Chapter - 1

Purification and characterization of Buffalo liver cystatin
[IV] RESULTS

4.1 PURIFICATION AND CHARACTERIZATION OF BUFFALO LIVER CYSTATIN (BLC)

4.1.1 PURIFICATION OF BUFFALO LIVER CYSTATIN

In the present work a thiol proteinase inhibitor (cystatin) has been purified from buffalo liver as detailed in the methods section. The procedure involved three steps after homogenisation, alkaline treatment (pH 11.0), ammonium sulphate precipitation (20-60%) and affinity chromatography using CNBr activated sepharose 4B. The crude homogenate contained free cathepsins, so presumably the inhibitors were entirely complexed. The alkaline treatment destroys the lysosomal cysteine proteases liberating the inhibitors in assayable form. A large amount of inactive proteins were precipitated during the readjustment to pH 7.5 and could be removed by centrifugation. The proteins obtained after alkaline treatment were precipitated between 40-60% ammonium sulphate saturation. The precipitate thus obtained was dissolved in 0.5 M sodium sulphate buffer, pH 7.5 and dialyzed against the same buffer containing 0.15 M NaCl. This process resulted in 6.70 fold purification with an yield of 35.15% (Table 2.1).

4.1.2 AFFINITY CHROMATOGRAPHY

Dilaysed protein from liver source was subjected to affinity chromatography on Cm-papain-Sepharose gel equilibrated in 50 mM sodium phosphate buffer (pH 6.5)/0.5 M NaCl and 1 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. This procedure gave an activity yield of 32.8% and a 438.82 fold purification (Table 2.1).
The precipitate obtained from 40 to 60% ammonium sulphate saturation (after alkaline fractionation) was dissolved and dialyzed against several changes of 50 mM sodium phosphate buffer, pH 7.5 containing 0.15 M NaCl. The sample was applied on activated cm-papain sepharose (20 × 1.5 cm) and fractions were eluted with the elution buffer containing 50 mM sodium phosphate buffer, pH 11.0. Fractions of 4ml were collected and immediately mixed with 0.5M sodium phosphate buffer, pH 4. The fractions were monitored for casienolytic activity of papain. Fractions were pooled for further studies. Protein concentration in the fractions was determined by talking absorbance at 660 nm.
TABLE 1.5: PURIFICATION CHART OF CYSTATIN FROM BUFFALO LIVER

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Steps of purification</th>
<th>Total Volume (ml)</th>
<th>Total Protein content (mg)</th>
<th>Total Activity (units)</th>
<th>Specific activity (Units/mg Protein)</th>
<th>Fold Purification</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Liver extract</td>
<td>140</td>
<td>7600</td>
<td>1430</td>
<td>0.188</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Alkaline treatment</td>
<td>105</td>
<td>5437</td>
<td>4840</td>
<td>0.89</td>
<td>4.73</td>
<td>338.46</td>
</tr>
<tr>
<td>3</td>
<td>Ammonium Sulphate Fractionation (40-60%)</td>
<td>80</td>
<td>3225</td>
<td>4068</td>
<td>0.26</td>
<td>6.70</td>
<td>35.15</td>
</tr>
<tr>
<td>4</td>
<td>Affinity chromatography</td>
<td>3</td>
<td>5.70</td>
<td>470.25</td>
<td>82.5</td>
<td>438.82</td>
<td>32.8</td>
</tr>
</tbody>
</table>

Protein concentration was determined by the method of Lowry et al (1951).

One unit of enzyme inhibitory activity is defined as the method of inhibitor bringing about 0.001 changes in O.D/ml/min.
4.1.3 HOMOGENEITY OF THE PURIFIED INHIBITOR

As observed in fig 2.2, the inhibitor eluted as a single symmetrical peak with constant specific activity suggesting a homogenous preparation. In addition, the preparation did not inhibit bovine trypsin, chymotrypsin or pepsin. Physical evidence for homogeneity was further provided by gel electrophoresis under non-denaturing conditions. The electrophoretic pattern of BLC is shown in figure, lane e. The inhibitor moved as a single band.

4.1.4 REDUCING AND NON-REDUCING SDS-PAGE

Purified BLC was analyzed by SDS-PAGE under non-reducing (in the absence of β-mercaptomaethanol and reducing conditions (in the presence of β-mercaptomaethanol). In both the conditions cystatin migrated as a single band with different mobilities, suggesting a double subunit structure with subunits held together by non-covalent forces (fig 2.3).

4.1.5 PROPERTIES OF THE PURIFIED LIVER CYSTATIN

Molecular weight determination

The molecular weight of buffalo liver cystatin (BLC) was determined under denaturing conditions was calculated from its mobility in SDS-PAGE by the procedure of Weber and Osborn (1969). The mobilities of the marker proteins were plotted against the logarithm of their molecular weights. The least square analysis of the data indicated a linear relationship between log M and relative mobility (Rm) of the marker proteins. The position of migration of BLC corresponds to 18 KDa respectively (Fig 2.4 A and 2.4 B).

Carbohydrate content

Type I and Type 2 cystatins, generally, lack carbohydrate content. In contrast to this buffalo liver cystatin was found to possess 1.7 % carbohydrate content.
Figure 2.2: Gel electrophoresis of BLC during various stages of purification

Electrophoresis was performed on 7.5% acrylamide gel as described in methods section. Lane (a) contained liver homogenate, lane (b) homogenate after alkaline treatment, lane (c) is fraction obtained after dialysis of the fraction after ammonium sulphate fractionation, lane (d) liver cystatin after affinity chromatography fraction. 60 µg of protein was applied in each lane (a-d).
Figure 2.3: **SDS Polyacrylamide gel electrophoresis of purified liver cystatin under non-reducing and reducing conditions.**

Electrophoresis was performed on 7.5% gels as described in methods section. SDS PAGE was performed with and without βME. Lane a: non-reducing conditions (in the absence of β-mercaptoethanol), lane b: reducing conditions (in the presence of β-mercaptoethanol). Lane a and b each contained 60 µg of the inhibitor, respectively.
**Sulphydryl groups**

The sulphydryl groups in liver cystatin were titrated against DTNB. Colourless solution was obtained indicating the absence of sulphydryl groups in the purified inhibitor.

**Effect of pH on the activity of buffalo liver cystatin**

Effect of pH on the thiol proteinase inhibitory activity of BLC was examined at various pH values. Figure 2.5 shows that the inhibitor is stable in the pH range 3-10 with maximum activity at pH 7.5.

**Effect of temperature on the activity of buffalo liver cystatin**

Stability of BLC was also investigated as a function of temperature between 25 and 85°C in 50 mM, sodium phosphate buffer, pH 7.5, by means of inhibitory activity assay. Liver cystatin retained considerable activity within temperature range of 25-85°C (fig 2.6).

**4.1.6 IMMUNOLOGICAL PROPERTIES**

**Antibody titer**

The liver cystatin caused good immune response leading to a high titre of the antibody. The resulting antiserum had a titer of 31622.80 as determined by direct binding ELISA in rabbit serum (fig 2.7).

**Cross-reactivity**

Liver cystatin was immunogenic and readily induced antibody formation in rabbits. Figure shows that the antiserum raised against purified inhibitor has cross reactivity with the inhibitor as indicated by a single precipitin line on immunodiffusion plate exhibiting immunogenic purity and homogeneity of the inhibitor.
Figure 2.4: Molecular weight determination of liver cystatin by the SDS-PAGE electrophoresis

(A) Electrophoresis was performed on 12.5% polyacrylamide gel. Lane a contained 60 µg BLC without β-mercaptoethanol and lane b contained 60 µg of β-mercaptoethanol treated purified inhibitor. Lane c contained the molecular mass standards: 1, phosphorylase b (97.4 KDa); 2, bovine serum albumin (68 KDa); 3, ovalbumin (45 KDa); 4 carbonic anhydrase (29.1 KDa); 5, soyabean trypsin inhibitor (20 KDa); 6, lysozyme (14.3 KDa).

(B) Plot of log M (Molecular weight) vs relative mobility (Rm) calculated from figure (A). Data was plotted using leastsquare analysis.
preparation. The antiserum exhibited immunogenic identity with goat liver cystatin as a single precipitin line was obtained (Fig 2.8). Experiments also showed that the antisera had no immunogenic identity with goat pancreatic cystatin purified in our lab. This indicates that the epitopes of buffalo liver cystatin are different from goat pancreatic thiol proteinase inhibitor.

### 4.1.7 KINETIC PROPERTIES OF BUFFALO LIVER CYSTATIN

**Stiochiometry of Inhibition**

The inhibition of proteinases was studied by varying the molar concentration of proteinases at a fixed molar concentration of cystatin. The remaining activity of proteinase showed that as the concentration of proteinase increased from 0.01-0.06 µM it is progressively inhibited by cystatin (0.06 µM) showing a stiochiometric ratio of 1:1, therefore one molecule of cystatin inhibits one molecule of active protease. Stiochiometric ratios of 1:1 were also obtained for ficin and bromelain.

**Inhibition of different proteinases by BLC**

The inhibitory activity of liver cystatin towards thiol proteinases, papain, ficin, bromelain and serine proteinases, trypsin and chymotrypsin was examined using casein as substrate. Liver cystatin inactivated papain and ficin very efficiently and bromelain to a slightly extent. The order of inhibition was papain > ficin > bromelain. However it failed to inhibit bovine trypsin and chymotrypsin (fig 2.9).

**Ki determination**

Dissociation equilibrium constants (measured as ki), for the binding of liver cystatin to plant proteinases papain, ficin and bromelain, were determined by monitoring the loss of enzyme activity and after lowering the respective inhibitor and proteinase concentrations, which favour the dissociation of the complex. Ki values
Figure 2.5: Effect of pH on the activity of Buffalo liver cystatin

50 µg of the inhibitor was incubated in 50 mM sodium acetate buffer, pH 3.0-6.0, sodium phosphate buffer, pH 7.0-8.0, tris-HCl buffer, pH 9.0, for 30 min at 37°C. After the incubation the pH of the mixture was neutralized and then 50 µg of activated papain was added and the mixture was further incubated for 60 min at 37°C. The following procedure was same as described in methods section for assaying the inhibitor using casein as substrate.

Figure 2.6: Effect of temperature on the activity of Buffalo liver cystatin

50 µg of the inhibitor was incubated in 50 mM sodium phosphate buffer, pH 7.5, at various temperatures for 30 min and then rapidly cooled. 50 µg of activated papain was added and kept for 60 min at 37°C. The remaining % activity was analyzed by caseinolytic method of Kunitz (1947).
Figure 2.7: Direct binding ELISA

Serially diluted antiserum and pre-immune serum were incubated with 0.5 µg/100 µl antigen. The procedure has been described in methods. The curve with red square is for pre-immunized sera, whereas the curve with blue square is for post-immunized sera.
were determined using the steady state equation derived by Krupka and Laidler (1959).

\[
\frac{[I]_0}{1 - \left(\frac{v_1}{v_0}\right)} = K_i \left[1 + \left(\frac{[S]_0}{K_m}\right)\frac{v_1}{v_0} + [E]_0\right] 
\]

The increasing values of Ki (app) with an increase in the substrate concentration (figure 3.0, 3.1 and 3.2) suggested a competitive mechanism of inhibition. The true Ki were obtained from the replot of Ki (app) versus substrate concentration. The Ki values obtained for papain, ficin and bromelain are 2.3×10⁻⁹ M, 4.2×10⁻⁸ M and 7.2×10⁻⁸ M, respectively, implying the highest affinity of the inhibitor for papain.

**Dissociation rate constant (Kₐ⁻¹)**

The conditions for the dissociation were taken such that the enzyme-inhibitor complex obeys first order kinetics during the initial part of the reaction. In this case the integrated form of the equation is given by,

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

(5)

\[
\log\left\{\frac{[EI]}{[EI]_0}\right\} = t \times -K_{-1} / 2.303 
\]

Figures 3.3, 3.4 and 3.5 show the respective plots for papain, ficin and bromelain. The Kₐ⁻¹ values obtained for papain, ficin and bromelain are 2.2×10⁻⁴ s⁻¹, 4.9×10⁻³ s⁻¹ and 3.5×10⁻³ s⁻¹ respectively.
Figure 2.8: Ouchterlony immunodiffusion

Anti-liver cystatin antiserum was raised in rabbits. For the immunodiffusion study, the antiserum was allowed to react with goat liver cystatin and goat pancreatic cystatin. The central well contained the anti BLC serum whereas the well (a) contained purified BLC, well (b) contained goat liver cystatin and well (c) contained goat pancreatic cystatin.
Figure 2.9: **Inhibitory activity of liver cystatin with different proteinases**

40 µg of thiol proteinases papain, ficin, bromelain and serine proteinases, trypsin and chymotrypsin were incubated with varying concentrations of liver cystatin (0-40 µg) for 30 min. The inhibitory activity of liver cystatin towards proteinases was measured by using 2% casein as substrate in methods section.
Association rate constant \((K_{+1})\)

The association rate constant \((K_{+1})\) for the three thiol proteinases can be determined by monitoring the time dependence of the association under second order reaction conditions. \(K_{+1}\) is calculated assuming that enzyme \([E]\) and inhibitor \([I]\) react in such a way that dissociation constant \(K_{-1}\) or reverse rate is negligible in the initial part of the process. Thus initial concentration of \([E]_0\) and inhibitor \([I]_0\) are identical.

Association rate constants can also be calculated from measured dissociation rate and dissociation equilibrium constants by the relation,

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

(4)

Or

\[
K_{+1} = \frac{K_{-1}}{K_i}
\]  

(7)

Association rate constant and hence the affinity of the inhibitor for proteinases is in the following order: papain \((1.1\times10^5 \text{ M}^{-1} \text{ s}^{-1})\) > ficin \((4.3\times10^4 \text{ M}^{-1} \text{ s}^{-1})\) > bromelain \((1.9\times10^4 \text{ M}^{-1} \text{ s}^{-1})\). The results of kinetic constants obtained on interaction of liver cystatin which proteinases papain, ficin and bromelain are summarized in Table 1.6.

Half life of the complex

Half life of the complexes may be calculated by rearranging the equation

\[
t_{1/2} = \frac{0.693}{K_{-1}}
\]  

(6)

The half life values of BLC calculated were found to be \(1.1\times10^3\) s, \(1.9\times10^3\) s and \(3.8\times10^3\) s for papain, ficin and bromelain, respectively.
Figure 3.0: Determination of inhibition constant (Ki) with papain

Papain was used at final concentration of 0.06 μM with increasing amounts of liver cystatin (0.01-0.24 μM) and measurements of residual activity were made as described in methods using casein as substrate. Four different substrate concentrations were used, i.e. 0.5 Km, 1 Km, 2 Km, and 3 Km with Km = 2.4 mM. For the sake of clarity only the result obtained for [S] = 0.5 Km are shown. The inset shows the replot of experimental Ki (app) values versus [S]. Intercept on the ordinate gives the true Ki.
Figure 3.1: Determination of inhibition constant (Ki) with ficin

Ficin was used at final concentration of 0.06 μM with increasing amounts of liver cystatin (0.01-0.24 μM) and measurements of residual activity were made as described in methods using casein as substrate. Four different substrate concentrations were used, i.e. 0.5 Km, 1 Km, 2Km, and 3 Km with Km = 2.4 mM. For the sake of clarity only the result obtained for [S] = 0.5 Km are shown. The inset shows the replot of experimental Ki (app) values versus [S]. Intercept on the ordinate gives the true Ki.
Figure 3.2: Determination of inhibition constant (Ki) with bromelain

Bromelain was used at final concentration of 0.06 μM with increasing amounts of liver cystatin (0.01-0.24 μM) and measurements of residual activity were made as described in methods using casein as substrate. Four different substrate concentrations were used, i.e. 0.5 Km, 1 Km, 2Km, and 3 Km with Km = 2.4 mM. For the sake of clarity only the result obtained for [S] = 0.5 Km are shown. The inset shows the replot of experimental Ki (app) values versus [S]. Intercept on the ordinate gives the true Ki.
TABLE – 1.6: KINETIC CONSTANTS FOR THE INTERACTION OF BUFFALO LIVER CYSTATIN WITH DIFFERENT PROTEINASES – PAPAIN, FICIN AND BROMELAIN.

<table>
<thead>
<tr>
<th>Proteinase</th>
<th>$K_i$ (M)</th>
<th>$K_{+1}$ (M$^{-1}$S$^{-1}$)</th>
<th>$K^{-1}$S$^{-1}$</th>
<th>Half life of complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papain</td>
<td>2.3±0.1×10^{-9}</td>
<td>1.1±0.1×10^{5}</td>
<td>2.2±0.2×10^{-4}</td>
<td>3.15±0.1×10^{3}</td>
</tr>
<tr>
<td>Ficin</td>
<td>4.2±0.3×10^{-8}</td>
<td>4.3±0.1×10^{4}</td>
<td>4.9±0.3×10^{-3}</td>
<td>1.41±0.3×10^{2}</td>
</tr>
<tr>
<td>Bromelain</td>
<td>7.2±0.1×10^{-8}</td>
<td>1.9±0.2×10^{4}</td>
<td>3.5±0.1×10^{-3}</td>
<td>1.98±0.3×10^{2}</td>
</tr>
</tbody>
</table>

Results represent the mean ± SEM calculated from three independent experiments.
Figure 3.3: Determination of dissociation rate constant ($K_{i1}$) with papain

Papain-BLC complex (1 µM) was preincubated for 30 min at 37°C before excess substrate was added to the mixture. Appearance of papain activity was recorded as a function of time. Inset shows plot of the data as described in methods.
Figure 3.4: Determination of dissociation rate constant ($K_{-1}$) with ficin

Ficin-BLC complex (1 µM) was preincubated for 30 min at 37°C before excess substrate was added to the mixture. Appearance of papain activity was recorded as a function of time. Inset shows plot of the data as described in methods.
Figure 3.5: Determination of dissociation rate constant ($K_{-1}$) with bromelain

Bromelain-BLC complex (1 µM) was preincubated for 30 min at 37°C before excess substrate was added to the mixture. Appearance of papain activity was recorded as a function of time. Inset shows plot of the data as described in methods.
4.1.8 ANTIBACTERIAL ACTIVITY OF BLC

The results of cystatin subjected to different bacterial strains to study their biological
effect have been summarized in table 2.3. As is evident from the table, the growth of
*Escherichia coli* was inhibited to a greater extent by cystatin than *Pseudomonas
fluorescens*. With a dose of around 50µg/ml and above there was a considerable
increase in the zone of inhibition (11-13 mm) against E.coli. Moreover, cystatin was
also able to inhibit the growth of *Pseudomonas fluorescens* with the zone of inhibition
of around 7-8 mm at 100 µg/ml concentration of cystatin.

4.1.9 SPECTRAL ANALYSIS

The nature of interaction of thiol proteinases with the isolated inhibitor was observed
through the spectroscopic changes that accompanied the binding mode of thiol
proteinase with liver cystatin in their specific stiochiometric ratio. These binding
interactions were followed by UV-absorption difference and fluorescence emission
spectroscopies.

**Ultraviolet absorption difference spectra of buffalo liver cystatin with
papain**

Absorption difference spectra between the cystatin and cystatin-papain complex
reveals that a pattern of changes in ultraviolet absorption and in the aromatic
wavelength region accompanies the interaction (fíg 3.6). The spectrum shows a
trough around 240 nm and a peak around 250 nm. The peak around 250 nm may be
partly due to the perturbation around phenylanaline residues and may also contain
contribution from other aromatic residues. These results suggest that the environment
of several aromatic amino acids has been effected by this interaction.
TABLE-1.7: ANTI-BACTERIAL EFFECTS OF BUFFALO LIVER CYSTATIN

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Inhibitor 25 µg</th>
<th>Inhibitor 50 µg</th>
<th>Inhibitor 100 µg</th>
<th>Inhibitor 300 µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.coli</td>
<td>(+)</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>P.fluorescence</td>
<td>-</td>
<td>(+)</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

Inhibition zone diameter: - = no zone; (+) = 11-12 mm; + = 13-14 mm; ++ = 15-16 mm.
Figure 3.6: UV absorption difference spectra measured for cystatin-papain complex

Liver cystatin (2.66 μM) was incubated with activated papain for 30 min and an absorbance difference spectrum was calculated between 200-300 nm. Liver cystatin and papain were in a molar ratio of 1:1.
Figure 3.7: Fluorescence spectra of cystatin alone and cystatin in complex with papain

Fluorescence spectra of the inhibitor alone liver cystatin, papain and papain-liver cystatin complex was measured at excitation wavelength ($\lambda_{ex}$) of 280 nm and emission range ($\lambda_{em}$) of 300-400 nm. The concentration of buffalo liver cystatin was 2 $\mu$M. The fluorescence of complex of BLC with papain was measured at a molar ratio of 1:1. The slit width was 5 nm for excitation and 5 nm for emission beams.
Fluorescence spectra of buffalo liver cystatin in complex with papain

Fluorescence emission spectra showed maxima at 337 nm for buffalo liver cystatin. Upon formation of papain inhibitor complex the fluorescence intensity increased comparative to the native inhibitor with a blue shift of 5 nm (fig 3.7). These changes are indicative of alterations in conformation of either one or both the proteins involved in complex formation.

Circular Dichroism spectra of native BLC and its complex with papain

Far UV-CD spectra (fig. 3.8) depicts the contribuation of secondary structure of the protein. The α-helical structure of the protein in the far UV region is characterized by negative peaks at 208-210 nm and 222 nm and the positive peaks between 190 nm and 192 nm. In the present study we measured far UV-CD spectra of the purified inhibitor at a concentration of 0.2 mg/ml, the spectra shows a significant peak at 222 nm and a small peak at 210 nm also. The results were expressed as MRE (mean residue ellipticity) in deg. cm$^{2}$dmol$^{-1}$, which is defined as

$$MRE = \theta_{obs} / (10 \times n \times l \times Cp)$$

Where $\theta_{obs}$ is the CD in milli degrees, $n$ is the number of amino acid residues, $l$ is the path length of the cell and $Cp$ is the mole fraction. Helical content of BLC was calculated from the MRE values at 222 nm using the following equation as described by (Chen et al., 1997).

$$\% \alpha\text{-helix} = (MRE_{222nm} - 2340/30300) \times 100$$

The results show BLC has 30.89 % α-helical content.

Far UV-CD analysis of BLC with papain (Fig. 3.8) indicated the change in the conformation of BLC upon complex formation with papain.
Figure 3.8: CD spectra of cystatin alone and cystatin in complex with papain