Results
And
Discussion
CHAPTER 1

Purification and Characterization of Goat Lung cystatins
4. RESULTS

4.1 PURIFICATION AND CHARACTERIZATION OF GOAT LUNG CYSTATINS

4.1.1 PURIFICATION

The purification procedure used in the present study is a combination of ammonium sulphate fractionation and ion exchange chromatography. The proteins obtained after alkaline treatment were precipitated between 40-60% ammonium sulphate saturation. The precipitate thus obtained was dissolved in 0.1 M sodium phosphate buffer, pH 7.5 and dialyzed against the same buffer containing 0.15 M NaCl. This resulted in 1.396 fold purification with an yield of 56.6% (Table 1.3).

4.1.2 ION EXCHANGE CHROMATOGRAPHY

The proteins obtained after dialysis was subjected to ion exchange chromatography on DEAE-cellulose column (2×7cm) equilibrated with 0.05 M sodium phosphate buffer pH 8.0. Fractions were eluted by running a salt gradient of 0-0.5 M NaCl. Two protein peaks with papain inhibitory activity were obtained (Fig. 2.0). These peaks have been represented as goat lung cystatin I and II (GLC-I and GLC-II). First peak (GLC-I) was purified with a fold purification of 75.9 and yield of 11.25% while second peak (GLC-II) was obtained with a fold purification of 343.5 and yield of 8.3%. The purification of both GLC-I and GLC-II has been summarized in Table 1.3.

4.1.3 HOMOGENEITY

Homogeneity of GLC-I (peak I) and GLC-II (peak II) eluted from DEAE-cellulose ion exchange column was determined by polyacrylamide gel electrophoresis (PAGE) in the absence of SDS. Figure 2.1 shows the native PAGE of GLC-I and II during various stages of purification. Purified inhibitors GLC-I and II showed a single band on PAGE.
TABLE 1.3: PURIFICATION CHART OF CYSTATINS FROM GOAT LUNG

<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>Lung extract</td>
<td>150</td>
<td>6900</td>
<td>855</td>
<td>0.154</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Alkaline treatment</td>
<td>110</td>
<td>4950</td>
<td>1023</td>
<td>0.206</td>
<td>1.337</td>
<td>79.7</td>
</tr>
<tr>
<td>3</td>
<td>Ammonium Sulphate fractionation (40-70%)</td>
<td>90</td>
<td>3375</td>
<td>726.3</td>
<td>0.215</td>
<td>1.396</td>
<td>56.6</td>
</tr>
<tr>
<td>4</td>
<td>Ion exchange chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Peak I</td>
<td>9</td>
<td>8.19</td>
<td>95.85</td>
<td>11.70</td>
<td>75.9</td>
<td>11.25</td>
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<tr>
<td></td>
<td>Peak II</td>
<td>15</td>
<td>1.78</td>
<td>88.2</td>
<td>52.9</td>
<td>343.5</td>
<td>8.3</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 amount of inhibitor bringing about 0.001 changes in O.D/ml/min.
The protein obtained by precipitation between 40-60 % ammonium sulphate saturation was dissolved in 0.1 M sodium phosphate buffer, pH 7.5 and dialyzed against several changes of the same buffer containing 0.15 M NaCl. The protein was further purified by ion exchange chromatography on DEAE cellulose column (2x7 cm) equilibrated with 0.05 M sodium phosphate buffer of pH 8.0. The elution was performed in 3 ml fraction using a linear gradient of 0-0.5 M NaCl at a flow rate of 37 ml/hr. Each fraction was assayed for thiol proteinase inhibitory activity (●) and protein concentration for which absorbance was taken at 660 nm (○).
Figure 2.1: Gel electrophoresis of the purified GLC-I and II during various stages of purification

Electrophoresis was performed on 7.5% non-denaturing polyacrylamide gel at 25 °C as described in methods section. Lane (a) contained lung homogenate, lane (b) contained homogenate after alkaline treatment, lane (c) contained dialyzed fraction after ammonium sulphate fractionation and lane (d) and (e) contained purified inhibitor GLC-I and GLC-II respectively after ion exchange chromatography on DEAE cellulose column. 60μg of protein was applied in each lane (a to c) and inhibitors were added in a concentration of 40 μg (lane d and e).
4.1.4 REDUCING AND NON REDUCING SDS-PAGE

Purified GLC-I and GLC-II were also analysed by SDS-PAGE under non-reducing (in the absence of β-mercaptoethanol) and reducing conditions (in the presence of β-mercaptoethanol). Appearance of single band of the purified inhibitors (GLC-I and GLC-II) under reducing and non-reducing conditions represent the monomeric nature of the purified inhibitors (Fig. 2.2 A and 2.2 B).

4.1.5 PROPERTIES OF THE PURIFIED INHIBITORS (GLC-I & GLC-II)

Molecular Weight

The molecular weight of GLC-I and II 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 GLC-I and II corresponds to 63 and 73 kDa respectively (Fig. 2.3 A and 2.3 B).

Mass Spectrometry (MALDI-TOF analysis)

MALDI-TOF analysis is one of the most recent and sophisticated techniques through which accurate molecular mass can be determined easily in a short time period. Fig. 2.4 and 2.5 shows the molecular mass determination of GLC-I and II, respectively by mass spectrometry. Molecular mass of GLC-I and II as analysed by mass spectrometry is found to be 66426.70 and 76425.62 Da respectively.

Carbohydrate content

GLC-I and II exhibited low glycosylation that is 0.07% and 2.3%, respectively as determined by Dubois method (Dubois et al., 1956).
Figure 2.2: (A) SDS PAGE of GLC-I under non reducing and reducing conditions

Electrophoresis was performed on 10% acrylamide gel in the presence of SDS with and without βME as described under method section. Lane (a) and (b) contained 50µg of GLC-I under non reducing and reducing conditions, respectively.

(B) SDS PAGE of GLC-II under nonreducing and reducing conditions

SDS PAGE of GC-II was performed on 10% acrylamide gel under non reducing and reducing conditions as described under methods. Lane (a) and (b) contained 50µg of GLC-II in the absence and presence of βME, respectively.
Figure 2.3: Molecular weight determination of GLC-I and GLC-II by SDS PAGE

(A) Electrophoresis was performed on 10% acrylamide gel. Lane (a) 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) and 6: lysozyme (14.3 kDa). Lane b and c contained 60 µg of purified inhibitor (GLC-I) in the absence and in the presence of β-mercaptoethanol respectively. Lane d and e contained 60 µg of purified inhibitor (GLC-II) in the absence and in the presence of β-mercaptoethanol respectively.

(B) The Log M (Molecular weight) of the marker proteins were plotted against their relative mobility (Rm) using least square analysis. Molecular weight of GLC-I and II is indicated by an arrow.
and was analyzed by the procedure given in methods. 1.2 ml (1 mg/ml) of the inhibitor GCL-1 was applied on the sample plate. 0.1% sinapinic acid (1:1, v/v). This preparation was then vortexed and the samples were dissolved in 1.0% trifluoroacetic acid and

**Spectrometry**

**MALDI-TOF**

**Figure 2.4**: Molecular weight determination of GCL-1 by mass
The samples were dissolved in 1.0% trifluoroacetic acid and 0.1% sinapinic acid (1:1, v/v). This preparation was then vortexed and 1.2 ml (1 mg/ml) of the inhibitor GLC-II was applied on the sample plate and was analysed by the procedure given in methods.
**Sulphydryl content**

The sulphydryl groups of GLC-I and II were titrated against DTNB as described in the methods section. Colourless solutions were obtained in the native and denatured forms indicating that no sulphhydryl groups are present in the purified GLC-I and GLC-II.

**pH stability of GLC-I and GLC-II**

Both GLC-I and II are found to be quite stable in the pH range of 4-10. Both the cystatins (GLC-I and II) retained about 71% inhibitory activity at pH 4.0 as shown in Fig. 2.6. However their inhibitory activity decreased significantly below pH 4.0 and above pH 10.0.

**Temperature stability of GLC-I and GLC-II**

Both the inhibitors were stable upto 75°C. However at temperature above 90°C, GLC-I and GLC-II lost 28% and 22% of their inhibitory activity, respectively as shown in Fig. 2.7. Both the inhibitors were maximally active within the temperature range of 25-75°C.

**4.1.6 IMMUNOGENIC PROPERTIES**

**Antibody Titre**

Owing to its high molecular weight and consequent high immunogenicity, GLC-I led to a high titre of the antibody (25,600) in the serum of rabbits as determined by direct binding ELISA (Fig. 2.8).

**Cross Reactivity**

Goat lung cystatin (GLC-I) was highly immunogenic and readily induced antibody formation in rabbits. Fig.3.0 shows that the antisera raised against purified GLC-I has cross reactivity with the inhibitor as indicated by single precipitin line on immunodiffusion plate exhibiting immunogenic purity and homogeneity of the inhibitor preparation. GLC-I also exhibited immunogenic identity with goat brain cystatin and GLC-II indicating the similarity in epitopes (Fig.2.9).
Figure 2.6: Effect of pH on GLC-I and GLC-II

The pH stability of both the inhibitors was investigated from pH 2-12. Fifty micrograms of the inhibitors was incubated with tris glycine buffer (pH 2-6, 50mM), sodium phosphate buffer (pH 7.0 and 8.0) and tris-NaOH buffer (pH 9.0, 10.0, 11.0 and 12.0) for 30 min. at 37°C. After the incubation, the pH of the inhibitor solutions was neutralized and then 50μg of activated papain was added and the mixture was further incubated for 60 min. at 37°C. Following the inhibitory activity was determined as detailed in methods section. The activity of papain in the absence of inhibitor at pH 7.5 was taken as 100%.
Fifty micrograms of each inhibitor in 50mM sodium phosphate buffer (pH 7.5) was incubated at various temperatures in the range of 25-100°C for 30 min. These samples were rapidly cooled in ice-cold water bath and checked for residual inhibitory activity against 50 microgram of papain. Inhibitory activity was assayed by the method of Kunitz as described in the methods section. The inhibitory activity of the GLC-I and GLC-II at 37°C was taken as 100%. 

Figure 2.7: Effect of temperature on GLC-I and GLC-II
Figure 2.8: Direct binding ELISA

Serially diluted antiserum and pre immune serum were incubated with 0.5μg/100ml antigen (GLC-I). The procedure has been described in methods. The curve with solid square (■) denotes preimmunized serum while post immunized serum is presented by solid triangles (▲).
Anti GLC-I antiserum was raised in albino rabbits. In the immunodiffusion experiment cystatin antisera was allowed to react with GLC-I, GLC-II and goat brain cystatin. The central well contained the GLC-I antiserum, whereas the wells 1, 2 and 3 contained 20μg of GLC-I, GLC-II and goat brain cystatin, respectively.
Inhibition of Proteinases by GLC-I and GLC-II

The inhibitory activity of GLC-I and GLC-II towards thiol proteinases papain, ficin, bromelain and serine proteinases, trypsin and chymotrypsin was examined using casein as substrate. Both the cystatins inhibited thiol proteinases in the order papain > ficin > bromelain. However both GLC-I and GLC-II did not show any significant inhibition of serine proteinases like trypsin and chymotrypsin (Fig. 3.0a and b)

4.1.7 KINETICS OF INHIBITION

Stoichiometry of Inhibition

The stoichiometry of association of purified GLC-I and II was determined by incubating a fixed concentration of papain (0.06μM) with increasing concentration of inhibitors (0.01μM-0.06μM). Caseinolytic activity of papain using casein as a substrate was determined in the presence of GLC-I and II. A linear increase in the inhibitory activity was observed for both the inhibitors indicating a complete association between the enzymes and inhibitors under these conditions. GLC-I and II interacted with papain with a molar ratio of 1:1 which implicates that 1 mole of GLC-I and II combines with one mole of papain. Stoichiometric ratio of 1:1 was also obtained for ficin and bromelain.

Kₚ Determination

Kₚ values have been determined after lowering the papain and inhibitor concentrations, which favour the dissociation of the complex. Kₚ values were determined using the steady state equation derived by Krupka and Laidler (1959),

\[
\frac{[I]_0}{1 - (v_i/v_o)} = \frac{K_i \left[ \frac{[S]_0}{K_m} \right]}{v_i + [E]_0} \tag{1}
\]

Fig.3.1 shows that the Kₚ (app) increases as a function of the substrate (casein) concentration. The true Kₚ value may be obtained from a replot of Kₚ (app) against substrate concentration [S]. The Kₚ (app) values for GLC-I obtained from the plot with papain was 1.5×10⁻⁹M. Fig.3.2 depicts the Kₚ (app) value determination for GLC-I using the proteinase ficin. GLC-I exhibited a Kₚ of 1.3×10⁻⁸ M for ficin. However, the Kₚ values obtained with bromelain was found to 2.6×10⁻⁸ M (Fig.3.3). The Kₚ values was found to be lowest for papain hence it has highest affinity for the goat lung cystatin.
Figure 3.0: Inhibitory activity of GLC-I and GLC-II with different proteinases

40μg of thiol proteinases papain, ficin, bromelain and serine proteinases, trypsin and chymotrypsin were incubated with varying concentrations of GLC-I and GLC-II (0-40μg) for 30 min. The inhibitory activity of GLC-I (A) and GLC-II (B) towards different proteinases was measured by using 2% casein as substrate as described in methods.
Concentration of GLC-I in µg

Concentration of GLC-II in µg
Figure 3.1: Determination of inhibition constant \((K_i)\) with papain

Papain was used at final concentration of 0.06\(\mu\)M with increasing amounts of GLC-I (0.01-0.24\(\mu\)M) 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\(K_m\), 1\(K_m\), 2\(K_m\) and 3\(K_m\) with \(K_m = 2.4\)mM. For the sake of clarity only the result obtained for \([S] = 0.5K_m\) are shown. The inset shows the replot of experimental \(K_i\) (app) values versus \([S]\). Intercept on the ordinate gave the true \(K_i\).
Figure 3.2: Determination of inhibition constant (Ki) with ficin

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

Figure 3.3: Determination of inhibition constant (Ki) with bromelain
**Association Rate Constant (K<sub>i</sub>)**

Association rate constant (K<sub>i</sub>) values for the three thiol proteinases were determined by monitoring the time dependence of the association under second order reaction conditions. K<sub>i</sub> was calculated assuming that enzyme [E] and inhibitor [I] react in such a way that dissociation constant K<sub>i</sub> is low enough to neglect the reverse reaction during the initial part of the process. Thus, initial concentration of [E]<sub>0</sub> and inhibitor [I]<sub>0</sub> are identical. The association rate is given by the equation:

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

(4)

Tight binding inhibitors have high association rate constants. Among 3 thiol proteinases, papain gave highest K<sub>i</sub> of 3.06×10^4 M<sup>-1</sup>Sec<sup>-1</sup> followed by ficin (8.33×10<sup>4</sup> M<sup>-1</sup>Sec<sup>-1</sup>) and bromelain (8.0×10<sup>4</sup> M<sup>-1</sup>Sec<sup>-1</sup>) (Table 1.4).

**Dissociation Rate Constants (K<sub>-1</sub>)**

For the dissociation rate constant, the conditions for maximal association between proteinase and either inhibitor was achieved before the reaction was shifted towards dissociation by adding excess substrate which also binds the entire free enzyme. Dissociation of EI complex obeys first order kinetics. Thus, integrated form of the dissociation rate equation is given by:

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

(5)

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

K<sub>-1</sub> values of GLC-I with different proteinase were calculated as 4.6×10<sup>-4</sup>Sec<sup>-1</sup>, 1.09×10<sup>3</sup>Sec<sup>-1</sup> and 2.1×10<sup>3</sup>Sec<sup>-1</sup>, for papain, ficin and bromelain, respectively (Fig 3.4; 3.5 and 3.6).
TABLE- 1.4: KINETIC CONSTANTS FOR THE INTERACTION OF GOAT LUNG CYSTATIN (GLC-I) 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>$K^*$ (S$^{-1}$)</th>
<th>Half life of complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papain</td>
<td>1.5±0.2×10$^{-9}$</td>
<td>3.06±0.1×10$^5$</td>
<td>4.6±0.4×10$^{-4}$</td>
<td>1.5±0.2×10$^3$</td>
</tr>
<tr>
<td>Ficin</td>
<td>1.3±0.2×10$^{-8}$</td>
<td>8.33±0.2×10$^4$</td>
<td>1.09±0.1×10$^{-3}$</td>
<td>6.3±0.1×10$^2$</td>
</tr>
<tr>
<td>Bromelain</td>
<td>2.6±0.3×10$^{-8}$</td>
<td>8.0±0.4×10$^4$</td>
<td>2.1±0.2×10$^{-3}$</td>
<td>3.3±0.3×10$^2$</td>
</tr>
</tbody>
</table>

Results represent the mean ± SEM calculated from three independent experiments.
Figure 3.4: Determination of dissociation rate constant \( (K_{\text{diss}}) \) with papain

Papain-GLC-I complex (1µM) 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 ($K_{diss}$) with ficin

Ficin-GLC-I complex (1μM) 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 3.6: Determination of dissociation rate constant ($K_{diss}$) with Bromelain

Bromelain – GLC-I complex (1μM) 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.
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 GLC-I calculated were found to be $1.5 \times 10^3$ Sec, $6.3 \times 10^2$ Sec and $3.3 \times 10^2$ Sec for papain, ficin and bromelain, respectively. Among all the three proteinases bromelain cystatin complex has the shortest half life.

4.1.8 SPECTRAL ANALYSIS

The nature of interaction of thiol proteinases with the isolated inhibitors was observed through the spectroscopic changes that accompanied the binding of model thiol proteinase papain with the lung cystatins in their specific stoichiometric ratio. These binding interactions were followed by UV– difference spectroscopy, fluorescence emission spectra and far UV-CD spectra.

Ultraviolet absorption difference spectra of GLC-I and GLC-II with papain

The UV difference spectrum shows a peak at 260 nm and another peak at 295-300 nm while minima at around 280 nm for both the inhibitor-papain complexes (Fig. 3.7). This is indicative of perturbation in the environment of several aromatic amino acids residues in proteins induced upon complexation.

Fluorescence spectra of GLC-I and GLC-II in complex with papain

Fluorescence emission spectra showed maxima at 325 nm and at 330 nm for GLC-I and GLC-II, respectively. Upon formation of papain inhibitor complexes in both cases, fluorescence intensity increased comparative to the native inhibitors with red shift of 5 nm and 10 nm for GLC-I and GLC-II, respectively (Fig.3.8 and 3.9). These changes are indicative for alterations in conformation of either one or both the proteins involved in complex formation.
Figure 3.7: UV-Absorption difference spectra measured for goat lung cystatin–papain complex

GLC-I and II (2.66 μM) were incubated with activated papain for 30 min and an absorbance difference spectrum was calculated between 240nm to 320nm. GLC-I (○) and GLC-II (▲) and papain were in a molar ratio of 1:1.
Figure 3.8: Fluorescence spectra of GLC-I in complex with papain

Fluorescence spectra of the inhibitor alone (GLC-I), papain and papain – GLC-I complex was measured at excitation wavelength ($\lambda_{ex}$) of 280 nm and emission wavelength ($\lambda_{em}$) of 300 - 400nm using xenon arc lamp as the light source and cells of 1cm path length were used. The concentration of GLC-I was 2 $\mu$M. GLC-I was prepared in sodium phosphate buffer (50 mM, pH 7.5). The fluorescence of complex of GLC-I with papain was measured at a molar ratio of 1:1. The slit width was 5nm both excitation and emission beams.
Fluorescence spectra of the inhibitor alone (GLC-II), papain and papain-GLC-II complex were measured at excitation wavelength ($\lambda_{ex}$) of 280 nm and emission wavelength ($\lambda_{em}$) of 300 – 400 nm using xenon arc lamp as the light source and cells of 1 cm path length were used. The concentration of GLC-II was 2μM. GLC-II was prepared in sodium phosphate buffer (50 mM, pH 7.5). The fluorescence of complex of GLC-II with papain was measured at a molar ratio of 1:1. The slit width was 5 nm both excitation and emission beams.
Fluorescence Intensity vs. Wavelength (nm)

- □ - Papain
- ● - GLC-II
- ▲ - Papain + GLC-II
Circular Dichroism spectra of native GLC-I and its complex with papain

Far UV-CD spectra (Fig. 4.1) depict the contribution 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 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\(^2\).d.mol\(^{-1}\), which is defined as

\[
MRE = \frac{\theta_{obs}}{(10 \times n \times l \times Cp)}
\]

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

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

The results show GLC-I have 48.8% α-helical content.

Far UV-CD analysis of GLC-I with papain (Fig. 4.0) indicated that the change in the conformation of GLC-I upon complex formation with papain.
Figure 4.0: Far UV-CD Spectra of GLC-I in complex with papain

Far UV-CD spectra of native GLC-I alone and papain-GLC-I complex. The concentration of GLC-I was 200μg/ml and GLC-I and papain molar ratio was 1:1. The complex was obtained after incubation at 37°C for 30 minutes in 50 mM sodium phosphate buffer, pH 7.5. Cells of 1 mm path length were used.
DISCUSSION

Endogenous thiol proteinase inhibitors constitute the ultimate level of regulation of the overall cellular activity of cysteine proteinases (Sotirpoulou et al., 1997). Because of their involvement in various important physiological functions, they demand significant attention. A proteinase inhibitor is of significant importance because inhibition is achieved at physiological concentration of the inhibitor in a sufficiently short time with negligible dissociation of the complex (Beith, 1980). They perform several important functions in the body and are associated with several pathological conditions including rheumatoid arthritis (Trabandt et al., 1991), purulent bronchitis (Buttle et al., 1990), osteoporosis (Delaisse et al., 1991), renal failure (Servais, 2008; Kabanda et al., 1995), septic shock (Assfal-Machleidt et al., 1988), cardiovascular diseases (Lutgens et al., 2007), metastasizing cancer (Lee et al., 2008), Alzheimer's disease (Levy, 2008) and microbial invasion (North et al., 1990) resulting due to imbalance of endogenous cysteine proteinases and their inhibitors. Extensive work has been carried out for the purification and characterization of these inhibitors using various isolation procedures. Cystatins have been purified and characterized from various sources including bovine muscle (Bige et al., 1985), human spleen (Jarvinen, 1982), human liver (Green et al., 1984), sheep plasma (Baba et al., 2005), human placenta (Rashid et al., 2006), skin of Atlantic Salmon (Synnes, 1998) and bovine brain (Aghajanyan et al., 1988). Very recently some cystatin forms have been characterized from goat tissues (Priyadarshini and Bano, 2009; Sumbul and Bano, 2006; Zehra et al., 2005). The lack of existing information on cystatins from a very important tissue that is lung inventoried the present work, to the best of our knowledge it describes for the first time the purification and properties of cystatins from goat lung. It was envisaged that a thorough and systematic study of CPI from this new source will shed some light towards indepth understanding about the cystatins of mammalian system and it will be useful in comparing its properties with other known mammalian cystatins.

In the present work purification of goat lung cystatin was achieved using a three step procedure including alkaline treatment, ammonium sulphate precipitation and ion exchange chromatography. The procedure is efficient and simple with a percent yield and fold purification of 11.25% and 75.9 for GLC-I and 8.3% and 343.5 for GLC-II respectively. Purification of CPIs from other sources has been reported
using a combination of several techniques like affinity chromatography, chromatofocussing, gel filtration and ion exchange chromatography (Rashid et al., 2006; Sumbul and Bano, 2006; Baba et al., 2005; Zehra et al., 2005; Evans and Barrett, 1987; Anastasi et al., 1983). Our simple three step purification procedure for goat lung cystatin has given better yield and fold purification as compared to the values reported in literature for other species (Baba et al., 2005; Aghajanyan et al., 1988, Bige et al., 1985).

The molecular weight of the two isolated inhibitors as determined by SDS-PAGE is found to be 53.1 (GLC-I) and 63.1 (GLC-II) kDa under non-reducing conditions and it was found to be 63.1 (GLC-I) and 73.1 (GLC-II) under reducing conditions (Fig. 2.3). The discrepancy may be accounted for by the fact that lung cystatins on denaturation under reducing conditions exhibits hydrophobic aggregate tendency and the unfolded protein might have non-covalently aggregated to give a higher molecular weight. This result is supported by the work of Ekiel (Ekiel et al., 1997). Slower mobility of GLC on SDS-PAGE might be also due to the more exposure of hydrophobic regions in reducing conditions (SDS-β-mercaptoethanol complex) as is evident by the fact that proteolysis cleaved a number of hydrophobic residues which in turn bind more SDS than hydrophilic regions and consequently slower mobility in the presence of β-mercaptoethanol. Furthermore anomalous increase in the molecular weight determination of GLC by SDS PAGE under reducing and non-reducing conditions may also be explained on the basis of the hypothesis that all proteins bind fairly equal amounts of detergents and that the complexes formed by protein and detergent adopt the same shape and conformation such that the electrophoretic mobility is function of the molecular weight and pore size of the gel. If a protein does not show such behaviour then molecular weight determination by this method becomes erroneous. Known examples of such proteins include polypeptide with unusual charge (Panyim and Chalkley, 1971; Tung and Knight, 1971), conformation (Bjork et al., 1972), or with unreduced disulphide bridges (Trayer et al., 1971) and glycoprotein’s (Mitchell et al., 1973). The ambiguity of this method in case of GLC could be that this protein deviates from the general behaviour of proteins in dodecylsulphate solution. In order to rule out the discrepancy in molecular weight of GLC, we did sensitive and accurate method of molecular weight determination by MALDI-TOF analysis (Fig. 2.4 & 2.5) and the MW was found to be 66.42670 and
76.42562 for GLC-I and GLC-II, respectively which is very close to the molecular weight determined by SDS-PAGE under reducing conditions.

Cystatins from mammalian tissues are usually small molecular weight inhibitors having molecular mass in the range of 11-25 kDa (Rashid et al., 2006; Turk et al., 1995; Zabari et al., 1993; Green et al., 1984; Hiriado et al., 1981). However, high molecular mass inhibitors have also been reported from the skin of Atlantic salmon (Synnes, 1998), Goat brain (Sumbul and Bano, 2006), Goat pancreas (Priyadarshini and Bano, 2009) and goat kidney (Zehra et al., 2005).

High stability of the purified inhibitors (GLC-I and GLC-II) in broad temperature and pH ranges (75°C & pH 4-10) (Fig.2.7 and 2.6) are the properties consistent with other cystatins isolated from bovine muscle (Bige et al., 1985) and chicken cystatin (Saxena and Tayyab, 1997). GLC-I is found to be devoid of carbohydrate content while GLC-II possess 2.3% carbohydrate content. Presence of carbohydrate content has been reported in some type II cystatins like rat cystatin C isolated from urine is slightly glycosylated (Esnard et al., 1988) and cystatin isolated from goat kidney had also found to be slightly glycosylated (Zehra et al., 2005). Ni et al (1997) found carbohydrate attachments in cystatin E. Glycosylation has also been demonstrated in cystatin F from the immune cells and cystatin M isolated from primary tumour cell lines (Ni et al., 1998; Sotirpoulou et al., 1997). Thus, glycosylation in GLC-II is in accordance with cystatins isolated from above mentioned sources as well as those isolated from goat kidney and brain (Zehra et al., 2005; Sumbul and Bano, 2006). Sulphydryl content was found to be absent in both the inhibitors, which is in accordance with result previously reported by (Warwas and Sawicki, 1985) and others (Sumbul and Bano, 2006). The lung cysteine proteinase inhibitors GLC-I and II isolated in this study resemble more with other cystatins of type I isolated from various species with respect to carbohydrate content, disulphide linkages, pH and thermal stability, but the anomalous molecular weight of GLC-I obtained indicates that it does not resemble type I cystatins however shows some similarity with this class and hence can be placed as a variant of cystatin type I of the cystatin super family.

High molecular mass goat lung cystatin showed immune response and gave a high titre of antibodies in rabbits owing to its high molecular weight. The titre of antibodies determined by direct binding ELISA in rabbit serum was 25,600 (Fig. 2.8).
The antibodies raised against purified GLC-I gave a single precipitin line on immunodiffusion (Fig. 2.9) indicating that the GLC-I has immunogenic purity and homogeneity. The antisera exhibited immunogenic identity with GLC-II and goat brain cystatin indicating identity in epitopes.

Stoichiometry of the binding of GLC-I with papain, ficin and bromelain was determined by the titration of the proteinases with the inhibitor. A stoichiometric ratio of 1:1 was obtained for both GLC-I and GLC-II with papain, ficin and bromelain. This suggests that both the forms of goat lung cystatins are tight binding inhibitors of these thiol proteinases. Anastasi et al (1983) and Nicklin and Barrett (1984) reported equimolar complexes of cystatin with cysteine proteinases. The result is also consistent with the findings of Abrahamson et al (1987).

The goat lung cystatins exhibited a $K_i$ of $1.5 \pm 0.2 \times 10^{-9} \text{M}$, $1.3 \pm 0.2 \times 10^{-8} \text{M}$ and $2.6 \pm 0.3 \times 10^{-8} \text{M}$ for papain (Fig. 3.1), ficin (Fig. 3.2) and bromelain (Fig. 3.3), respectively (Table 1.4). A comparison of $K_i$ (app) values indicates that GLC-I has highest affinity for papain, then ficin and least with bromelain. It has been reported that cystatin isolated from other sources generally do not inhibit bromelain but in the present study bromelain is found to be inhibited by the purified inhibitor (GLC-I) as reported earlier for goat kidney (Zehra et al., 2005), human placental cystatin (Rashid et al., 2006; Warwas and Sawicki, 1985) and goat brain cystatin (Sumbul and Bano, 2006). The increasing value of $K_i$ for lung cystatin with an increase in the substrate concentration suggests the inhibition to be competitive. This finding is supported by the apparent results of Nicklin and Barrett (1984) for the inhibition of human cathepsin B by chicken cystatin and also strengthens by the work of Abrahamson et al (2003).

Tight binding inhibitors have high association rate constants. Among the three thiol proteinases, as expected papain has the highest $K_{+1}$ (association rate constant) of $3.06 \pm 0.1 \times 10^5 \text{M}^{-1}\text{sec}^{-1}$. The $K_{+1}$ values of $8.33 \pm 0.2 \times 10^4 \text{M}^{-1}\text{sec}^{-1}$ and $8.0 \pm 0.4 \times 10^4 \text{M}^{-1}\text{sec}^{-1}$ were found for ficin and bromelain, respectively (Table 1.4). Again the affinity was in the order of papain > ficin > bromelain.

The dissociation rate constant ($K_{-1}$) for the enzyme-inhibitor complex was determined by displacement procedure, in which the inhibitor released from the complex, was trapped by excess substrate (casein) with the increase in time. The
amount of enzyme released from the complex was monitored by continuous measurement of enzyme activity. The respective $K_{-1}$ values of GLC-I obtained for papain (Fig.3.4), ficin (Fig.3.5) and bromelain (Fig.3.6) were $4.6\pm0.4\times10^{-4}$ sec$^{-1}$, $1.09\pm0.1\times10^{-3}$ sec$^{-1}$ and $2.1\pm0.2\times10^{-3}$ sec$^{-1}$, respectively.

The values of $K_{+1}$ and $K_{-1}$ are consistent with rate constant of chicken cystatin with papain (Nicklin and Barrett, 1984). Low $K_{-1}$ and high $K_{+1}$ and half life values suggest the stability of the enzyme inhibitor complex (EI) and rapid complex formation. Hence data obtained gives comprehensive information about the kinetics of the purified goat lung cystatin with papain, ficin, and bromelain.

UV difference spectra between cystatin–papain complexes revealed the pattern of changes in ultraviolet absorption in the aromatic wavelength region. The peaks observed at 260 nm may have the contributions of cysteine or phenylalanine residues or may be due to tryptophan also (Donovan, 1969). Similarly, the peaks around 295 nm for GLC-I and GLC-II–papain complex are suggestive of the involvement of aromatic residues in the binding with papain. Minima at around 280 nm indicate changes around tyrosine residues (Donovan, 1973; 1969) (Fig.3.7), the spectra is similar to the cystatin purified from goat brain (Sumbul and Bano, 2006). The negative peaks in difference spectra for GLC-II are indicative of changes around tyrosine residues (Donovan, 1973), an attribute similar to the UV spectra of kininogen purified from sheep plasma (Baba et al., 2005).

The complexes of GLC-I and GLC-II with equimolar papain showed a 5 nm and 10 nm red shift and increase in fluorescence intensity (Fig.3.8 and 3.9). This suggests that the interaction of the two proteins occur in a manner leading to the exposure of absorbing groups to the polar environment or tryptophan has come near the charged groups (Friefelder, 1982).

CD spectra of GLC-I in the far-UV region shows a peak at 222 nm and a small peak at 210 nm (Fig.4.0). The $\alpha$-helical content is found to be 48.8% which is higher as compared to other previously reported cystatins like human placental cystatin and chicken cystatin which have 21.08%, and 20% helical content, respectively (Rashid et al., 2006; Schwabe et al., 1984).

It is interesting to speculate on the physiological role of these endogenous proteinase inhibitors. It seems likely that these endogenous inhibitors would at least
serve a protective function against inappropriate proteolysis both within the cell and outside the cell. The lysosomal proteinases of leukocytes and alveolar macrophages are believed to contribute to the destruction of lung tissue, which occurs in emphysema. It has been shown that α1-antitrypsin inactivates cathepsin B (James et al., 1979), tending to weaken lung defence against other proteinases. Thus, GLC-I and GLC-II could represent second line of defense against emphysema. Recently evidence has been implicated for cystatins in the machinery working against microbes of the trachea (North et al., 1990). Thus, due to the importance and myriad crucial functions that cystatins performs in the mammalian body, the study is of great significance as it sheds light on the physiochemical properties of cystatins in one of the most important mammalian organ, the lung.
CHAPTER-2

(A) Unfolding Studies of Goat Lung Cystatin Using Urea and Guanidine Hydrochloride as Denaturants

(B) Acid Induced Unfolding of Goat Lung Cystatin, a Cause of Amyloid Fibril Formation
4.2 RESULTS

(A) UNFOLDING STUDIES OF CYSTATIN IN THE PRESENCE OF DENATURANTS

4.2.1 INHIBITORY ACTIVITY OF GLC-I IN THE PRESENCE OF GdnHCl and UREA

Effect of Guanidium hydrochloride (GdnHCl) on the inhibitory activity of GLC-I

Changes in the inhibitory activity of goat lung cystatin (GLC-I) after incubation for 2 hours at different concentrations of guanidine hydrochloride are shown in Fig. 4.1 A. At 1 M concentration of GdnHCl, GLC-I lost its 50% inhibitory activity. The inhibitor (GLC-I) was found to be completely inactivated at 5-6 M of GdnHCl.

Effect of urea on the inhibitory activity of GLC-I

Changes in the inhibitory activity of (GLC-I) after incubation for 2 hours at different concentrations of urea are shown in Fig.4.1 B. The activity of GLC-I increased 1.5 fold when the urea concentration was increased to 2 M. The inhibitory activity then decreased with further increase of the urea concentration.

4.2.2 INTRINSIC FLUORESCENCE STUDIES OF GLC-I IN THE PRESENCE OF DENATURANTS

Effect of GdnHCl on intrinsic fluorescence of GLC-I

Intrinsic fluorescence is an excellent spectroscopic probe to investigate conformational changes in tertiary structure of proteins. The modification of microenvironment of aromatic residues of GLC-I due to denaturants has been studied by monitoring the changes in intensity and wavelength of emission maxima as a function of denaturant concentration. Fluorescence spectra of GLC-I in the presence of GdnHCl shows unfolding of the inhibitor as manifested by the red shift (15 nm) of the emission maximum (Fig. 4.2 A).
Figure 4.1 A: Guanidine hydrochloride induced inactivation of GLC-I

Native GLC-I (1.066μM) was incubated with increasing concentration of GdnHCl (0.5-6 M) for 2 h at room temperature. GLC-I was assayed for loss of inhibitory activity by caseinolytic assay of Kunitz (1947) as described in methods. Values are mean of four independent determinations.

Figure 4.1 B: Urea induced inactivation of GLC-I

Native GLC-I (1.066μM) was incubated with increasing concentration of urea (1-8 M) for 2 h at room temperature. GLC-I was assayed for loss of inhibitory activity by caseinolytic assay of Kunitz (1947). Values are mean of four independent determinations.
Effect of urea on intrinsic fluorescence of GLC-I

Fluorescence spectra of GLC-I in the presence of urea showed different behaviour of unfolding in comparison to GdnHCl. At lower concentration of urea (0-3 M) blue shift (5 nm) was observed followed by red shift (5 nm) at higher concentration (Fig. 4.2 B).

4.2.3 EXTRINSIC FLUORESCENCE STUDIES OF GLC-I IN THE PRESENCE OF DENATURANTS (GdnHCl / Urea)

Effect of GdnHCl on extrinsic fluorescence of GLC-I

The fluorescence emission of 1-anilinonaphthalene-8-sulphonic acid (ANS) is known to increase when the dye binds to the hydrophobic regions of the protein (Stryer, 1965). Fig. 4.3A shows the extrinsic fluorescence spectra of GLC-I in the presence of GdnHCl. There is an increase in the ANS fluorescence when the GdnHCl concentration increased from 0.5M to 1M while from 1M to 6M, there is decrease in the extrinsic fluorescence with red shift of 26 nm.

Effect of urea on extrinsic fluorescence of GLC-I

Changes in the ANS fluorescence are generally used to detect non-native conformation of globular proteins (Semisotonov et al. 1991). The hydrophobic fluorescent dye, ANS was used to probe intermediate conformations of goat lung cystatin (GLC-I). Fig. 4.3B showed the ANS fluorescence spectra of GLC-I in the presence of different concentration of urea. Upon pre-incubation of GLC-I with urea, the ANS fluorescence intensity of the inhibitor was increased at low concentrations of urea (0-2 M) with a blue shift of 5 nm. Further increase in the concentration of denaturant was accompanied by a sharp decrease in ANS fluorescence intensity with a red shift of 16 nm.
Figure 4.2 A: Intrinsic fluorescence analysis of GLC-I on interaction with various concentrations of GdnHCl

The concentration of GLC-I was 2μM. It was pre incubated for 2 hrs at room temperature in 50mM sodium phosphate buffer, (pH 7.5) containing the indicated concentrations of GdnHCl (0.5-6M). Fluorescence was measured at an excitation wavelength of 280nm in the emission range of 300-400nm with a slit width of 5nm. Fluorescence spectra of GLC-I was measured in different concentrations of GdnHCl (0.5-6M). Spectra of native, 0.5, 1, 2, 3, 4, 5, and 6M GdnHCl are shown in the Fig.4.2 A. Inset shows dependence of wavelength maxima (λmax) of GLC-I on interaction with different concentration of GdnHCl.

Figure 4.2 B: Intrinsic fluorescence analysis of GLC-I on interaction with various concentrations of urea

The concentration of GLC-I was 2μM. It was pre incubated for 2 hrs at room temperature in 50mM sodium phosphate buffer, (pH 7.5) containing the indicated concentrations of urea (1-8M). Fluorescence was measured at an excitation wavelength of 280nm in the emission range of 300-400nm with a slit width of 5nm. Fluorescence spectra of GLC-I was measured in different concentrations of urea (1-8M). Spectra of native, 1, 2, 3, 4, 5, 6, 7 and 8M urea are shown in the Fig.4.2 B. Inset shows dependence of wavelength maxima (λmax) of GLC-I on interaction with different concentration of urea.
Figure 4.3 A: Extrinsic fluorescence analysis of GLC-I in the presence of GdnHCl

The concentration of GLC-I was 2\( \mu \text{M} \). It was pre incubated for 2 hrs at room temperature in 50mM sodium phosphate buffer, (pH 7.5) containing the indicated concentrations of GdnHCl (0.5-6M). Fluorescence was measured at an excitation wavelength of 380nm in the emission range of 400-600nm with a slit width of 5nm. The molar ratio of protein to ANS was taken as 1:50. ANS fluorescence at 480 nm was measured in different concentrations of GdnHCl (0.5-6M). Spectra of native, 1, 2, 3, 4, 5, and 6M GdnHCl at 480 nm are shown in the Fig.4.3 A. Inset shows dependence of wavelength maxima (\(\lambda_{\text{max}}\)) of GLC-I on interaction with different concentration of GdnHCl.

Figure 4.3 B: Extrinsic fluorescence analysis of GLC-I in the presence of urea

The concentration of GLC-I was 2\( \mu \text{M} \). It was pre incubated for 2 hrs at room temperature in 50mM sodium phosphate buffer, (pH 7.5) containing the indicated concentrations of urea (1-8M). Fluorescence was measured at an excitation wavelength of 380nm in the emission range of 400-600nm with a slit width of 5nm. The molar ratio of protein to ANS was taken as 1:50. ANS fluorescence at 480 nm was measured in different concentrations of urea (1-8M). Spectra of native, 1, 2, 3, 4, 5, 6, 7 and 8M urea at 480 \text{ nm} are shown in the Fig.4.3 B. Inset shows dependence of wavelength maxima (\(\lambda_{\text{max}}\)) of GLC-I on interaction with different concentration of urea.
A

[Graph showing ANS Fluorescence intensity vs. Concentration of GdnHCl (M)]

B

[Graph showing ANS Fluorescence intensity vs. Concentration of urea (M)]
4.2.4 CD MEASUREMENTS

Effect of guanidium hydrochloride on secondary structure of GLC-I

Far UV-CD spectroscopy was used to monitor the changes in the secondary structure content of GLC-I upon GdnHCl induced denaturation. Fig. 4.4 A shows the effect of increasing concentrations of GdnHCl on the ellipticity of native protein (GLC-I). Ellipticity decreased with increasing concentration of GdnHCl and the protein is nearly unfolded at 6 M GdnHCl.

Effect of urea on secondary structure of GLC-I

Fig. 4.4 B shows the far UV-CD measurements of GLC-I in absence and presence of urea. The obtained results for urea shows, increase in ellipticity at 222 nm as compared to the native form in the range of 0-3 M, whereas its ellipticity decreased with further increase in the concentration of urea.

(B) ACID INDUCED UNFOLDING OF GOAT LUNG CYSTATIN:
A CAUSE OF AMYLOID FIBRIL FORMATION

4.2.5 Extrinsic fluorescence studies of GLC-I in the presence of acid

GLC-I was allowed to undergo acid denaturation by subjecting it to buffers of varying pH range from pH 1.0 to pH 7.0. Acid denaturation studies of model protein (GLC-I) highlights that the inhibitor retains tertiary structure (hydrophobic patches) upto pH 4.0 as indicated by ANS (Fig. 4.5 A). However at pH 3.5 there is a slight increase in ANS binding and with concomitant decrease in ellipticity which indicates partial unfolding of GLC-I at pH 3.5. The inhibitor unfolds further upon decreasing the pH below 3.5.

4.2.6 CD measurements of GLC-I in the presence of acid

Secondary structure of GLC-I was carried out in the presence of different pH range varying pH range from pH 1.0 to pH 7.0. The obtained results (Fig.4.5 B) showed that GLC-I retains its secondary structure upto pH 4.0. However at pH 3.5, it slightly loosens its ellipticity, which inflicts partial unfolding of GLC-I at pH 3.5. Further decrease in pH leads to unfolding of the inhibitor.
Figure 4.4 A: Secondary structure analysis of GLC-I in the presence of GdnHCl

Far UV-CD spectra of GLC-I in the presence of varying concentrations of GdnHCl (0-6M) were taken. Each spectrum is the average of at least 4 scans. 0.2mg/ml of GLC-I (dissolved in 50 mM sodium phosphate buffer, pH 7.5) was incubated with increasing concentrations of GdnHCl for 24 hrs. CD spectra were recorded in the range of 200-250 nm.

Figure 4.4 B: Secondary structure analysis of GLC-I in the presence of urea

Far UV-CD spectra of GLC-I in the presence of varying concentrations of urea (0-8M) were taken. Each spectrum is the average of at least 4 scans. 0.2mg/ml of GLC-I (dissolved in 50 mM sodium phosphate buffer, pH 7.5) was incubated with increasing concentrations of GdnHCl for 24 hrs. CD spectra were recorded in the range of 215-250 nm.
A

Wavelength (nm)

CD (mdeg)

5M
6M

205 210 215 220 225 230 235 240 245 250

B

Wavelength (nm)

CD (mdeg)

1M
2M
3M
4M
5M
6M
7M

215 220 225 230 235 240 245 250
Figure 4.5 A: Extrinsic fluorescence study of GLC-I at different pH values

For ANS study 1μM of GLC-I was incubated with glycine/HCl buffer (pH 1.0-2.0), sodium acetate buffer (pH 3.0-5.0) and sodium phosphate buffer (pH 6.0-7.0) at 20mM concentration for 4 hrs at 37°C. Fluorescence was measured at an excitation wavelength of 380nm in the emission range of 400-600nm with a slit width of 5nm. The molar ratio of protein to ANS was taken as 1:50.

Figure 4.5 B: CD spectra of GLC-I at different pH values

For Far UV-CD, 0.2mg/ml of GLC-I was incubated with glycine/ HCl buffer (pH 1.0-2.0), sodium acetate buffer (pH 3.0-5.0) and sodium phosphate buffer (pH 6.0-7.0) at 20mM concentration for 4 hrs at 37°C and CD spectra were recorded at 222 nm. Cells of 1mm path length were used.
A

![Graph A](image)

**ANS fluorescence intensity** against **pH**

B

![Graph B](image)

**CD (mdeg) at 222 nm** against **pH**
4.2.7 Fibril formation of goat lung cystatin (GLC-I)

There is increasing evidence that amyloid fibrils do not develop from the native conformations of proteins but from precursors which are only partially folded (Uversky and Fink, 2004). We decided, therefore to explore condition at which GLC-I is denatured but retains considerable amounts of residual structure. GLC-I (50µM) was incubated with buffers of varying pH range 1.0-7.0. Fibril formation was followed by ThT fluorescence and Congo red binding assay. Increase in ThT fluorescence and Congo red binding clearly indicates that GLC-I forms amyloid like structure under acidic conditions.

4.2.8 Thioflavin T Binding Studies

GLC-I (50µM) was incubated with buffers of varying pH range (pH 1.0-7.0). Five microlitres of the protein solution in which fibrils were growing, 600 µl of the ThT buffer was added (pH 7.5, 25 mM phosphate buffer, 20 µM ThT). ThT fluorescence at 480 nm was monitored at two different time intervals (2 and 14 days). Time course curve (Inset) of ThT binding was studied at regular time intervals for pH 4.0, 3.5 and 2.5 to follow the time period of fibrillation (Fig.4.6). ThT does not show any binding at pH 4.0. However at pH 3.5, it shows binding as indicated by increase in ThT fluorescence from zero to 10. At pH 2.5 ThT binding is more pronounced and increases with increasing time of incubation.

4.2.9 Congo red staining

Congo red dye binding to amyloid fibrils is a fibril specific probe. A change in colour from orange-red to rose is induced that corresponds to the characteristic shift in the Congo red absorbance spectrum (Fig.4.7). Congo red alone has an absorbance spectrum with a maximum at 470 nm. When fibrils are present the intensity increases and the absorption maximum red shifts to 490 nm. This enhancement in Congo red absorbance along with the concomitant 20 nm red shift in absorbance maximum reafirms the amyloid formation from GLC-I under acidic conditions.
Fluorescence emission spectra of thioflavin T (ThT) dye in the presence of goat lung cystatin (50μM) was incubated with buffers of different pH range varying from (pH 1.0-7.0) for different interval of time. Five microlitres of the stock solution (protein solution in which fibrils were growing) were dissolved in 600 μl of the ThT buffer (pH 7.5, 25 mM phosphate buffer, 20 μM ThT) just before the measurements. Excitation was at 440 nm and spectra were recorded at 480 nm. The slit width was 5nm for both excitation and emission beams. Solid triangle (▲) represent ThT fluorescence spectra of GLC-I under different pH condition after 14 days of incubation while hollow circle (○) represent ThT fluorescence after 2 days of incubation. Inset shows spectra after 2, 3, 4, 7 and 14 days of incubation at three different pH.
ThT Fluor at 480 nm

The graph shows the pH levels over time with points at 0, 10, 20, 30, 40, and 50 along the x-axis. The y-axis represents pH levels from 0 to 7. The graph includes multiple curves indicating different pH levels over time.
Figure 4.7: Absorbance spectra of Congo red treated with goat lung cystatin (GLC-I)

8.2 μM of GLC-I (diluted from fibrils making solution at pH 2.5) were treated with 0.006 mM Congo red (prepared in sodium phosphate buffer, pH 7.4 containing 150 μM NaCl) and their absorbance was recorded in the range of 400-600 nm.
4.2.10 Electron Microscopy

Electron micrographs from samples recovered after a period of 14 days reveal the presence of amyloid like aggregates at pH 4.0. Further, increase in acidity (pH 3.5) leads to organization of short amyloid type filament which at further low pH 2.5 value shows a network of filaments (Fig. 4.8). Sample at pH 7.5 does not show any aggregation (Fig. 4.8 a).
Figure 4.8 A: Electron micrographs of GLC-I incubated at pH 7.5

Electron micrograph of amyloid fibrils produced by GLC-I incubated for 14 days at pH 7.5. Reaction mixtures were spread on carbon-coated grids, and then stained with 1% phosphotungstic acid (pH 7.0), and finally examined with a Hitachi H-7000 electron microscope with an accelerated voltage of 10 Kv. Bar line (---) represents magnification.
Figure 4.8 B: Electron micrographs of GLC-I incubated at pH 4.0

Electron micrograph of amyloid fibrils produced by GLC-I incubated for 14 days at pH 4.0. Reaction mixtures were spread on carbon-coated grids, and then stained with 1% phosphotungstic acid (pH 7.0), and finally examined with a Hitachi H-7000 electron microscope with an accelerated voltage of 10 Kv. Bar line (---) represents magnification.
Figure 4.8 C: Electron micrographs of GLC-I incubated at pH 3.5

Electron micrograph of amyloid fibrils produced by GLC-I incubated for 14 days at pH 3.5. Reaction mixtures were spread on carbon-coated grids, and then stained with 1% phosphotungstic acid (pH 7.0), and finally examined with a Hitachi H-7000 electron microscope with an accelerated voltage of 10 Kv. Bar line (---) represents magnification.
Figure 4.8 D: Electron micrographs of GLC-I incubated at pH 2.5

Electron micrograph of amyloid fibrils produced by GLC-I incubated for 14 days at pH 2.5. Reaction mixtures were spread on carbon-coated grids, and then stained with 1% phosphotungstic acid (pH 7.0), and finally examined with a Hitachi H-7000 electron microscope with an accelerated voltage of 10 Kv. Bar line (---) represents magnification.
Discussion-2
DISCUSSION

There is a well known relationship between protein structure and function. A unique three dimensional conformation in a protein is a pre-requisite for its desired function. Protein denaturation is a highly cooperative process, which for small globular proteins may be approximately a two-state model and no significant intermediates are present during the transition N-D (where, N= native and D= denatured) (Aune and Tanford, 1969). However, several reports show that some intermediates exist during unfolding of proteins (Brems et al., 1990; Mitchinson and Pain, 1985). The intermediates between native and unfolded states have been referred to as molten globules in some cases (Ptitsyn, 1995). The characteristic features of a molten globule are (a) it is less compact than the native state, (b) it is more compact than the unfolded state, (c) it contains significant secondary structure, and (d) it has only loose tertiary structure without side-chain packing.

Increasing inclination in protein folding/unfolding is due to the recognition that failure of cellular protein folding mechanisms is associated with a variety of important human disorders ranging from cystic fibrosis to Alzheimer’s disease (Dobson, 2004). There is a growing body of evidence indicating a critical role for partially folded protein conformers in the process of conversion of normal cellular proteins into disease causing, proteinase-resistant protein aggregates of various morphologies (Dobson, 2004). There are various reports available which have shown different effects of GdnHCl and urea on protein unfolding like mushroom tyrosinase which shows different behaviour towards GdnHCl, urea and SDS in terms of different transition processes for these denaturants (Park et al., 2003). In this study an attempt has been made to gain an insight into the structure-function relationship of goat lung cystatin (GLC-I) to depict its behavior towards denaturants like GdnHCl and urea.

Activity measurements show that at 0.5 M GdnHCl concentration, the inhibitor was inactivated by 50% suggesting changed conformation of protein compared to fully active form. GdnHCl starts behaving as a classical denaturant at higher concentration where it leads to extreme unfolding of the protein, which ultimately causes complete loss of inhibitory activity of GLC-I at 4 M GdnHCl. The above result is supported by the fact that polar solvents such as water, with a high polarizability value, provide a change in the microenvironment of the fluorophore by
increasing solvation, reducing excited state complex energy, and producing a red shift as is occurring in the case of GLC-I denatured by GdnHCl. The charged molecule of GdnHCl allows the competition with ionic pairs, destroying them and forming new interactions with denaturing agent, leading to the unfolded protein. Intrinsic fluorescence, extrinsic fluorescence and CD spectroscopy results altogether indicate two state transition of GLC-I in the presence of GdnHCl.

In case of urea, the enhanced activity of GLC-I at lower denaturant concentration (2 M) seems to be due to conformational and dynamic changes in the GLC-I. This agrees with previously reported findings that activation of different enzyme by denaturants was associated with conformational changes in the tertiary structure (Das and Dasgupta, 1998; Wolosiuk and Stein, 1990) and secondary structure (Inui et al., 1999) of the enzymes, or the dynamics of enzymes active sites making them more active (Jaenicke and Bohm, 2001; Zhang et al., 1997).

The interesting observation was the blue shift in the fluorescence emission in the presence of low concentration of urea. The emission maximum suffers a blue shift of 5 nm toward lower wavelength in the presence of 3 M urea. This result can be attributed to the fact that the shift in the fluorescence emission maximum may be due to the burial of the already exposed tryptophan residues. Intrinsic fluorescence results clearly indicate the formation of intermediate and its stabilization at lower concentration of urea which is further confirmed by the increase of ellipticity at lower concentration of urea (0-3 M) followed by a sharp decrease in ellipticity as compared to native state. Stabilization of the purified inhibitor (GLC-I) at lower concentration of urea is in accordance with the other previously reported results (Johnson and Fresht, 1995; Mayr and Schmid, 1993). However, the mechanism of this type of stabilization in the presence of urea is not clear. As urea is a non-ionic denaturant, the effect of ionic interactions on the stabilization of intermediate state of GLC-I can be ruled out. Thus, the stabilizing effect of urea, at low concentrations, may be arising from an effect of urea on the structure of water. This creates unfavourable conditions for the exposed hydrophobic groups and may be pushing them into the interior of the molecule as observed by the 5 nm blue shift in the intrinsic as well as extrinsic fluorescence emission maximum. Elimination of water molecules from the interior of the protein leads to a decrease in the interior volume leading to increase in the
High concentration of urea leads to unfolding of (GLC-I) as revealed by 5 nm red shift with an increase in fluorescence intensity. ANS binding studies show decrease in ANS fluorescence intensity with red shift of 16 nm, which indicates disruption of hydrophobic patches due to unfolding of the inhibitor.

Taking into consideration all these results, mechanism of conformational alternation of GLC-I by urea and GdnHCl may be represented as:

\[
\begin{array}{c}
0.5-6 \text{ M GdnHCl} \\
\text{Native (GLC-I)} \rightarrow \text{Denatured} \\
(N) \rightarrow 0-3 \text{ M Urea} \rightarrow \text{Non-native (Intermediate)} \rightarrow 4-8 \text{ M Urea} \\
\end{array}
\]

Cystatins have important roles to play in normal body processes owing to their cysteine proteinase inhibitory activity and it is of utmost consequence that their conformation should be stable for maximum functional activity. The information obtained from GdnHCl and urea denaturation of proteins gives an idea about proteins stability and can be extrapolated to physiological processes. Thus the above observations have shed some light on the structural alterations and loss of function of proteins which can result due to their exposure to denaturants and thus effect the normal functioning of the protein.
Protein aggregation is essentially a self-association process in which many identical protein molecules form higher order conglomerates of low solubility that eventually precipitate. On the basis of their macroscopic morphology, they are generally classified as either ordered or disordered aggregates (Dobson, 2004; Lopez and Serano, 2004). Under physiological conditions, almost any protein can be induced at high concentration to form amorphous aggregates, under the same conditions, a much smaller set of proteins form highly ordered β-rich structure called as amyloid fibers (Dobson, 2003). Protein aggregation is a widespread phenomenon that arises from early folding intermediates through kinetic competition between proper folding and misfolding. It can occur under particular conditions or following certain mutations. The formation of aggregates depends on protein concentration, pH, temperature, ionic strength etc. (Yon, 1996). Their formation has often been considered as a nonspecific association of partially folded polypeptide chains through hydrophobic interactions.

There are approximately 20 known amyloid diseases representing a perplexing medical problem, including such diverse entities as Alzheimer’s disease (fibrils from the amyloid β-peptide, Aβ), the spongiform encephalopathies or prion diseases, including Creutzfeldt-Jakob disease in humans, scrapie in sheep and bovine spongiform encephalopathy (mad-cow disease) (fibrils from the prion protein, PrP), type 2 diabetes mellitus (fibrils from islet amyloid polypeptide or amylin), Parkinson’s disease (fibrils from α-synuclein) and Huntington’s disease (fibrils from huntingtin) (Rochet and Lansbury, 2000). Amyloid formation is also inflicted in lungs proteins like SP-C (surfactant protein C) which played an important role in preserving alveolar potency and in lung host defence (Whitsett and Weaver, 2002; Creuwels et al., 1997). In addition to pathogenesis caused by amyloid formation, it also has a protective role as shell formation in the silk moth (Iconomidou et al., 2000), and spiders’ web fibres have been proposed to have amyloid-like properties (Kenney et al., 2002).

The study was tailored to corroborate the “generic amyloid” hypothesis using cystatin of an important tissue, the lung as the model protein. Formation of amyloid-like fibrils is frequently considered to be a generic property of proteins including cystatins (Fandrich et al., 2001; Dobson, 1999) and is not limited to only disease related proteins (Kelly, 1996; Koo et al., 1996) or with any obvious sequence similarity. A cross β-sheet structure forming the core of fibrils is archetype of all
Amyloid fibrils (Sunde and Blake, 1997). These straight and unbranched amyloid fibrils range in width from 60-130 Å and in length from 1000 to 16000 Å (Sipe and Cohen, 2000). These structures can be detected through the binding of amyloid specific dyes such as thioflavin T (ThT) (Levine, 1999) or Congo red (Klunk et al., 1989).

A common observation is that fibrillization starts from an intermediate state; either partially unfolded or partially folded molten globule or native like intermediate (Uversky and Fink, 2004; Zerovnik et al., 2002). Such a “conformational change hypothesis” is widely supported by a large body of experimental data (Ahmad et al., 2004; Zerovnik et al., 2002). For fibril growth by GLC-I, this desideratum was met by choosing low pH conditions.

Acidic denaturation studies of model protein (GLC-I) highlights that the inhibitor retains its secondary as well as tertiary structure (hydrophobic patches) upto pH 4.0 as indicated by ANS and CD measurements (Fig. 4.5 A and 4.5 B). However at pH 3.5 there is slightly increase in ANS binding and cooperatively, its ellipticity also decreases which indicate partial unfolding of GLC-I at pH 3.5. The inhibitor unfolds further upon decreasing the pH below 3.5.

ThT fluorescence at 480 nm was monitored at regular time intervals for pH 4.0, 3.5 and 2.5 to follow the time course of fibrillation (Fig. 4.6). ThT does not show any binding at pH 4.0 which is supported by the fact that, GLC-I is not in partially unfolded state (upto pH 4.0), which is prerequisite for fibril formation. However at pH 3.5, it shows binding which is consistent with the partially unfolding of the inhibitor at this pH. Thus, pH 3.5 is a highest fibrillogenic starting pH for GLC-I. At pH 2.5 ThT binding is more pronounced and increases with increased time of incubation. This finding is supported by the fact that the relatively unfolded but compact intermediate readily forms amyloid fibrils, whereas the native like structure preferentially leads to amorphous aggregates (Khurana et al., 2003). Minimum binding of ThT at lower pH (< 2.5) is attributed to the fact that, at this pH inhibitor looses its maximum conformation as shown in figure 4.5 A and 4.5 B, thereby indicating less chances of self assembly. Thus, above results clearly indicate that partially unfolded state readily forms fibril as compared to completely disordered structure.
Electron micrographs of lung cystatin grown under different solvent conditions (pH value, 4.0, 3.5, & 2.5) were analyzed. Only aggregates were observed at pH 4.0 (Fig. 4.8 B). At pH 3.5 sharp fibril is observed and at 2.5 fibrillar network is observed (Fig. 4.8 C and D). Thus above results clearly indicate that acidic pH induces fibrillization and this fibrillization is more rapid and clear at lower pH (below 4).

The findings reported here, along with the previous observation of fibrils formed by SH3 domain (Zurdo et al., 2001), BSA (Holm et al., 2007), and stefin B (Zerovnik et al., 2002) substantiate that amyloid fibril formation is not unique to few disease causing proteins found in the amyloid plaques that accumulate in vivo.

The present results suggest that, provided appropriate conditions are maintained over prolonged periods of time, the formation of ordered amyloid protofilaments and fibrils could be an intrinsic property of many polypeptide chains, rather than being limited to very few aberrant sequences. GLC-I has not yet been implicated as the cause of any diseased state in the lung. However the conditions which predispose it to aggregate may provide an opportunity to understand protein folding in general as well as to design therapeutic approaches to combat such an aggregation as deviation from physiological pH under stressful environment may occur in cells.
CHAPTER 3

Preventive Effect of Curcumin and Quercetin Against Nitric Oxide Mediated Modification of Goat Lung Cystatin
4.3 RESULTS

4.3 NITRIC OXIDE INDUCED DAMAGE AND PREVENTIVE EFFECT OF CURCUMIN AND QUERCETIN ON GLC-I

4.3.1 Nitric oxide production from sodium nitroprusside

Nitric oxide was generated from sodium nitroprusside (SNP) (nitric oxide donor) and measured by the Griess reaction (Green et al., 1982). SNP in aqueous solution at physiological pH spontaneously generates NO, which interacts with oxygen to produce nitrite ions which can be estimated using Griess reagent (1% sulphanilamide and 0.1% naphthylenediamine dihydrochloride in 2% H₃PO₄). Incubation of solutions of SNP in phosphate buffered saline of pH 7.4 at 25°C resulted in linear time-dependent nitrite production (Fig. 4.9).

4.3.2 Functional inactivation of GLC-I by nitric oxide

GLC-I on incubation with SNP, lost its inhibitory activity (Table 1.5). More than 47% loss in activity was observed within 60 minutes of incubation with SNP (NO donor). With in 180 minutes, inhibitory activity trailed off to zero.

4.3.3 Structural modification of GLC-I by nitric oxide

Intrinsic fluorescence is an excellent spectroscopic probe to investigate conformational changes in tertiary structure of proteins. The modification of microenvironment of aromatic residues of GLC-I in the presence of NO has been studied by monitoring the changes in intensity and wavelength of emission maximum as a function of SNP concentration. Fluorescence spectra of GLC-I in the presence of NO shows quenching of fluorescence intensity coupled with 15 nm red shift (Fig. 5.0 A). Fig. 5.0B shows that NO also causes conformational alterations in GLC-I in a time dependent manner.

Structural changes in GLC-I by NO were investigated using polyacrylamide gel electrophoresis. As the concentration of SNP is increased from 0.05mM to 10mM, there is formation of aggregates as indicated by the shift of protein band to a upper position in the gel as compared to native protein band (Fig.5.1).
Figure 4.9: Time-dependent nitric oxide production from a solution of 10 mM sodium nitroprusside

10 mM of SNP was prepared by dissolving the powder in PBS, pH 7.4. At 30 min intervals, 1 ml of incubation solution was removed and reacted with 1 ml of Griess reagent as described in methods section. The absorbance was read at 540 nm.
TABLE- 1.5: EFFECT OF VARYING TIME OF INCUBATION WITH 10 mM SNP ON ANTIPROTEOLYTIC ACTIVITY OF GLC-I

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Treatment &amp; Time of Incubation</th>
<th>% Remaining Activity</th>
<th>% Loss of inhibitory Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Native GLC-I</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>2.</td>
<td>GLC-I+SNP (0 min)</td>
<td>98.29±1</td>
<td>1.71</td>
</tr>
<tr>
<td>3.</td>
<td>GLC-I+SNP (15 min)</td>
<td>89.20±1.5*</td>
<td>10.80</td>
</tr>
<tr>
<td>4.</td>
<td>GLC-I+SNP (30 min)</td>
<td>83.52±2.1*</td>
<td>16.48</td>
</tr>
<tr>
<td>5.</td>
<td>GLC-I+SNP (45 min)</td>
<td>60.77±2.2*</td>
<td>39.23</td>
</tr>
<tr>
<td>6.</td>
<td>GLC-I+SNP (60 min)</td>
<td>52.94±1.3*</td>
<td>47.06</td>
</tr>
<tr>
<td>7.</td>
<td>GLC-I+SNP (90 min)</td>
<td>38.51±1.5*</td>
<td>61.49</td>
</tr>
<tr>
<td>8.</td>
<td>GLC-I+SNP (120 min)</td>
<td>29.19±2.1*</td>
<td>70.81</td>
</tr>
<tr>
<td>9.</td>
<td>GLC-I+SNP (150 min)</td>
<td>18.06±1.1*</td>
<td>81.94</td>
</tr>
<tr>
<td>10.</td>
<td>GLC-I+SNP (180 min)</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

- Activity of native GLC-I was taken as 100
- Control used for calculations was native GLC-I
- *: p<0.001 by one way ANOVA
Figure 5.0 A: Effect of varying concentrations of NO on fluorescence spectra of GLC-I

GLC-I was incubated with different concentrations of SNP (0.05mM, 1mM and 10mM) for 30 min. as described in methods. The protein concentration used was 1μM. Excitation wavelength was 280 nm and the emission was recorded in the range of 300-400 nm with a slit width of 5 nm.
Figure 5.0 B: Stability of GLC-I on incubation with 0.05 mM SNP

GLC-I (1µM) was incubated with 0.05 mM SNP for different time intervals (30-120 min). The conformational changes in goat lung cystatin induced by NO generated from SNP were detected by fluorescence measurements which were carried out on a Shimadzu spectrofluorimeter model RF-540 equipped with a data recorder DR-3. Excitation wavelength was 280 nm and the emission was recorded in the range of 300-400 nm. Slit width was 5 nm.
Figure 5.1: Polyacrylamide gel electrophoresis of GLC-I in the presence of nitric oxide generating chemical (SNP)

50μg of GLC-I incubated with different concentration of SNP prepared in phosphate buffer saline of pH 7.4 at 25°C as described in methods. Lane (a) is native, lanes (b), (c) and (d) contain GLC-I incubated with 0.05mM, 5mM and 10mM SNP respectively for 2 hrs.
4.3.4 Prevention of functional inactivation of GLC-I by curcumin and quercetin

Curcumin (0-50μM) and quercetin (0-250μM) were used to validate their scavenging capacity for NO and protection of GLC-I against this damage. It was found that 50μM curcumin caused 95% retrieval of inhibitory activity of GLC-I (Table 1.6) while much higher concentration of quercetin (250μM) was needed to gain back 93.8% of activity (Table 1.7).

4.3.5 Preventive effect of curcumin and quercetin on structural modification of GLC-I

When GLC-I treated with 10 mM SNP (prepared in phosphate buffer saline pH 7.4 at 25°C) was run on PAGE in the presence of 50μM curcumin and 250μM quercetin concentration, no structural change of GLC-I was observed (Fig. 5.2). Loss in tertiary structure of GLC-I by NO is clearly prevented by curcumin (50μM) and quercetin (250μM) (Fig. 5.3).
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Treatment of Curcumin with GLC-I</th>
<th>% Remaining activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Native GLC-I</td>
<td>100</td>
</tr>
<tr>
<td>2.</td>
<td>GLC-I + SNP</td>
<td>18.06±1.1</td>
</tr>
<tr>
<td>3.</td>
<td>GLC-I + SNP + Curcumin (10µM)</td>
<td>27.60±1.5*</td>
</tr>
<tr>
<td>4.</td>
<td>GLC-I + SNP + Curcumin (20µM)</td>
<td>47.8±2.2*</td>
</tr>
<tr>
<td>5.</td>
<td>GLC-I + SNP + Curcumin (30µM)</td>
<td>74.29±2*</td>
</tr>
<tr>
<td>6.</td>
<td>GLC-I + SNP + Curcumin (40µM)</td>
<td>90.31±2.4*</td>
</tr>
<tr>
<td>7.</td>
<td>GLC-I + SNP + Curcumin (50µM)</td>
<td>95.87±2*</td>
</tr>
</tbody>
</table>

- Concentration of SNP used was 10mM
- Time of incubation of the reaction mixture was 2½ hrs.
- Control used for calculations was GLC-I treated with 10 mM SNP
- *: p<0.001 by one way ANOVA
TABLE 1.7: EFFECT OF VARYING CONCENTRATIONS OF QUERCETIN ON ANTIPROTEOLYTIC ACTIVITY OF SNP TREATED GLC-I

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Treatment of quercetin with GLC-I</th>
<th>% Remaining activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Native GLC-I</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>GLC-I+SNP</td>
<td>18.06±1.1</td>
</tr>
<tr>
<td>3</td>
<td>GLC-I+SNP+Quercetin(10µM)</td>
<td>18.74±1.5</td>
</tr>
<tr>
<td>4</td>
<td>GLC-I+SNP+Quercetin(20µM)</td>
<td>22.49±2.0</td>
</tr>
<tr>
<td>5</td>
<td>GLC-I+SNP+Quercetin(30µM)</td>
<td>28.85±2.5</td>
</tr>
<tr>
<td>6</td>
<td>GLC-I+SNP+Quercetin(40µM)</td>
<td>33.28±1.5</td>
</tr>
<tr>
<td>7</td>
<td>GLC-I+SNP+Quercetin(50µM)</td>
<td>35.78±2.2</td>
</tr>
<tr>
<td>8</td>
<td>GLC-I+SNP+Quercetin(100µM)</td>
<td>48.39±1.5</td>
</tr>
<tr>
<td>9</td>
<td>GLC-I+SNP+Quercetin(150µM)</td>
<td>64.97±2.5</td>
</tr>
<tr>
<td>10</td>
<td>GLC-I+SNP+Quercetin(200µM)</td>
<td>78.95±2.3</td>
</tr>
<tr>
<td>11</td>
<td>GLC-I+SNP+Quercetin(250µM)</td>
<td>98.83±1.9</td>
</tr>
</tbody>
</table>

- Activity of native GLC-I was taken as 100
- Concentration of SNP used was 10mM
- Time of incubation of the reaction mixture was 2½ hrs.
- Control used for calculations was GLC-I treated with 10 mM SNP
- *: p<0.001 by one way ANOVA
Figure 5.2: Polyacrylamide gel electrophoresis of GLC-I treated with NO and its scavengers curcumin and quercetin

GLC-I (50μg) incubated with 10 mM SNP (prepared in phosphate buffer saline of pH 7.4 at 25°C as described in methods) in the presence and in absence of curcumin and quercetin. Lane (a) is native while lanes (b) and (c) contain NO treated GLC-I in presence of 50μM curcumin and 250μM quercetin, respectively.
Figure 5.3: Intrinsic fluorescence study of SNP treated GLC-I in the presence of curcumin and quercetin

GLC-I was incubated with 5 mM SNP in the presence and in the absence of curcumin and quercetin. The intrinsic fluorescence spectra of native GLC-I and its treatment with NO and also in the presence of (50μM) curcumin and (250μM) quercetin were recorded after exciting the protein at 280 nm. The protein concentration used was 1μM. Excitation wavelength was 280 nm and their emissions were recorded in the range of 300-400 nm.
- Native (GLC-I)
- 5mM SNP+GLC-I
- SNP+GLC-I+Curcumin (50μM)
- SNP+GLC-I+Quercetin (250μM)
Discussion-3
DISCUSSION

The generation of reactive oxygen species (ROS) and other free radicals (R•) during metabolism is a necessary and normal process that ideally is compensated by an elaborate endogenous antioxidant system. However, due to many environmental, lifestyle, and pathological situations, there can be an excess of radicals resulting in a situation of oxidative stress, which has been implicated in cardiovascular disorders, cancer, and other chronic diseases accounting for a major cause of deaths today. Proteins are major targets for damage by free radicals because of their abundance in biological system. They are modified at the backbone or at the side chain groups. As many proteins are catalytic in nature, modifications by free radicals can have an amplified effect on their activity. They can enhance susceptibility to proteolysis, and aggregation. Davies (2005) reported that proteins, which have been exposed to certain radicals, exhibit altered primary, secondary and tertiary structure, and can undergo spontaneous fragmentation or increased proteolytic susceptibility.

Cystatins are endogenous thiol proteinase inhibitors and are responsible for the regulation of activity of cysteine proteinases, which may otherwise cause a number of diseases including rheumatoid arthritis (Trabandt et al., 1991), purulent bronchitis (Buttle et al., 1990), osteoporosis (Delaisse et al., 1991), renal failure (Servais, 2008; Kabanda et al., 1995), septic shock (Assfalq-Machleidt et al., 1988), cardiovascular diseases (Lutgens, 2007), metastasizing cancer (Lee et al., 2008), Alzheimer’s disease (Levy, 2008) and microbial invasion (North et al., 1990) resulting due to imbalance of endogenous cysteine proteinases and their inhibitors.

The treatment of cystatin purified from goat lungs with the NO-generating compound SNP causes concentration-and time-dependent loss of enzyme activity (Table 1.5 & Figure.5.0 A & B). This functional inactivation may arise due to modification of active site amino acids or may also be due to oxidation of critical tryptophan residues as shown by the quenching of fluorescence coupled with 15 nm red shift suggesting the complete unfolding of protein (GLC-I) in the presence of NO. This result is in accordance with previous pronouncement that NO may reversibly inhibit enzymes with transition metals or with free radical intermediates in their catalytic cycle. NO in micro molar concentrations reversibly inhibited catalase and cytochrome P-450 (Brunelli et al., 2001; Wink et al., 1993). GLC-I treated with SNP,
exhibits mobility less than the untreated sample due to aggregation visible in non-denaturing PAGE (Fig. 5.1).

Considering that reactive species may act as toxins, mediators and modulators of inflammatory gene activation and potentially damage membranes and cellular workhorses, proteins, efforts have been directed to investigate antioxidant molecules as potential therapeutic agents (Corner and Grisham, 1996). In this context polyphenols, flavonoids and other natural products are becoming increasingly important (Packer et al., 1999; Groot and Reven, 1998; Korkinov and Afanasiv, 1997). Present study, thus investigated the protective role of well known antioxidants curcumin and quercetin against the damage produced by NO. Curcumin (diferuloylmethane), a widely used yellow curry powder from turmeric (Curcuma longa) in Indian and other countries, has been used as a therapeutic agent because of its attractive combination of properties that include anti-inflammatory (Rao et al., 1982), antioxidant (Kuchandy and Rao, 1990), antifibrotic (Kang et al., 2002) and anticancer qualities (Gescher et al., 1998). Quercetins are polyphenolic compounds widely distributed in dietary fruits, vegetables and wine. It has a range of activities including antioxidant (Filipe et al., 2004), inhibit nitric oxide pathway (Mu et al., 2001), and have anti-inflammatory, anticancer and antihistamine activities (Mertens-Talcott and Percival, 2005; Wadsworth et al., 1999; Marozzi et al., 1970).

From the result of this study, it was found that curcumin (50μM) prevented NO induced functional and structural damage of GLC-I while for reclamation to similar extent approximately 5 times concentration of quercetin was required as indicated by activity and fluorescence measurements (Table 1.6 & 1.7). This observation could be explained in part by the fact that curcumin is a specific NO quencher. NO scavenging effects of curcumin have been reported earlier also by various groups (Frade et al., 2005; Man-Ying Chan et al., 2005; Punithavathi et al., 2003). Our results obtain support from above findings. Oxidative stress plays a major role in the pathogenesis of various diseases including cerebral ischemia-reperfusion injury, haemorrhage and shock, neuronal cell injury, hypoxia and cancer (Halliwell and Gutteridge, 2007), it was reported that curcumin exhibits strong anti-oxidant properties, comparable to vitamin E and C (Toda et al., 1985). Dietary supplementation of curcumin was also found to be beneficial in neurodegenerative diseases such as Alzheimer’s disease (Yang, 2005; Calabrese, 2003). Inhibitory
activity of curcumin on inflammation has been shown by many studies including inhibition of inflammation induced by carrageenan (Reddy and Lokesh, 1994; Srimal and Dhawan, 1973) and acute lung injury induced by cyclophosphamide (Venkatesan and Chandrakasan, 1995). Asthma and bronchitis are allergic reactions which are found to suppressed by the use dietary flavonoids especially quercetin (Chaabi et al., 2007). There are several reports which showed that animals pre-treated with quercetin or related compounds have reduced reactions to chemicals that trigger asthma attack (Jiang et al., 2007; Rogerio et al., 2007). Our results corroborate the possible involvement of NO in lung diseases and their cure by the use of curcumin and quercetin.

The results of the study are of great significance as proteins are the major targets of reactive species. It has been emphasized earlier that modifications of proteins by reactive species such as reactive oxygen species and reactive nitrogen species render them more susceptible to enhanced proteolysis, inactivation and denaturation. The present study documents the suppression of NO induced inactivation of GLC-I by curcumin and quercetin. Also, structural changes in SNP treated GLC-I are minimized by curcumin and quercetin. The widespread dietary consumption of curcumin, quercetin (and many other polyphenols) in varying proportions and amounts by the human population provides a rather inexpensive therapeutic option against oxidative injury. However, mechanism by which curcumin and quercetin prevent protein damage in vitro, evaluation of their efficiency in this context in vivo, and elucidation of molecular basis of their action summons future research.
CHAPTER 4

Effect of Drugs on Goat Lung Cystatin
1. Methotrexate
2. Benzo (a)pyrene
4.4 RESULTS

(A) EFFECT OF ANTICANCER DRUG METHOTREXATE (MTX) ON GLC-I

4.4.1 DRUG-PROTEIN INTERACTION (Binding of MTX with GLC-I)

Methotrexate is a chemotherapy drug used to treat leukemia, lymphomas, and osteosarcoma. It is also used in the treatment of AIDS and rheumatoid arthritis. The common side effects of MTX include liver dysfunction, immunosuppression, neurotoxicity and lung damage (Hughes et al., 1987). Drugs mainly exert effects by binding to proteins. And off-target binding of drugs to undesired proteins can have ill-consequences. For macromolecules, the fluorescence measurements can give some information about the binding of small molecules with proteins, such as the binding constants, binding sites and binding mechanism. Fluorescence intensity of a compound can be decreased or quenched by a variety of molecular interactions, such as excited-state reactions, molecular rearrangements, energy transfer, ground state complex formation and collisional quenching. The fluorescence spectra of GLC-I (1μM) in the presence of different concentrations (0.25-7 μM) of MTX were recorded in the range of 300-400 nm upon excitation at 280 nm. MTX caused quenching of the intrinsic fluorescence of GLC-I (Fig. 5.4) accompanied by a red shift (11 nm) of fluorescence maximum at 7μM. These results indicated that there were interactions between MTX and goat lung cystatin (GLC-I) and the binding reactions resulted in a non-fluorescent complex. The fluorescence quenching data was analysed by the Stern-Volmer equation:

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

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 constant and \([Q]\) is the concentration of quencher. Fig. 5.5 shows the Stern-Volmer plot for the binding of GLC-I-MTX complex. The \( K_{SV} \) values obtained at three different temperatures are shown in Table 1.8.
Figure 5.4: Intrinsic fluorescence study of GLC-I in the presence of MTX

GLC-I (1μM) was incubated with various concentrations of MTX varying from 0.25μM to 7μM for 2 hours. Fluorescence measurements were carried out on a Shimadzu Spectrofluorimeter model RF-540 equipped with a data recorder DR- 3 at 25°C. 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 5 nm for excitation and emission. The path length of the sample was 1 cm.
<table>
<thead>
<tr>
<th>Parameters</th>
<th>298K</th>
<th>308 K</th>
<th>318 K</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_{sv} ) (M(^{-1}))</td>
<td>2.8±0.04\times10^5</td>
<td>2.6±0.03\times10^5</td>
<td>2.3±0.04\times10^5</td>
</tr>
<tr>
<td>( K ) (M(^{-1}))</td>
<td>4.26\times10^5±0.02</td>
<td>4.0\times10^5±0.042</td>
<td>3.82\times10^5±0.05</td>
</tr>
<tr>
<td>( n )</td>
<td>1.11</td>
<td>0.98</td>
<td>0.97</td>
</tr>
<tr>
<td>( (\Delta G^\circ) ) KJmol(^{-1})</td>
<td>-32.0</td>
<td>-33.0</td>
<td>-33.9</td>
</tr>
<tr>
<td>( (\Delta H^\circ) ) KJmol(^{-1})</td>
<td></td>
<td>-3.8</td>
<td></td>
</tr>
<tr>
<td>( (\Delta S^\circ) ) J mol(^{-1})K(^{-1})</td>
<td></td>
<td></td>
<td>94.97</td>
</tr>
</tbody>
</table>

- \( K_{sv} \) represents Stern-Volmer constant
- \( K \) is binding constant
- \( n \) is number of binding sites
- \( \Delta G^\circ \) is Standard free energy change
- \( \Delta H^\circ \) is Standard enthalpy change
- \( \Delta S^\circ \) represent Standard entropy change
Determination of binding constant (K) and number of binding sites (n)

When small molecules bind independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecules is given by the equation (Feng et al. 1998; Gao et al. 2004):

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

Where K and n are the binding constant and the number of binding sites, respectively. Thus, a plot of \( \log \frac{F_0 - F}{F} \) versus [Q] can be used to determine K as well as n. The values of K and n obtained at three different temperatures are shown in Table 1.8.

Type of interaction between MTX and GLC-I

The forces of interaction between drugs and biomolecules include hydrophobic forces, electrostatic interactions, van der Waals interactions and hydrogen bonds. In order to identify the interacting forces between MTX with GLC-I, the thermodynamic parameters, i.e., free energy change (\( \Delta G^0 \)), enthalpy changes (\( \Delta H^0 \)), and entropy change (\( \Delta S^0 \)) of the interactions were calculated from the following equations:

\[
\ln K = \frac{-\Delta H^0}{RT} + \frac{\Delta S^0}{R}
\]

also \( \Delta G^0 = -RT \ln K = -\Delta H^0 + T \Delta S \)

The results obtained are shown in Table (1.8). The positive entropy change occurs because the water molecules that are arranged in an orderly fashion around the ligand and protein acquire a more random configuration as a result of hydrophobic interactions. The negative \( \Delta H^0 \) and positive \( \Delta S^0 \) values in case of MTX, therefore showed that both hydrogen and hydrophobic and interactions play a role in the binding of MTX to GLC-I.
Figure 5.5: Stern-Volmer plot for the binding of GLC-I with MTX at different temperatures

GLC-I (1μM) was incubated with various concentration of MTX varying from 0.25μM to 7μM for 2 hrs at temperatures 298K, 308K and 318K and their fluorescence spectra were recorded between 300-400 nm after exciting GLC-I at 280 nm. The fluorescence quenching data was further analysed by the Stern-Volmer equation as described in methods. The plot of Fo/F vs concentration of MTX gives binding constant (K) and the number of binding sites (n) between MTX-GLC-I complex.
4.4.2 UV-vis absorption studies of MTX-GLC-I complex

The interaction between MTX-GLC-I was studied from UV-vis absorption spectral data (Fig. 5.6). The UV absorption intensity of GLC-I increased with the variation of MTX concentration. The addition of drug results in the distinct shift of MTX-GLC-I spectrum toward longer wavelength (red shift). These results evince interaction and complex formation between MTX and GLC-I (Cui et al. 2004; Hu et al. 2004).

4.4.3 Inhibitory activity of GLC-I in the presence of MTX

Changes in the inhibitory activity of goat lung cystatin (GLC-I) after incubation at different time intervals (2-6 hrs) with increasing concentration of MTX is shown in Table 1.9. The result showed that GLC-I lost significant amount of inhibitory activity at 7 µM concentration after 6 hours of incubation. The obtained data also indicates that inactivation of goat lung cystatin by MTX is concentration as well as time dependent.

4.4.4 FT-IR measurements of GLC-I in the presence of MTX

Additional evidence regarding the MTX-GLC-I interaction comes from FT-IR spectroscopy, showing drug-protein complexes. Infrared spectra of proteins exhibit a number of the so-called amide bonds, which represent different vibrations of the peptide moiety. This vibration mode originates from the C = O stretching vibration of the amide group (coupled to the in-phase bending of the N–H bond and the stretching of the C–N bond) and gives rise to infrared bands in the region between approximately 1600 and 1700 cm⁻¹ (Wiltod et al. 1993). The protein amide bands have a relationship with the secondary structure. Figure 5.7 shows the FT-IR spectra of the free MTX and MTX-bound with GLC-I. The spectrum in figure 5.7 was obtained by subtracting the absorption of phosphate buffer from the spectrum of inhibitor solution. The evident peak shift of amide I band from 1652.4 to 1635.4 cm⁻¹ indicates that the secondary structure of GLC-I is changed when MTX was added.
Figure 5.6: UV-vis spectroscopy of GLC-I in the presence of MTX

(GLC-I) concentrations was fixed at 1μM while the MTX concentration was varied from 0.25μM-7μM. Absorption spectra of native GLC-I and in presence of MTX were recorded in the range of 200-300 nm on a double beam Shimadzu UV-vis spectrophotometer UV-1700 using a cuvette of 1 cm path length.
The graph shows the absorption spectra of MTX (Methotrexate) at different concentrations and native GLC-I (Glyceraldehyde 3-Phosphate Dehydrogenase). The x-axis represents the wavelength (nm), ranging from 200 to 300 nm. The y-axis represents absorbance, ranging from 0 to 4.

- MTX at various concentrations:
  - 1 µM MTX
  - 3 µM MTX
  - 7 µM MTX

- Native GLC-I (Glyceraldehyde 3-Phosphate Dehydrogenase)
- 0.25 µM MTX
TABLE-1.9: ANTIPROTEOLYTIC ACTIVITY OF GLC-I IN THE PRESENCE OF MTX AFTER INCUBATION AT VARIOUS TIME INTERVALS

<table>
<thead>
<tr>
<th>Concentration of MTX (μM)</th>
<th>2 hrs of incubation</th>
<th>4 hrs of incubation</th>
<th>6 hrs of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 μM</td>
<td>83.1±2.2</td>
<td>78.2±1.5</td>
<td>68.2±2.5</td>
</tr>
<tr>
<td>1 μM</td>
<td>79.4±1.4</td>
<td>75.3±2.0</td>
<td>65.4±2.2</td>
</tr>
<tr>
<td>1.25 μM</td>
<td>76.2±2.0</td>
<td>70.7±2.3</td>
<td>59.5±1.5</td>
</tr>
<tr>
<td>2 μM</td>
<td>71.2±2.5</td>
<td>64.5±1.5</td>
<td>53.3±2.5</td>
</tr>
<tr>
<td>3 μM</td>
<td>63.1±2.5</td>
<td>55.3±2.5</td>
<td>42.7±2.0</td>
</tr>
<tr>
<td>5 μM</td>
<td>51.5±1.9</td>
<td>45.5±2.2</td>
<td>28.4±1.2</td>
</tr>
<tr>
<td>7 μM</td>
<td>42.3±1.5</td>
<td>34.2±2.2</td>
<td>15.2±2.2</td>
</tr>
</tbody>
</table>

- The inhibitory activity of GLC-I in the presence of MTX was assessed by its ability to inhibit caseinolytic activity of papain as described in methods.
Figure 5.7: FT-IR spectra of GLC-I in the presence of MTX

GLC-I (1μM) was incubated with different concentration of MTX (0-7μM) for 2 hrs. The absorbance spectra were taken in the region 1800-1400cm⁻¹ as described in methods.
(B) EFFECT OF BENZO (a) PYRENE ON GLC-I

4.4.5 Intrinsic fluorescence studies of GLC-I in the presence of benzo (a) pyrene

Benzo (a) pyrene is a polycyclic aromatic hydrocarbon (PAH) which is an effective mutagen, together with its metabolites formed as a product of its metabolism in the liver. Polynuclear aromatic hydrocarbons (PAHs) are ubiquitous contaminants of the environment. A number of PAHs are carcinogenic to laboratory animals and some are also suspected human carcinogens. In the assessment of exposure to carcinogens, the use of DNA and protein adducts have been well documented. Since proteins are very important in transferring pharmaceuticals and other compounds, their interaction with PAHs is perhaps one of the ways for transferring PAHs in the biosystem. Moreover, PAHs adducts with biomolecules are used as biomarkers in order to assess human exposure to PAHs. Furthermore, since no repair occurs in protein so the adduct level is a total sum of human exposure to PAHs which takes place during the lifetime of proteins. Altered function or level of cystatin has been implicated in the lung cancers. Thus to the above facts, present study was designed to explore the effect of carcinogenic agent (benzo (a) pyrene) on GLC-I.

The fluorescence spectra of GLC-I in the presence of different concentrations of benzo (a) pyrene were recorded in the range of 300-400 nm upon excitation at 280 nm. Benzo (a) pyrene caused unfolding of the GLC-I as indicated by enhancement in fluorescence intensity accompanied by the red shift of 5 nm (Fig. 5.8).

4.4.6 UV-vis absorption studies of benzo (a) pyrene complex with GLC-I

The UV absorption intensity of GLC-I decreased with the increasing concentration of benzo (a) pyrene. Decrease in absorption intensity may arise due to disruption or perturbation of absorbing groups (Fig. 5.9).
4.4.7 Inhibitory activity of GLC-I in the presence of benzo (a) pyrene

Changes in the inhibitory activity of goat lung cystatin (GLC-I) with increasing concentration of benzo (a) pyrene is shown in Table (2.0). The results showed that GLC-I lost significant amount of inhibitory activity at 10 μM concentration of benzo (a) pyrene.
Figure 5.8: *Intrinsic fluorescence studies of GLC-I in the presence of benzo (a) pyrene*

GLC-I (1μM) was incubated with various concentrations of benzo (a) pyrene (1μM - 10μM) for 2 hrs. Fluorescence measurements were carried out on a Shimadzu Spectrofluorimeter model RF-540 equipped with a data recorder DR- 3 at 25°C. 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 5 nm for excitation and emission. The path length of the sample was 1 cm.
Figure 5.9: UV-vis spectroscopy of GLC-I in the presence of benzo(a) pyrene

(GLC-I) concentration was fixed at $1\mu$M while benzo(a) pyrene concentration was varied from $1\mu$M-$10\mu$M. Absorption spectra of native GLC-I and in presence of benzo (a) pyrene were recorded in the range of 200-300 nm on a double beam Shimadzu UV-vis spectrophotometer UV-1700 using a cuvette of 1 cm path length.
**TABLE- 2.0: EFFECT OF BENZO (a) PYRENE ON INHIBITORY ACTIVITY OF GOAT LUNG CYSTATIN (GLC-I)**

<table>
<thead>
<tr>
<th>Concentration of Benzo(a) Pyrene (µM)</th>
<th>% Inhibitory activity of GLC-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>86±2</td>
</tr>
<tr>
<td>1</td>
<td>72±1</td>
</tr>
<tr>
<td>5</td>
<td>40±2</td>
</tr>
<tr>
<td>10</td>
<td>No activity</td>
</tr>
</tbody>
</table>
Discussion 4
DISCUSSION

Methotrexate is a folate antagonist which is used not only as an anti-tumour drug but also as an anti-rheumatoid and antineoplastic drug (Miyazono et al., 2004). The cancer chemotherapy of MTX is often accompanied by toxic side effects, which complicates the drug’s usage as anticancer agent. Most common side effects include abnormal liver function, immunosuppression, neurotoxicity and lung damage (Hughes et al., 1987). It has also been reported that methotrexate induces pulmonary fibrosis (Bedrossian et al., 1979).

The analysis of pharmacokinetics and pharmacodynamics of drugs used in the leukaemia multidrug therapy is of great importance. The multidrug therapy from high effectiveness carries the risk of side effects and an increase of therapeutic action of free concentration of drugs. This can be dangerous when exhibiting toxic effect. Thus, the present study was designed to explore, the side effects of methotrexate on cysteine proteinase inhibitor isolated from goat lung.

The role of cysteine proteinases in chronic obstructive pulmonary diseases (COPD) has not been defined, although they do contribute to the elastolytic activity of alveolar macrophages in COPD patients (Russell et al., 2002). The pathophysiological significance of proteinases in lung disease is exemplified by α1-anti-trypsin deficiency, for example the loss of proteinase-inhibitor function leads to emphysema. However other proteinases, elastase and cathepsins may also contribute to the pathogenesis of emphysema (Zheng et al., 2000) where an increased expression of cathepsins B, D, H, L and S is reported (Wang et al., 2000). It is known that cathepsin inhibitors markedly reduce emphysema. Therefore, proteinase-antiproteinase balance must be retained in order to avoid COPD.

The quenching of intrinsic fluorescence of goat lung cystatin (GLC-I) is accompanied by the red-shift of fluorescence maximum (Fig. 5.4). This indicates the increase in polarity of the fluorophore environment, probably due to the hydrogen bonds formed between MTX and NH₂, OH and SH groups in the inhibitor (GLC-I) which stabilizes the complex (Bures et al., 1990).
Negative value of $\Delta G$ and the number of binding sites (n) (Table 1.8) showed that complex formation between MTX and GLC-I occur spontaneously and there is only one independent binding site of interaction. Our finding is similar to the binding of drug with human serum albumin (Khan et al., 2008).

Inhibitory activity assay and FT-IR result of goat lung cystatin in the presence of methotrexate (Fig. 5.7) clearly indicates that methotrexate the drug causes functional and structural inactivation of cysteine proteinase inhibitor (GLC-I). The obtained result is in accordance with the binding of sodium diethyl dithiocarbamate with phytocystatin (Sharma et al., 2005) and is further supported by similar results (Khan et al., 2008) in which the authors have showed conformational change in proteins (from $\alpha$ helix to $\beta$-sheet) in the presence of drug.

From the study, it can be concluded that lung cancer or in the chronic inflammatory conditions such as asthma, emphysema, and idiopathic pulmonary fibrosis might not be only due to oversecretion of cathepsins but also due to inactivation of their inhibitor (GLC-I).

Cigarette smoking is a risk factor for atherosclerotic cardiovascular disease, pulmonary emphysema, and cancer (Howard et al., 1998; Leone, 1995). Among the toxic compounds contained in tobacco smoke are the reactive oxygen species (ROS), polycyclic aromatic hydrocarbons such as benzo(a)pyrene (BaP), cadmium, and nicotine (Benowitz and Gourlay, 1997; Jaakkola et al., 1997). Cigarette smoke was reported to contain approximately 5 to 80 ng BaP per cigarette in the mainstream smoke while much higher concentrations of approximately 25 to 200 ng BaP per cigarette was in the sidestream smoke. The concentration of benzo (a) pyrene in a cigarette smoke-polluted environment can be from 400 to 760,000 ng/m$^3$. Cigar and pipe smoke can contain from 18 to 51 ng/g and 85 nanogram/g of benzo (a) pyrene, respectively (Tremolada et al., 2009). Food is a significant source of benzo (a) pyrene in Europe due to PAHs in oils, fats and cereals which represent a high percent of the European diets (Guillen and Sopelana, 2004).

Benzo (a) pyrene is a polycyclic aromatic hydrocarbon (PAH) widely distributed class of environmental contaminants implicated in the etiology of some human cancers (Heidelberger, 1975), it is a cause of cancer in laboratory animals
(Wattenberg and Leong, 1970; Wynder and Hoffmann, 1959) and cell death, mutation, and transformation in cultured mammalia cells (Huberman and Sach, 1974). Generation of this biological damage depends on metabolic activation of the hydrocarbon (Gelboin et al., 1970) and is thought to result from the covalent interaction of reactive intermediates with critical cellular macromolecules. Although DNA modification, leading to somatic mutation, for example, is an obvious candidate for the initial step in the critical process, transformation may represent a stable change in gene regulation. Such a change could result from functional alteration of specific regulators of gene activity due to interaction with poly aromatic hydrocarbon (PAH). No specific gene regulators have as yet been identified in eukaryotes, and current theories suggest both RNA and protein as potential elements of the regulatory apparatus (Davidson and Britten, 1979). Thus, to understand the molecular mechanisms involved in chemical carcinogenesis, detailed studies of the interactions of PAH with all classes of cellular macromolecules are necessary. Moreover, it is found that regulation of cathepsins and their inhibitor cystatins play an important role in lung tumourogenesis (Panayotis et al., 1996). The present study was designed to explore the effect of carcinogenic agent i.e benzo (a) pyrene on goat lung cystatin (GLC-I).

Intrinsic fluorescence studies of GLC-I in the presence of benzo (a) pyrene shows enhancement in fluorescence intensity accompanied by a red shift of 5 nm which indicates perturbation in the environment of aromatic residues and unfolding of the GLC-I in the presence of benzo (a) pyrene (Fig. 5.8). Unfolding of the inhibitor or change in tertiary structure of GLC-I causes functional inactivation of GLC-I.

Cathepsin D, B, and L belong to a group of proteolytic enzymes, which are mostly localized to lysosomes under physiological conditions. In various human and animal tumors, altered expression and trafficking of cathepsins has been observed (Kane and Gottesman, 1990). The final control of their activity by endogenous inhibitors may be compromised in tumors (Knoch et al., 1994). The complex mechanisms of cathepsins regulation in malignant cells still remain to be elucidated but it seems that there are differences in some of their regulatory pathways (Sloane et al., 1990). In human tumors, increased expression of various cathepsins compared to the surrounding nonmalignant tissues have been observed in several organs. The
expression of cathepsin D in some other human tumors was found to be elevated (Sloane et al., 1990). There is evidence that level of cathepsin B was increased by 4.4 fold in lung tumours (Knoch et al., 1994). Moreover, the ratio of Cathepsin B and the inhibitory activity of endogenous cystatins (CPIs) was significantly shifted in favor of increased Cathepsin B activity. Thus, keeping in view above facts, our results reaffirms that cathepsin-GLC-I imbalance may be a cause of lung cancer.