Materials & Methods

2. MATERIALS

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

Genei Banglore, India

Molecular weight markers-PMW-L, Fruend's Complete and Incomplete Adjuvants.

Merck-Schereherdt, Germany

2-Mercaptoethanol, magnesium sulphate, magnesium chloride, sodium suphate

Sigma Chemicals Co. USA

Anti-rabbit alkaline phosphatase (conjugate), Bromelain, CAPS buffer, Chymotrypsin, Cytochrome C, Ficin, Insulin, Malathion, Curcumin, Thioflavin T, Papain, p-nitro phenyl phosphate, PVDF membrane,CNBr activated sepharose 4B, Iodoacetic acid Ribonuclease, Sephadex G_{50-80} , Sorbitol, Soyabean Trypsin Inhibitor,TFE, Trypsin.

Qualigens Fine Chemicals, India

Ammonium persulphate, ammonium sulphate, bromophenol blue, disodium hydrogen phosphate, ethyl alcohol, glacial acetic acid, glycerol, hydrochloric acid, methanol, isopropanol, mannitol, monosodium dihydrogen phosphate, potassium iodide, sodium carbonate, sodium bicarbonate, sodium chloride, sodium hydroxide, sodiun lauryl sulphate, sodium potassium tartarate, sulphuric acid, trichloroacetic acid, TEMED, (Tris (hydroxymethyl aminomethane).

Sisco Research Lab (SRL), India

Acrylamide, N' N' methylene bis-acrylamide, bovine serum albumin, casein, L-cysteine, commassie brilliant blue-R250, cytochrome C, DEAE cellulose, EDTA, Folin ciocalteau's phenol reagent, GdnHCl, glycine, phenol, Urea.

3. METHODS

PURIFICATION OF BUFFALO LIVER CYSTATIN

Fresh liver tissue (100g) was homogenized in 50 mM sodium phosphate buffer of pH 7.5 (200 ml)containing a mixture 1% (w/v) NaCl, 2% (v/v) butanol and 3 mM EDTA. After centrifugation at 6000 rpm for 15 mins at 4°C in Beckman J-21 cooling centrifuge, pellet having cell debris was discarded and the supernatant was further processed.

Alkaline Treatment

The supernatant was adjusted to pH 11.0 by 3N NaOH and then incubated at 4°C for 30 minutes. The precipitated proteins were removed by centrifugation at 8000 rpm for 30 mins at 4°C for in a Beckman J-21 cooling centrifuge. After this, the pH of the solution was brought back to 7.5 with glacial acetic acid.

Ammonium Sulphate Fractionation

The supernatant was fractionated with 40% saturation of ammonium sulphate with gentle stirring at 4°C. After 3 h the precipitate was removed by centrifugation at 8000 rpm for 30 minutes and the supernatant thus collected was made 60% saturated with ammonium sulphate. The solution was further made to stand at 4°C overnight, the precipitate obtained by further centrifugation (8000rpm for 30 minutes) was then dissolved in minimum volume of 50mM sodium phosphate buffer, pH 7.5. The fraction thus obtained was extensively dialyzed against several changes of the same buffer at 4°C containing 1% NaCl to remove ammonium sulphate.

Affinity chromatography

Cm-papain-Sepharose for affinity chromatography was prepared as follows (Anastasi et al., 1983). Papain (30mg) was activated with 2 mM-cysteine and 1 mM-disodium

EDTA in 7.5 ml of 0.1 M-sodium phosphate buffer, pH6.0, for 10min at 200C, and allowed to react with 10mM (final concn.) -iodoacetic acid. The solution was dialysed against 0.1 M-NaHCO3, and stirred overnight with 18g (wet weight) of CNBr activated Sepharose 4B. The gel was then treated with 0.1 M-glycine for 1 h and washed at pH 5 and pH 9. The amount of protein coupled to the gel was determined by the method of Lowry et al. (1951) to be 0.6 mg/g of hydrated gel.

Dilaysed protein from liver source was dissolved in (5 ml) 0.05 M sodium phosphate buffer, pH 6.5, containing 1 mM EDTA, followed by the addition of water (5 ml). The resulting sample was mixed with 18 ml of Cm-papain-Sepharose gel equilibrated in 50 mM sodium phosphate buffer (pH 6.5)/0.5 M NaCl and1 mM EDTA and incubated for 2 h at room temperature. The gel was then poured into a column (1.6 cm-20 cm) and washed with the equilibrium buffer until the A_{280} was close to zero. Bound material was eluted with 0.05 M $K_2 HPO_4$ /NaOH, pH 11.5, until A_{280} approached zero. Fractions (2 ml each) were collected in glass tubes containing 1 ml of 0.25 M $KH_2 PO_4$, pH 4.5, to bring the pH to neutral. Fractions containing bound material were pooled and dialysed against 50mM sodium Phosphate buffer and concentrated by freeze drying. Further fractions were assayed for protein and papain inhibitory activity. Homogeneity of the preparation was analyzed by 12.5 % PAGE.

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 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 min of incubation at room temperature with 0.5 ml of 1.0N Folin-Ciocalteu's phenol reagent. The tubes were instantly vortexed. The color developed was read after 30 min at 660nm against a reagent blank. A standard curve was prepared using BSA. Protein in the column fractions was monitered at 280nm in Campspec Spectrophotometer Model M330 B.

Carbohydrate Estimation

The procedure described by Dubois (1956) was followed. Two milliliter aliquots containing 10-70 µg of protein was pipetted in to a test tube and 0.05ml of 80% phenol was added. This was followed by the addition of concentrated sulphuric acid. The tubes were allowed to stand for 10 min, thoroughly mixed and again incubated for 20 min at 30°C. The color intensity was measured at 490nm for the quantitation 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 cystatin. SDS and β-mercaptoethanol induced appearance of free thiol group in the cystatin was followed by titration with DTNB reagent. Appropriate aloquots of 0.2ml native, SDS and β-mercaptoethanol treated cystatin were mixed with 0.1ml of DTNB reagent (prepared by dissolving 40mg in 100ml of 0.05M Tris-EDTA buffer, pH 8.0) in a total volume of 3.1ml. The absorbance was read after 15 min at 412nm in Campspec 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 standard plot was prepared using cysteine.

Assay of Cystatin Inhibitory Activity

Inhibitory assay of cystatin was performed as described by kunitz (1947). BLC was examined for its ability to prevent thiol proteinases from digesting casein. For determination of inhibitory activity, papain was activated in presence of 0.2 m EDTA and 0.5M cysteine for 10 min prior to incubation of papain cystatin complex for 30 min at 37°C in 20mM sodium phosphate buffer, pH 7.0. The enzyme inhibitor complex was further incubated with casein for 30 min at 37°C and the reaction was stopped by addition of 10% TCA. Acid insoluble material was removed by centrifugation at 2500 rpm for 15 min. The supernatant was analysed for acid soluble peptides with Folin

phenol reagent as described by Lowry 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 activatred at $37^{o}C$ for 10 min in the presence of 20mM cysteine. Then the volume was made upto 1ml by sodium phosphate buffer pH 6.0. One ml of 0.5 % casein was added and incubated for 30 min at $37^{o}C$. The reaction was stopped by addition of 1.0ml of 5% TCA. Acid insoluble material was removed by centrifugation at 2500rpm for 15min. The supernatant was analyzed for acid soluble peptides by Folin phenol reagent by the method of Lowry et al (1951).

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% bisacrylamide 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 glass plates (8.5×10 cm) separated by 1.5mm thick spacers. Bubbles and leaks were avoided. A comb providing template for seven sample wells was inserted into the stacking gel solution before the polymerization began. The polymerization was complete in about 30 min after which the comb was removed and wells overlaid with running buffer. Routinely, a 12.5% acrylamide gel was used. Samples containing 20-40µg of protein 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 192mM glycine and 25mM Tris-HCl pH 6.8 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% biscarylamide and 1.5M Tris, pH 8.8 were mixed in appropriate proportions to give the final required percentage. 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-mercaptoethanol and 0.001% (w/v) bromophenol blue. The samples were incubated at $100^{o}C$ for 5 min. 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 192 mM glycine and 25mM Tris-HCl, pH 6.8.

STAINING OF THE GEL

Commassie Briliant Blue Staining

After electrophoresis was complete the gels were removed and the protein bands were visualized by staining the gel with five gel volumes of 0.25% commassie brilliant blue R-250 in 50% methanol and 10% acetic acid for atleast 4 hours. For destaining, the gels were incubated with shaking in 5% methanol and 7.5% acetic acid at room temperature.

MOLECULAR WEIGHT DETERMINATION

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

Molecular weight of BLC 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 Ovalbumin (43 KDa), Carbonic Anhydrase (29KDa), Soyabean Trypsin

Inhibitor (20.1KDa), Lysozyme (14.3 KDa) and Aprotinin (6.9KDa). The analysis of the data indicated a linear relationship between logM and relative mobility (Rm) and the plot was used in calculating the molecular weight of BLC.

IMMUNOLOGICAL STUDIES

Immunization of rabbits

Antibodies against human placental cystatin were raised in healthy male albino rabbits. Purified inhibitor (300µgs/0.5 ml of normal saline) was emulsified with equal volume of Fruends Complete adjuvant and injected subcutaneously. Two weeks later 150µgs of BLC was injected along with Fruends Incomplete adjuvant.The injection was repeated every week and the rabbit was bled every second week. The blood collected was allowed to coagulate at 22^0C for 3h.The antisera was decomplimented at 57^0C for 30 minutes and stored at -20^0C in small aliquots.

Direct binding ELISA

The generation of antigen specific antibody was measured in the sera of BLC immunized rabbits by the technique of Direct binding ELISA as given by Voller et.al (1976). Ninety six wells of micro-titre plate (immulon 2HB,Dynex,USA) were coated overnight with 100µl of antigen at 4^0C.The plate was washed three times with TBS-T buffer (Tris buffered saline Tween-20, pH 7.4, 20mM Tris,14.3mM sodium chloride,200mg potassium chloride and 5ml Tween 20 dissolved in 1L of distilled water and pH adjusted to 7.4 by 1N HCl).The unoccupied sites were saturated by incubation with 150µgs/200ml of 1.5% milk in TBS(Tris buffered saline pH 7.4,20mM Tris,150mM sodium chloride) for 5-6 h at room temperature. Plates were washed twice with TBS-T. The test and the control wells were then loaded with 100µl of serially diluted serum. The plate was incubated for 2hrs at room temperature and then overnight at 4^0C. 100µls of appropriate conjugate of anti-rabbit alkaline phosphatase (1: 3000) is coated in each well and kept for

2hrs at room temperature. After regular washing with TBS-T and distilled water, the substrate p-nitro phenyl phosphate (5µgs /100 ml of bicarbonate buffer-pH 9.5, 50mM containing 0.02% sodium azide) was added in each well and incubated for 30-45 minutes. The reaction was stopped by addition of 100µl of 3M NaOH in each well. The absorbance of each well was monitored at 405nm on a Qualigens ELISA reader.

Immunodiffusion

Immunodiffusion was performed essentially by the method of Ouchterlony (1962). Agarose (1%) in normal saline containing 0.2% sodium azide was poured on glass plates and allowed to solidify at room temperature. Required number of wells was cut and the slides were stored at 4°C. Fifteen microlitres of suitably diluted antiserum and required amount (60µgs) of antigen prepared in normal saline were added in different wells. The reaction was allowed to proceed for 12-24 h in a moist chamber at room temperature. The Sheep HMW Kininogen (Baba et al., 2005) and Phaseolus mungo cystatin (Sharma et al., 2005) used for immunodiffusion studies were purified in our laboratory.

ANTIBACTERIAL ACTIVITY OF CYSTATIN

The following bacterial strains were used:

- *Escherichia coli*

- *Pseudomonas fluorescens*

Nutrient broth was composed of nutrient agar (1.3g/l). Ingredients of softagar included agar agar (0.8g/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, 0.03ml of fresh culture was overlayed with soft agar on nutrient agar plates to aid the formation of bacterial lawns. Whatman filter discs were put in 25µg/ml, 50 µg/ml, 100

μg/ml and 300μg/ml of inhibitor for the inhibitor to be absorbed on the discs. After 2 hrs the disca 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 - = no zone; (+) = 11-12 mm; + = 13-14 mm; ++ = 15-16 mm based on the inhibition zone diameter. An absence of antibacterial activity is denoted by the symbol (-) in the table.

KINETICS OF INHIBITION

Stoichiometry of Proteinase Inhibition

Papain was used for the titration of BLC.The inhibitory activity of cystatin was assessed by its ability to inhibit caesinolytic activity of papain by the method of Kunitz (1947). The concentration of papain was varied from 0.01-0.06 μM, where as the inhibitor concentration was fixed at 0.06μM. Papain was activated on incubation with 2mM EDTA and 50 mM cysteine for 10 minutes at 37^0C. The inhibitor was added and the volume was diluted to 1 ml by 20 mM phosphate buffer, pH-7.0 and further incubated for 30 min at 37^0C. One ml of 2% caesin 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 (2500 rpm for 15 minutes) the acid soluble peptides were quantitated by the method of Lowry et.al (1951). Identical experiments were carried out for the BLC with other proteinases, ficin and bromelain using casein as substrate (Kunitz, 1947; Murachi and Neurath, 1960).

Inhibiton Constant (K_i) Determination

K_i 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 0.05 μM to react with the inhibitor varying from 0.011 to 0.211 μM and measurement of residual activity was made as described by Kunitz (1947) using casein as substrate. Four different substrate concentrations were used that is, 0.5 Km, 1Km, 2Km and 3Km and with Km = 2.4mM and the results were analysed by the steady state equation of Krupka

and Laidler (1959). The linear equation is presented as follows (Henderson, 1972).

$$\frac{[I]_0}{1 - \dfrac{V_i}{V_0}} = K_i \left[1 + \frac{[S]_0}{Km} \right] \frac{V_i}{V_0} + [E]_0 \qquad (1)$$

Where $[I]_0$, $[E]_0$ and $[S]_0$ are the initial concentration of Inhibitor, Enzyme and Substrate respectively. V_0 is the velocity without Inhibitor. The plot of $[I]_0 / (1 - V_i/V_0)$ against V_0/V_i is a straight line, the slope of which gives,

$$K_i \, (app) = K_i \left[1 + \frac{[S]_0}{Km} \right] \qquad (2)$$

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

Determination of Association Rate Constant (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 (0.05 µM) were mixed in a total volume of 1 ml of the same buffer, pH 7.5 at 37 ^{0}C and residual activity was assayed as a function of time after 0, 2, 5, 10, 15, 20, 25 and 30 minutes as described above using caesin as substrate for papain, ficin and bromelain.

Association rate constant (K_{+1}) was calculated assuming that Enzyme [E] and either Inhibitor [I] react according to equation (3) and considering dissociation constant (K_{-1}) to be low enough to neglect the reverse reaction during the initial part of the process.

$$E + I \quad \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} \quad EI \qquad (3)$$

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

$$\frac{1}{[E]} = \frac{1}{[E]_0} + K_{+1}\, t \tag{4}$$

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

Determination of Dissociation Rate Constants (K_{-1})

Conditions for maximal association between the protease and inhibitor were achieved before the reaction was shifted towards dissociation by adding either $\alpha_2 M$ which forms stable and enzymatically active complex with free enzymes or excess of substrate which also binds all the free enzyme (Beith et al., 1980). Substrate induced dissociation was monitered with identical enzyme inhibitor complex incubated for 30 min at 37^OC. Excess substrate (6% casein) was added to the mixture for different time periods and then assayed for their enzymatic activity.

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

$$\ln\left[\frac{[EI]}{[EI]_0}\right] = K_{-1}.t \tag{5}$$

From which half life of the complex may be calculated by rearranging the equation as follows

$$t_{1/2} = 0.693/\ K_{-1} \tag{6}$$

Similar experiments for ki determination, association and dissociation rate constants were done for ficin and bromelain with BLC using their respective substrates.

SPECTRAL ANALYSIS

Absorption Difference Spectra

An ultraviolet absorption difference spectrum was measured for BLC (2.66µM) along with activated papian with a molar ratio of 1:1 at 25 ± 2°C. Spectra were recorded by measuring the absorption between 200-350 nm on a Campspec spectrophotometer model M330 B in a cuvette of 1cm path length. Appropriate controls of the solvent buffer were run and corrections were made wherever necessary.

Fluorescence Spectroscopy

Fluorescence measurements of cystatin, papain and cystatin-papain complex were taken by measuring intrinsic fluorescence at 25 ± 2°C in Shimadzu spectrofluorimeter model RF-540 equipped with a data recorder DR-3. The protein was excited at 280nm (λ_{ex}) and the emission range was 300-400nm. 2µM cystatin and 2µM of papain in a total volume of 1ml was used for the study. Cells with 1cm pathlength were used and samples were continuously stirred during measurements. Corrected emission spectra were recorded with an excitation and emission band width of 5nm. Appropriate controls were run and corrections made wherever necessary.

Circular Dichroism (CD) Spectroscopy

Circular Dichroism (CD) measurements were carried out at 25^{0}C on a Jasco spectropolarimeter model J-720 using a SEKONIC XY plotter (model SPL-430 A) with thermostatically controlled cell holder attached to a NESLAB water bath model RTE 110 with an accuracy of $\pm 0.10^{0}$C. The instrument was calibrated with d-10-Camphorsulphonic acid. The spectra were recorded with a scan speed of 20nm min^{-1} and with a response time of 4 seconds. The concentration of inhibitor and papain for far UV-CD spectral analysis was (0.2mg/ml) and path length was 0.1 cm. Each spectrum was recorded as an average of five scans. The emission wavelength range was 210-250nm. Changes in the secondary structure of BLC on interaction of activated papain

(activated with 20mM EDTA and 50mM cysteine) with a molar ratio of 1:1 were monitored. The α helical content of the cystatin was calculated from the MRE value at 222nm using the following equation (Chen Et al., 1972).

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

GLYCATION OF PURIFIED BLC WITH REDUCING SUGARS

Fluorescence Measurements in the presence of Glucose, Fructose and Ribose.

The fluorescence was recorded in wavelength region 300-400 nm after exciting the protein solution at 280 nm for total protein fluorescence. The slits were set at 5nm for excitation and emission. The path length of the sample was 1cm. The protein concentration used in the measurement was 2μM. BLC in 50mM sodium phosphate buffer pH 6.0 was incubated in the presence Glucose, fructose and Ribose (0.5M) for 28 days at 37^0C and fluorescence was recorded after intervals of day1, day 3, day 7, day14, day 21, day 28 for the three sugars respectively. Appropriate controls containing the Sugars for the study were run and corrections made wherever necessary. Each spectrum was average of three scans.

Circular Dichroism (far-UV) measurements of BLC in presence of Glucose, Fructose and Ribose

Secondary structural changes of BLC on incubation with concentrations of Glucose (0.5M), Fructose (0.5M) and Ribose (0.5M) incubated at $37\ ^o$C and spectrum was taken on 3rd, 7th, 14th, 21st and 28th day. Each spectrum was recorded as an average of five scans. The concentration of BLC was 5μM and path length was 1mm. The spectra obtained were normalized by subtracting the base line recorded for the buffer having the same concentration of sugars under similar conditions.

Assay of Inhibitory Activity of BLC in the presence of Glucose, Fructose and Ribose

Under native conditions the inhibitory activity of BLC was assessed by its ability to inhibit caesinolytic activity of papain by the method of Kunitz, 1947. The inhibitor (2µM) was incubated with glucose, fructose and ribose (0.5M each) at 37^0C for different time intervals (days) before the activity was measured. Untreated BLC activity was taken as 100%.

UNFOLDING STUDIES OF BLC

I. Denaturation of BLC at low pH and in the presence of TFE

BLC was allowed to undergo acid denaturation by subjecting it to buffers of varying pH range from pH 7.0 to pH 2.0. The buffers used were 50mM solution of glycine/HCl (PH 2.0), Sodium acetate (pH 3.0−5.0) and Sodium phosphate (pH 6.0 − 7.0).pH measurements were carried on a Metzer Optical Instruments (Pvt. Ltd., India), pH meter model 603M with a least count of 0.01 pH unit. BLC was incubated with the respective buffer of desired pH at 4^0C and allowed to equilibrate for 4 h before any spectrophotometric measurements were taken. For comparison of acid induced state and completely unfolded state, BLC was completely denatured in presence of 6M GdnHCl, a stock solution of GdnHCl was made and required volume added to the given sample of BLC to get final molar concentration of 6M GdnHCl.

The protein concentration was determined by the method of Lowry et al. 1951. ANS binding has been widely used to detect the molten globule states of different proteins (Stryer 1965, Engelhard and Evans 1995). A stock solution (10mg/5ml) of ANS in distilled water was prepared and its concentration was determined using an extinction coefficient of $5000M^{-1} cm^{-1}$ at 350mm (Mulqueen and Kronman 1982). The molar ratio of protein to ANS was 1:60.

UV measurements at different pH and TFE concentrations

BLC was incubated with buffers of varying pH range from pH 7.0 to pH 1.0. The concentration of BLC used was 2 μM in presence of 50mM buffers of different pH (pH 7.0 to pH 1.0). Further BLC (2 μM) was incubated with TFE (0-60%) v/v at pH 2.0. Absorption spectra was recorded on a double beam Shimadzu UV-vis spectrophotometer UV-1700 using a cuvette of 1cm path length.

Fluorescence and circular dichromism spectroscopy

BLC was allowed to undergo acid denaturation by subjecting it to buffers of varying pH range from pH 7.0 to pH 1.0. Further at pH 2.0 BLC was incubated with TFE (0-60%) v/v. BLc was incubated with respective buffers of desired pH at 4^0C and allowed to equilibrate for 4 h before any spectroscopic measurements were taken. Fluorescence spectra of BLC (2 μM) at varying pH and also in presence of 0-60% TFE at pH 2.0 was studied by exciting the protein at 280nm and the emission spectra was recorded from 300-400 nm wavelength range with a 5 nm slit width for excitation and emission.

ANS binding to BLC (2 μM) at pH 2.0 in presence of 0-60% TFE was studied by exciting the the dye at 380 nm and the emission spectra was recorded from 400-600 nm wavelength range with a 5 nm slit width for excitation and emission. ANS to protein molar ratio of 1:50 was used for each respective measurement.

BLC was incubated with the respective buffers of desired pH at 4^0C and allowed to equilibrate for 4 h before CD measurements were taken. Far UV-CD spectra of BLC at varying pH ranges were measured. The concentration of BLC was 0.2mg/ml for far UV-CD and ellipticity was recorded at 222 nm in 0.1 cm path length cuvette. Further far UV-CD spectra was also measured for BLC incubated with (0-60%) TFE at pH 2.0 after incubating the samples for 4 h at 4^0C.

AMYLOID FIBRIL FORMATION OF BLC

BLC (50 μM) was incubated with buffers of different pH range varying from 1-7 containing 0.15 M sodium chloride and 0.01% sodium azide in presence and abence of TFE v/v (30%). Before incubation each sample was passed through a 0.2 μ m filter to remove traces of aggregated material.

Thioflavin T staining

Staining of BLC by Thioflavin T was done by the method of Nilsson (2004). Five microlitres of the protein solution in which fibrils were growing were dissolved in 600 μl of the ThT buffer (pH 7.5, 50mM phosphate buffer, 0.15 M NaCl, 20μM ThT) just before the measurements. Fluorescemce measurements were carried out on shimadzu spectrophotometer model RF-540 equipped wuth a data recorder DR-3 at 25^0C. Excitation was at 440nm and spectra was recorded from 455-580 nm. The spectra was also recorded after (2, 3, 4, 7, 14 and 28 days) of incubation.

Atomic Force microscopy (AFM)

Tapping mode AFM experiments were performed using commercial etched silicon tips as AFM probes by exposing the nanomatrix with the same protein-free buffer as the protein-contacted surfaces with typical resonance frequency of ca. 300 Hz (RTESP, Veeco, Japan).

INTERACTION OF BILIRUBIN WITH BLC

Spectroscopic studies

BLC concentration was fixed at 2 μM for each experiment with bilirubin. Bilirubin solution was prepared. Stock solution of bilirubin was prepared by dissolving 2mg bilirubin in 2 ml 0.05 M sodium phosphate buffer, pH 7.5, containing 1 M sodium carbonate and 1 mM EDTA and immediately diluting it to the desired volume with the above buffer. The solution was filtered and stored in the dark. The

concentration of bilirubin was determined spectrophotometrically by taking the absorbance of the bilirubin solution at 440 nm using a molar extinction coefficient of 47,500 M-1 cm-1 (Jacobsen and Wennberg 1974). The solution was prepared fresh and used within 2 h. All experiments involving bilirubin were done in dark.

Absorption spectroscopy

Absorption spectra of cystatin as well as cystatin bound to BR were taken on UV–vis spectrophotometer in the wavelength range 220–400 nm using a cell holder with 1 cm path length.

Assay of Inhibitory Activity of BLC in the presence Bilirubin

Under native conditions the inhibitory activity of BLC was assessed by its ability to inhibit caesinolytic activity of papain by the method of Kunitz, 1947. The inhibitor (2µM) was incubated with increasing concentration of Bilirubin (0.25µM to 20 µM) at 37 ºC for 30 mins. Untreated BLC activity was

Fluorescence spectroscopy

Fluorescence measurements were made on a Shimadzu spectrofluorometer (25ºC) using a quartz cell with 1 cm path length. The excitation and emission slits were set at 5 and 10 nm, respectively. The fluorescence of BR bound to cystatin was recorded in the wavelength range 300–400 nm after exciting the complex at 280 nm. Photochemical experiments were carried out to measure photo-induced changes in the optical properties of the BR– cystatin complex. Samples were exposed to 40-W white fluorescent light (FTL; Anchor Electronics and Electricals Ltd., India) for various time periods. The distance of the sample from the light source was 2 cm. Photo-induced changes in the BR–cystatin complex were measured by fluorescence and absorption measurements.

Fourier Transform Infrared Spectroscopy

Infrared spectroscopy was done to see the conformational changes observed during cystatin and bilirubin interaction at relevant concentrations. The spectra was truncated

between 1900 cm^{-1} and 1220 cm^{-1} and baseline corrected. The equipment used was NICOLET (ESP) 560 spectrophotometer equipped with a transmission OMNIC ESP 5.1 software and a DTGS detector; data was analyzed and quantitated using Grams 32 software. Original spectra of native BLC along with BLC co-incubated with Bilirubin at 37°C were taken with a fixed concentration of cystatin (2 µM) and an increasing concentration of Bilirubin (0.25-5µM) with a resolution of 4 cm^{-1} and 128 scans. The changes in peak frequency and intensity were then assigned to conformational changes within the protein.

EFFECT OF CERTAIN LIGANDS HAVING EASY ACCESS TO LIVER TISSUES

Drug protein interaction (Rifampin and Atorvastatin)

BLC (2 µM) was incubated with increasing concentration of Rifampin (0.1µM to 20µM) in 50mM sodioum phosphate buffer (pH 7.5), in a final reaction volume of 1500 µl at room temperature for 2 hours. Rifampin solutions were prepared in same buffer. Fluorescence measurements were performed on a spectrofluorimeter model RF-5301PC (Shimadzu, japan) equipped with a 150W xenon lamp at 298K, 308K and 318K. The fluorescence was recorded in wavelength region 300-400 nm after exciting the protein at 280 nm. The slits were set at 5 nm for excitation and emission. The path length of the sample was 1 cm.

In case of Atorvastatin concentration used was 0.1µM to 5 µM). Both drugs were made to interact with protein separately.

Stern-Volmer equation

The fluorescence quenching data was analysed by the Stern-Volmer equation:

$$F_0/F = 1 + K_{sv}[Q]$$

(7)

Where F_0 and F are the steady-state fluorescence intensities in the absence and presence of quencher, respectively, K_{sv} the stern-volmer quenching constatnt and [Q] is the concentration of the quencher.

UV measurements of BLC in the presence of Rifampin and Atorvastatin

The UV measurements of Buffalo liver cystatin in the presence and absence of Rifampin and atorvastatin were mde in the range of 200-300 nm and the inhibitor BLC concentration was fixed at 1 μM while the drug concentration was varied from 0.1μM to 20 μM in case of rifampin and 0.1μM to 5μM in case of atorvastatin. Absorption spectra were recorded on a double beam Shimadzu UV-vis spectrophotometer UV-1700 using a cuvette of 1 cm path length.

Activity measurements of BLC in the presence of Rifampicin and atrovostatin

The inhibitory activity of BLC was assessed by its ability to inhibit caesinolytic activity of papain by the method of Kunitz (1947). The inhibitor was incubated with increasing concentration of Rifampin (0.1μM to 20 μM) for different time intervals at 25 ºC before the activity was measured. Similar procedure was followed in case of drug atorvastatin taken with increasing concentration of (0.1μM to 5μM).

INTERACTION OF BLC WITH PESTICIDE (MALATHION)

Intrinsic Fluorescence studies of BLC with Malathion

BLC (2 μM) was incubated with increasing concentration of Malathion (0.1 ppm to 50 ppm) in 50mM sodioum phosphate buffer (pH 7.5), in a final reaction volume of 1500 μl at room temperature for 2 hours in presence and absence of varying concentration of (10-50 μM) curcumin and (10-210 μM) Quercetin. Malathion solutions were prepared in same buffer. Fluorescence measurements were performed on a spectrofluorimeter model RF-5301PC (Shimadzu, japan) equipped with a 150W

xenon lamp at 298K, 308K and 318K. The fluorescence was recorded in wavelength region 300-400 nm after exciting the protein at 280 nm. The slits were set at 5 nm for excitation and emission. The path length of the sample was 1 cm.

UV measurements of BLC with Malathion

The UV measurements of Buffalo liver cystatin with Malathion in the presence and absence of curcumin (50µM) and quercetin (210µM) were made in the range of 200-300 nm and the inhibitor BLC concentration was fixed at 1 µM while the pesticide concentration was varied from 0.1ppm to 50ppm. Absorption spectra were recorded on a double beam Shimadzu UV-vis spectrophotometer UV-1700 using a cuvette of 1 cm path length.