sodium azide as preservative. Substrate: 5mg p-nitrophenyl phosphate (in 10ml bicarbonate buffer).

*Procedure:* Polystyrene microtitre plates were taken. The test wells were coated with 100μl of the antigen (IIInd isoform, 0.5μg/100ml of bicarbonate buffer). The plate was inoculated for 2h at room temperature and then overnight at 4°C. The plate was washed three times with TBS-T buffer.

The unoccupied sites were saturated by incubation with 150μg/200μl of 1.5% milk in TBS for 5-6h at room temperature. Wash it twice with TBS-T. The test and control wells were then loaded with 100μl of serially diluted serum. Each dilution was in TBS buffer. Serially diluted blanks corresponding to each dilution
should be there. Then the plate was incubated for 2h at room temperature and overnight at 4°C. Wash five times with TBS-T buffer. Bound antibodies were assayed with an appropriate conjugate of anti-rabbit alkaline phosphatase (1:3000). 100µl of it is coated in each well and kept for 2h at room temperature. Wash the plate with TBS-T five times and twice with distilled water. Add p-nitrophenyl phosphate (50µg/100µl) in each well and incubate at 37°C for 30-45min. The reaction was stopped by the addition of 100µl of 3M NaOH in each well. The absorbance of each well was monitored at 405nm on a Labsystems ELISA Reader.

Inhibition ELISA

The specificity of ELISA was ascertained by inhibition experiments (Ali and Ali, 1986). Polystyrene microtitre plate was incubated with 100µl of the IIId isoform antigen (0.5µg/100µl) for 2h at room temperature and then overnight at 4°C. The plate was washed three times with TBS-T buffer. Free sites were blocked by adding 150µg/100µl of 1.5% milk in TBS buffer for 6h at room temperature. Immune complex was made by adding varying amounts of the isoforms of inhibitor (.01-50µg) with a constant amount of antibody (90µg/100µl). The mixture was incubated for 2h at room temperature. 100µl of the immune complex was coated and kept for 2h at room temperature then overnight at 4°C. Plate was washed with TBS-T four to five times. Then 100µl of antirabbit conjugate alkaline phosphatase (1:9000 dilution) was coated to quantitate bound antibodies. The mixture was kept for 2h at room temperature. The plate was washed with TBS-T five times and twice with distilled water. Now add para-nitrophenyl phosphate (5mg/100µl) in each well and keep it for 30min at 37°C. Stop the reaction by adding 100µl 3N NaOH in each well. The absorbance
of each well was monitored at 405nm on a Labsystems ELISA reader. The results were expressed as percent inhibition

\[
\% \text{ inhibition} = \frac{\text{inhibition}}{\text{uninhibited}} \times 100
\]

N-TERMINAL ANALYSIS

Sequencing was performed on Shimadzu PPSQ-21 sequencer which employs Edman degradation to sequentially cleave and identify amino acids starting at the amino terminus (N-terminus) of the protein. The highly purified protein was transferred to the PVDF membrane before the analysis.

ANTIBACTERIAL ACTIVITY OF CYSTATIN

The following bacterial reference strains were used:

- *Staphylococcus aureus*
- *Escherichia coli*
- *Streptococcus hemolyticus*
- *Bacillus subtilis*
- *Pseudomonas fluorescens*

Nutrient broth was composed of nutrient agar (1.3g/l). Ingredients of soft agar included agar agar (0.8g/100ml) and nutrient agar (1.3g/100ml)

The antibacterial activity of cystatin isoform was checked by determination of inhibition zone diameter (Kasprzykowski, 2000). The bacterial strains were allowed to grow overnight in nutrient broth at 37°C, 0.3ml of the fresh culture were overlayed with soft agar on nutrient agar plates to aid the formation of the bacterial lawns. Whatman filter paper discs were put in 25\(\mu\)g/ml, 50\(\mu\)g/ml, 100\(\mu\)g/
ml and 1mg/ml of inhibitor for the inhibitor to be absorbed on the discs. After 2h the discs were placed carefully on the bacterial lawn. The plates were then kept in the incubator at 37°C overnight. The antibacterial effect was classified as (+), +, ++ or +++ based upon the inhibition zone diameter of 11-12mm, 13-14mm, 15-16mm and 17mm and more than 17mm respectively. An absence of antibacterial activity is denoted by the symbol “—” in the table.

SPECTRAL ANALYSIS

UV Absorption Spectroscopy

The UV absorption spectra of native and papain treated cystatin was taken by measuring the absorption between 200-350nm in a Camspec spectrophotometer in a cuvette of 1cm path length. Appropriate controls of the solvent buffer were taken. 100μg of the inhibitor in a total volume of 1ml was taken for the spectra.

Fluorescence Spectroscopy

Native and papain (activated with 20mM EDTA and 50mM cysteine) treated complex of cystatin were analyzed by measuring intrinsic florescence at 25±0.2°C in Hitachi F 2000 spectroflurometer (Tokyo, Japan). The protein was excited (λexc) at 280nm. Corrected emission spectra were recorded with excitation and emission band widths of 10nm. Controls containing the reagents used for the treatment were run and corrections made wherever necessary.

To study the cumulative effect of tyrosine, tryptophan and phenylalanine, the protein was excited at 280nm. Denaturation of the inhibitor in the presence of 8M urea, 6M GdnHCl and 100mM β-mercaptoethanol was studied. Solutions of 1.2mM inhibitor with urea, GdnHCl and β-mercaptoethanol with final concentration of 8M, 6M and 100mM, respectively in 50mM sodium phosphate
buffer, pH 7.5, were mixed and incubated for 30 minutes. The temperature was maintained at 22°C by thermostating the cuvette housing during the measurement. The time of mixing was t=0. The fluorescence spectra were recorded after 30 min.

**Circular Dichroism (CD) Spectroscopy**

Circular dichroism spectra of native cystatin and that after various treatments were measured in a Shimadzu spectropolarimeter equipped with a temperature controlled sample cell holder. Spectra were collected with a scan speed of 20 nm/min and with a response time of 1 s. Measurements in the far UV (200-250 nm) were taken using 2.3 μM (about 0.15 g/l) cystatin, with a 1 mm pathlength cell whereas cell with 1 mm pathlength and cystatin concentrations of about 4.6 μM (0.3 g/l) were used in the near UV (250-300 nm) region. For each sample, a solution of buffer or papain of the same concentration was used as a blank.

The results were expressed as mean residue ellipticity (MRE) in deg. cm². dmol⁻¹ which is defined as

$$\text{MRE} = \frac{\theta_{\text{obs}} \text{ (m deg)}}{10 \times n \times 1 \times C_p}$$

where $\theta_{\text{obs}}$ is the CD in millidegree, n is the number of amino acid residues, l is the pathlength of the cell and $C_p$ is the mole fraction. The $\alpha$-helical content of cystatin was calculated from the MRE value at 222 nm using the following equation as described by Chen et al (1972)

$$\% \text{ helix} = \frac{[(\text{MRE}_{222}-2340)/30300] \times 100}{=}$$

The results in the near-UV CD were expressed as molar ellipticities.
KINETICS OF INHIBITION

Stoichiometry of Proteinase Inhibition

Papain was used for the titration of the two isoforms of cystatin. For this experiment activated papain (0.06 μM) was incubated for 10 min at 37°C with increasing amounts of either isoform in 50 mM sodium phosphate buffer, pH 7.5 in a final volume of 1 ml. 2 ml volume of 2 mM Bz-Arg-p-NA was added to the mixture containing cystatin and this was incubated at 40°C for 60 min. The reaction was stopped with 500 μl 0.3% (v/v) acetic acid and the liberated amine was assayed colorimetrically at 412 nm as described by Ganrot (1966).

Identical experiments were carried out for the inhibitors with other proteinases also such as bromelain and ficin using casein as a substrate for bromelain and ficin described by Kunitz (1947) and Murachi and Neurath (1960).

Ki Determination

Ki determination were carried out according to the same procedure but after lowering enzyme and inhibitor concentrations to obtain a non-linearity of dose response curves. Thus papain was used at a concentration of 0.05 μM to react with I and II isoforms varying from 0.011 to 0.17 μM and measurement of residual activity were made as described by Kunitz (1947) using casein substrate. Four different substrate concentrations were used, i.e. 0.5Km, 1Km, 2Km and 3Km, with Km=2.4 mM and the results were analyzed by the steady state equation of Krupka and Laidler (1959). This is linearized as follows (Henderson, 1972).

\[
\frac{[I_0]}{1-V/V_0} = K_i \left(1 + \frac{[S]_0}{K_m}\right) \frac{V_i}{V_0} + [E]_0
\]  

(1)
where \([I]_0\), \([E]_0\), and \([S]_0\) are the initial concentrations of inhibitor, enzyme and substrate, respectively. \(V_o\) is the velocity without inhibitor. The plot of \([I]_0/(1-\frac{V_i}{V_o})\) against \(\frac{V_i}{V_o}\) is a straight line, the slope of which gives

\[
K_{i(app)} = K_i \left(1 + \frac{[S]_0}{K_m}\right)
\]

(2)

True \(K_i\) was obtained from a replot of \(K_i\) (app) against \([S]_0\).

**Determination of Association Rate Constants (\(K_{+\text{i}}\))**

\(K_{+\text{i}}\) values were determined by monitoring the time-dependence of the association under second-order conditions. Equimolar amounts of enzyme and inhibitor (0.05 \(\mu\)M) in the final volume were mixed in a total volume of 1 ml of the same buffer, pH 7.5 at 37°C and residual activity was assayed as a function of time after 0, 2, 5, 10, 15, 20, 25 and 30 min as described above using casein as a substrate for papain, bromelain and ficin.

Association rate constant (\(K_{+\text{i}}\)) was calculated assuming that enzyme (E) and either inhibitor (I) react according to eqn (3) and considering \(K_{-\text{i}}\) to be low enough to neglect the reverse reaction during the initial part of the process:

\[
E + I \leftrightarrow EI
\]

(3)

Thus, when initial concentrations of enzyme, \([E]_0\) and inhibitor \([I]_0\) are identical, the integrated form of the equation giving association rate becomes:

\[
\frac{1}{[E]} = \frac{1}{[E]_0} + K_{+\text{i}}t
\]

(4)
where \([I]_0\), \([E]_0\) and \([S]_0\) are the initial concentrations of inhibitor, enzyme and substrate, respectively. \(V_o\) is the velocity without inhibitor. The plot of \([I]/(1-V/V_o)\) against \(V_o/V_i\) is a straight line, the slope of which gives

\[
K_{i(app)} = K_i \left(1 + \frac{[S]_0}{K_m}\right)
\]

(True \(K_i\) was obtained from a replot of \(K_i\) (app) against \([S]_0\).

**Determination of Association Rate Constants (\(K_{+1}\))**

\(K_{+1}\) values were determined by monitoring the time-dependence of the association under second-order conditions. Equimolar amounts of enzyme and inhibitor (0.05μM) in the final volume were mixed in a total volume of 1ml of the same buffer, pH 7.5 at 37°C and residual activity was assayed as a function of time after 0, 2, 5, 10, 15, 20, 25 and 30min as described above using casein as a substrate for papain, bromelain and ficin.

Association rate constant (\(K_{+1}\)) was calculated assuming that enzyme (E) and either inhibitor (I) react according to eqn (3) and considering \(K_{+1}\) to be low enough to neglect the reverse reaction during the initial part of the process:

\[
E + I \leftrightarrow EI
\]

Thus, when initial concentrations of enzyme, \([E]_0\) and inhibitor \([I]_0\) are identical, the integrated form of the equation giving association rate becomes:

\[
\frac{1}{[E]} = \frac{1}{[E]_0} + K_{+1} t
\]
Determination of Dissociation Rate Constants (K_d)

Conditions for maximal association between the proteinase and either inhibitor were achieved before the reaction was shifted towards dissociation by adding either α2M which forms stable and enzymically active complexes with free enzymes or excess of substrate, which also binds all the free enzyme (Beith, 1980). Substrate induced dissociation was monitored with identical enzyme inhibitor complexes incubated for 30min at 37°C. Excess substrate (6% casein) was added to the mixture for different time periods and then assayed for their enzymic activity.

On the basis of eqn (3) the dissociation of EI complex obeys first order kinetics during the initial part of the reaction, i.e. when there is almost complete association. In this case the integrated form of the dissociation rate equation is given by

\[ \ln \left( \frac{[\text{EI}]}{[\text{EI}_0]} \right) = K_d t \]  

From which K_d and half-life of complexes may be calculated by rearranging as follows:

\[ t_{0.5} = \frac{0.693}{K_d} \]

Similar experiments for Ki determination, association and dissociation rate constants for ficin and bromelain were carried out with either inhibitor using their respective substrates.
CYSTATIN DAMAGE BY RIBOFLAVIN

Cystatin damage and degradation by reactive oxygen species (ROS) was studied by riboflavin. Riboflavin was taken as a source of ROS on photoillumination.

**Time and Concentration Dependent Effect of Riboflavin on Cystatin**

1.76µM cystatin was incubated with 50µM, 100µM, 200µM, 300µM and 400µM concentration of riboflavin in 10mM sodium phosphate buffer, pH 7.5 in a final volume of 500µl for 2h. Fixed molar concentrations of riboflavin and cystatin were incubated for different time periods up to 5h in 10mM sodium phosphate buffer, pH 7.5. Incubation at room temperature (28-30°C) was performed under illumination of 500 flux from a fluorescent lamp. The reaction was stopped by adding the sample dye for PAGE. Aliquots of 100µl were loaded on a PAGE for analysis.

In another experiment the antiproteolytic activity of 1.76µM and 2.92µM cystatin was analysed by incubating with 100µM riboflavin for 60min. The activity was checked by taking 100µl aliquots after 5, 10, 20, 25, 30, 35, 40, 45, 50 and 60min respectively as described by Kunitz (1947).

**Effect of Copper (II) on the Activity of Cystatin**

The effect of Cu (II) in a final concentration of 100µM was studied by incubation with photoilluminated riboflavin and cystatin in the final volume of 300µl. Small aliquots from the reaction were taken for running PAGE (10%).

**Effect of Free Radical Scavengers**

To check the type of reactive oxygen species involved in the oxidation of cystatin scavengers of free radicals were used along with riboflavin. 25mM thiourea,
sodium formate, sodium azide, mannitol were used and incubated at room temperature under aerobic conditions. Retention of cystatin band in 10% PAGE in the presence of riboflavin showed the type of free radical involved in the reaction.