Chapter – 4

Acid induced unfolding of Buffalo Liver Cystatin, a cause of Amyloid Fibril Formation

4.4 RESULTS

(A) UNFLODING STUDIES OF BUFFALO LIVER CYSTATIN (BLC) IN THE PRESENCE OF ACIDIC pH CONDITIONS.

4.4.1 EFFECT OF pH ON THE INHIBITORY ACTIVITY OF BLC

Changes in the inhibitory activity of buffalo liver cystatin (BLC) after incubation for 2 hours at different pH values (pH 7.5 - pH 1.0) are shown in fig 6.4. At decreasing pH value there was a gradual loss in activity with a complete loss of activity at pH 1.0. The value at pH 7.5 showing no loss in the inhibitory activity was taken as control.

4.4.2 FLUORESCENCE STUDIES OF BLC AT DIFFERENT pH VALUES

Intrinsic Fluorescence Measurements

Intrinsic fluorescence is an excellent spectroscopic probe to investigate conformational changes in tertiary structure of proteins. The modification of microenvironment of aromatic residues of BLC due to denaturants has been studied by monitoring the changes in intensity and wavelength of emission maxima as a function of pH and chemical denaturant concentrations. Fluorescence spectra of BLC in the presence of gradually decreasing pH values (pH 7.5- pH 1) shows unfolding of the inhibitor as manifested by the blue shift (15 nm) of the emission maximum (Fig 6.5).

Fig. 6.5 shows the intrinsic fluorescence emission spectra of BLC in different conditions. In the native state BLC is characterized by a peak at 337 nm. The acid induced state (pH 7.5-1.0) has decreased fluorescence, however λ_{max} remains unchanged except for the pH conditions 3.0-1.0. Liver cystatin in the presence of 6M GdnHCl showed an increase in fluorescence accompanied by a red shift of emission maxima to 345 nm. GdnHCl is a known denaturant of proteins and it is used as a reference to compare unfolding or folding studies.

Figure 6.4: Inactivation of thiol proteinase activity of BLC at low pH

Native cystatin (1 μM) was incubated with buffers of different pH, 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 50mM concentration for 2 hours at 37°C. BLC was assayed for loss of inhibitory activity by caseinolytic assay of Kunitz (1947) as described in methods. Values are mean of three independent determinations.

Intrinsic fluorescence at varying pH values of BLC in the presence and absence of different concentrations of TFE

Trifluoroethanol (2, 2, 2–trifluoroethanol) is used as a solvent in organic chemistry. It is used in protein denaturation and stabilizing studies where it is used as a co-solvent in protein folding studies. Depending upon its concentration, TFE can strongly affect the three-dimensional structure of proteins. TFE denatures the protein and affects the tertiary and the quaternary structures of proteins (Radford et al. 1992). Fluorescence spectra of BLC in the presence of tri-fluoro ethanol (0-60% TFE) at low pH (pH 2.0) is shown in fig 6.6. The results indicate an (fig 6.6) increase in the fluorescence intensity with increase in the % TFE at pH 2.0. There is a no shift in the peak at 10% TFE and as the TFE (%v/v) is increased a red shift is observed and at around 20% TFE, the shift in the peak is almost 10 nm. At 40% (v/v) TFE there is a further shift in the peak (15 nm) towards longer wavelength (red shift) along with increase in fluorescence intensity. For further studies 20% TFE (v/v) was taken as standard. Fig 6.7 shows the effect of TFE (20%) on liver cystatin incubated at pH 1.0- pH 3.0. The spectra was also taken in the absence of TFE at these pH values. Results showed an increase in fluorescence intensity at all three pH values (1.0-3.0) in the presence of TFE indicating an unfolding of the protein.

4.4.3 EXTRINSIC FLUORESCENCE STUDIES OF BLC AT LOW pH CONDITIONS AND TFE

Effect of TFE on acid induced structural change of BLC

The fluorescence emission of 1-anilinonapthelene-8-sulphonic acid (ANS) is known to increase when the dye binds to the hydrophobic regions of proteins resulting in increase in fluorescence intensity which has been widely used to detect the molten globule states as well as random denatured state of different proteins (Stryer 1965, Engelhard and Evans 1995). Fig 6.8 shows the binding of ANS dye with BLC. As can be seen from this figure there is large increase in ANS binding at pH 3.0, pH 2.0 and pH 1.0 state as compared to that of native state accompanied by a blue shift from 500nm to 480nm indicating exposure of hydrophobic regions of the protein on acidification.

Figure 6.5: Intrinsic fluorescence spectra of BLC at different pH values

Livercystatin (1 μM) was preincubated with buffers of different pH,glycine/HCl buffer (pH 1.0-2.0), sodium acetate buffer (pH 3.0-5.0) and sodium phosphate buffer (pH 6.0-7.5) at 50mM concentration for 2 hours at 37°C. Fluorescence Spectra was measured at an excitation wavelength of 280nm in the emission range (300-400 nm) with a slit width of 5nm.

Figure 6.6: Intrinsic fluorescence analysis of BLC at low pH value in the presence of increasing concentration of TFE

Cystatin (1μM) was incubated at pH 2.0 with 50mM glycine/HCl buffer in presence of (0-60%) TFE (v/v) for 2 hours at 37°C. Fluorescence spectra was measured at an excitation wavelength of 280nm in the emission range (300-400 nm) with a slit width of 5nm. Cystatin without TFE was taken as control.

Figure 6.7: Intrinsic fluorescence spectra of BLC at different pH value in the presence and absence of TFE

Livercystatin (1 μM) was preincubated with buffers of different pH values,glycine/HCl buffer (pH 1.0-2.0), sodium acetate buffer (pH 3.0) in presence and absence of 20%TFE at 50mM concentration for 2 hours at 37°C. Fluorescence spectra was measured at an excitation wavelength of 280nm in the emission range (300-400 nm) with a slit width of 5nm.

ANS Binding at low pH values

ANS binding fluorescence spectra at 480 nm was determined for liver cystatin incubated at pH 3.0, pH 2.0 and pH 1.0 in the presence of 20% (v/v) TFE. Fig 6.8 shows the change in the fluorescence emission at different pH values in the presence of TFE (tri- fluoroethanol). Results indicate that in the presence of TFE there is decrease in the fluorescence intensity while in case of samples with no TFE, fluorescence intensity increases with decrease in pH. Thus increase in alcohol concentration leads to decrease in ANS fluorescence showing non–availability of hydrophobic regions for binding in presence of alcohols indicating secondary structural changes leading to burying of hydrophobic patches (Fig 6.8).

4.2.4 SECONDARY STRUCTURAL ANALYSIS OF BLC

Circular Dichroism (CD) measurements of BLC at low pH

Fig 6.9 shows the far UV-CD spectra of BLC at pH 7.5 (native state) to pH 1.0 and in 6M GdnHCl denatured state. The curve for native state shows presence of minima at 208 and 222nm suggesting presence of α–helical structure. The curve for the acid denatured state retains most of the features of secondary structure, although there was some decrease in the ellipticity value compared to the native state indicating loss of α–helical content. BLC in presence of 6M GdnHCl loses almost all the elements of secondary structure and forms a random coil as reported earlier.

Secondary structural analysis of BLC in the presence and absence of TFE

Figure 7.0 shows the effect of TFE (20 %) on the far UV-CD spectra of acid denatured state of BLC. The results reveal an increase in the helical content of protein as indicated by the increase in the ellipticity values. At 20 % TFE there is an increase in the ellipticity values of cystatin as compared to the absence of TFE in different acid induced states. The figure shows significant increase in the ellipticity values towards

Figure 6.8: Fluorescence emission spectra of ANS bound to BLC at pH 7.5, pH 3.0, pH 2.0, pH 1.0 alone and in the presenceof 20% TFE

Figure shows spectra of ANS bound to BLC at pH 7.0, at pH 3.0, at pH 2.0, at pH 1.0, in presence of 6M GdnHCl and in presence of 20% TFE at pH 3.0, pH 2.0 and pH 1.0 respectively. The ANS to protein molar ratio was 1:60. BLC was preincubated for 4h at 25^{o}C in buffers of pH 6.0 and pH 2.0 containing the indicated concentrations of GdnHCl, TFE and ANS for the respective fluorescencemeasurements. ANS Fluorescence was measured at an excitation wavelength of 380 nm in the emission range (400-600 nm) with a slit width of 5nm.

Figure 6.9: Secondary structure analysis of BLC at different pH values

For far UV-CD, 0.2mg/ml of BLC was incubated with glycine/HCl buffer (pH 1.0-2.0), sodium acetate buffer (pH 3.0-5.0) sodium phosphate buffer (pH 6.0-7.0) at 50mM concentration and in presence of GdnHCl for 4 hours at 37°C and CD spectra was recorded at 222 nm. Cells of 1 mm path length was used.

Figure 7.0: Effect of TFE on secondary structure of BLC at low pH values

Figure shows far UV-CD spectra of BLC at pH 3.0, at pH 2.0, pH 1.0 without TFE in 50mM glycine/HCl buffers, pH 3.0- 1.0and as a function of (40%) TFE (v/v), at 25^{o}C. The concentration of BLC was 0.2mg/ml and path length was 0.1 cm.

lower pH conditions at 20 % TFE. In case of pH 1.0, there is maximum increase in the helical content while lesser in case of pH 3.0. However, in absence of TFE the helical content of protein shows lower values at all pH states (3.0-1.0) indicating role of TFE in inducing secondary structural change.

Relative change in Ellipticity values and ANS fluorescence intensity

A comparison between the ANS fluorescence intensity at 480 nm and the ellipticity values for far UV-CD spectroscopy reveals the change in the secondary structure towards a more compact structure. Figure 7.1 (A), shows increase in the helical content in presence of TFE while Fig 7.1 (B), shows decrease in ANS fluorescence intensity towards pH 1.0 in the presence of TFE (20 %). There is no increase in ellipticity values in the absence of TFE from pH 3.0-1.0. These results reveal the helical content inducing property of TFE at low concentrations. Similarly the decrease in ANS intensity at pH 1.0 in the presence of TFE shows formation of a compact secondary structure while in absence of TFE, higher ANS fluorescence value at pH 1.0 indicates more random and denatured state of BLC. In case of pH 2.0 and pH 3.0, TFE induces the formation of helical structure, however, in the absence of TFE there is not much change in the ellipticity values (fig 7.1 A) or decrease in ANS intensity (fig 7.1 B).

Figure 7.1 (A): CD spectra of BLC in the presence of varying pH values at 25^{o}C.

Ellipticity values were monitored at 222nm. These values were obtained for pH 3.0 to pH 1.0 in the presence and absence of TFE (20% v/v). Data is taken from fig 4.6.

Figure 7.1 (B): pH dependence of ANS fluorescence intensity of BLC at 25^{o}C.

ANS fluorescence intensity at 480nm after excitation at380nm.These values were obtained for pH 3.0 to pH 1.0 in the presence and absence of TFE. Data is taken from fig 4.5.

(B) FIBRIL FORMATION OF BUFFALO LIVER CYSTATIN (BLC)

There is an 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). Therefore, it was decided to explore conditions at which BLC is denatured but retains considerable amounts of residual structure. For this purpose TFE was used as a solvent known to stabilize partially folded proteins (Thomas and Dill, 1993). BLC (50μM) was incubated with buffers of varying pH range 1.0-7.5, with and without 20% TFE at low pH separately. Fibril formation was analysed by Thioflavin T (ThT) fluorescence, FTIR spectroscopy, atomic force microscopy (AFM). Increase in ThT fluorescence clearly indicates that BLC forms amyloid like structure under acidic conditions.

4.4.5 Thioflavin T binding studies

Benzothiazole dye thioflavin T (ThT) is a sensitive probe for amyloid fibril detection. The ThT probing is based on its unique ability to form highly fluorescent complexes with amyloid and amyloid-like fibrils**.** ThT dye was used to determine amyloid like fibril formation at low pH and in the presence of Trifluoroethanol (TFE). BLC (50μM) was incubated with buffers of varying pH range (pH 1.0-7.5). In five microliters of the protein solution, 600 μl of the ThT buffer was added (25 mM phosphate buffer, pH 7.5, 20μM ThT). ThT binding was investigated in case of BLC incubated at low pH (3.0-1.0) without TFE (fig 7.2). ThT binding was also investigated in presence of TFE (20%) and low pH (fig 7.2). At pH 3.0, it shows binding as indicated by increase in the ThT fluorescence. At pH 2.0 ThT binding is more pronounced and increases with increasing time of incubation. In case of pH 1.0 binding is very strong. TFE is a known inducer of fibrils. ThT does not show any binding at pH 7.5 (fig 7.3 A, fig 7.3 B). ThT fluorescence at 480 nm was monitored at different time intervals (2, 7, 21, 28, 36 days) in case of BLC incubated without TFE and low pH 7.5, 3.0-1.0) buffers and the spectra was recorded from 445 nm to 580 nm (fig. 7.3 A) while in presence of TFE, fluorescence was recorded (fig 7.3 B) after intervals (2, 7, 21, 28 days).

4.4.6 Fourier Transform Infrared Spectroscopy (FTIR)

Further evidence regarding the change in secondary structure came from FT-IR spectroscopy, showing BLC undergoing secondary structure change. Infrared spectra of proteins exhibit a number of the so called amide bonds, which represent different vibrations of the peptide moiety. This vibration mode orginates 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 bonds have a relationship with the secondary structure. Figure 7.4 (A) Shows the FTIR spectra of BLC at low pH and in presence of TFE. The evident peak shift of amide I band from 1652.4 to 1628.4 cm^{-1} and a profound increase in the intensity at 1628cm^{-1} indicating that the secondary structure of BLC is changed at low pH and in presence of TFE. There is a change in the α-helix induced by the TFE into β- structure as evidenced from the peak shift from 1656 nm towards 1628 nm as the fibril formation is induced after incubation at low pH and TFE (20 %). Figure 7.4 (B) also shows a shift in the peak at pH 2.0 and 20% TFE from 1652.4 to 1625.6 cm^{-1}. However the intensity of peaks is lesser as compared to fig 7.4 (A). The results in fig 7.5, show a shift in the peak from 1652.3 $cm^{-1}$to 1629 cm^{-1}.

Figure 7.2: Staining of BLC with Thioflavin T at low pH and in the presence and absence of TFE

Fluorescence emission spectra of thioflavin T (ThT) dye in the presence of buffalo liver cystatin (50μM) was incubated with buffers of different pH range varying from (pH 1.0-3.0) and in presence of TFE (20%) after 21 days. Five microliters of the stock solution (protein solution in which fibrils were growing in presence of 20% v/v) were dissolved in 600 μl of the ThT buffer (pH 7.5, 50mM phosphate buffer, 20 μM ThT) just before the measurements. Excitation was at 440 nm and the spectra were recorded at 480 nm. The slit width was 5 nm for both excitation and emission beams.

Figure 7.3 (A): ThT fluorescence intensity peaks at low pH over a period of several days

Thioflavin T (ThT) dye in the presence of buffalo liver cystatin (50μM) incubated with buffers of different pH range varying from (pH 1.0-3.0), without TFE over a period of 36 days.The fluorescence intensity was measured after intervals. Excitation was at 440 nm and the spectra were recorded at 480 nm. The slit width was 5 nm for both excitation and emission beams.

Figure 7.3 (B): ThT fluorescence intensity peaks at low pH and TFE over a period of several days

Thioflavin T (ThT) dye was added to buffalo liver cystatin (50μM) incubated with buffers of different pH range varying from (pH 1.0-3.0), with TFE (20%) over a period of 28 days.The fluorescence intensity was measured after intervals. Excitation was at 440 nm and the spectra were recorded at 480 nm. The slit width was 5 nm for both excitation and emission beams.

Figure 7.4 (A): FTIR spectra of BLC in the presence of pH 1.0 and TFE

Cystatin incubated at pH 1.0 with50mM glycine/HCl buffer in presence of TFE (20% v/v) for different time intervals (given in Fig) at 37°C. The absorbance spectra were taken in the region 1800-1400 cm^{-1} as described in methods.The spectrum is the average of three individual scans.

Figure 7.4 (B): Conformational changes of BLC undergoing fibrillation at pH 2.0 and TFE as detected by FTIR spectroscopy

Cystatin incubated at pH 2.0 with50mM glycine/HCl buffer in presence of TFE (20% v/v) for different time intervals (given in Fig) at 37°C. The absorbance spectra were taken in the region 1800-1400 cm^{-1} as described in methods.The spectrum is the average of three individual scans.

Figure 7.5: Conformational changes of BLC undergoing fibrillation at pH 3.0 and TFE as detected byFTIR spectroscopy

Cystatin incubated at pH 3.0 with50mM glycine/HCl buffer in presence of TFE (20% v/v) for different time intervals (given in Fig) at 37°C. The absorbance spectra were taken in the region 1800-1400 cm^{-1} as described in methods.The spectrum is the average of three individual scans.

4.4.7 AFM

Atomic force microscopy (AFM) is a powerful tool for characterizing the structural properties of macromolecular complexes both physiological conditions (Yang *et al.* 2003). In the past two decades, the application of AFM has spread to many areas of biological sciences including studies of DNA (Hansma 2001), RNA (Bonin *et al.* 2000; Henn *et al.* 2001; Liphardt *et al.* 2001), proteins (Heymann *et al.* 1997; Isralewitz *et al.* 2001), lipids (Dufrene 2000; Balashev *et al.* 2001), carbohydrates (Misevic 1999; Dettmann *et al.* 2000; Marszalek *et al.* 2001), biomolecular complexes (Willemsen *et al.* 2000; Safinya 2001), organelles (Danker and Oberleithner 2000) and cells (Ohnesorge *et al.* 1997). This technique is used to investigate the morphology and mechanical properties of protein molecules and fibril strands. In the field of proteins to study unfolding, protein-protein interactions and cell adhesion.

In the Tapping Mode of the technique, AFM tip–cantilever assembly oscillates at the sample surface while the sample is scanned; thus, the tip lightly taps the sample surface while rastering and only touches the sample at the bottom of each oscillation. This prevents damage to soft specimens and avoids the "pushing" of specimens around on the substrate. Tapping Mode AFM can be performed on both wet and dry sample surfaces. AFM provides a number of advantages over conventional microscopy techniques. AFMs probes the sample and make measurements in three dimensions, *x, y,* and *z* (normal to the sample surface), thus enabling the presentation of three-dimensional images of a sample surface.

Atomic force microscopy (AFM) images recovered after a period of 21 days reveal the presence of amyloid like aggregates at pH 3.0, pH 2.0 and pH 1.0 in case of incubation with 20% TFE (v/v) (Fig 8.1, Fig 8.0 and Fig 7.8). There was no amyloid fibril formation at pH 3.0 after 21 days. Fibril formation was also seen at pH 2.0 and pH 1.0 in the absence of TFE but it took longer for fibrils to form and fibril formation was seen (Fig 7.9 and Fig 7.7) after approximately 35 days of incubation at these two pH values. No amyloids were formed at pH 3.0 though aggregation occurred as revealed by ThT fluorescence (Fig 7.2 and 7.3) where there is a clear increase in intensity at 490 nm and rough surface in fig 8.1. Sample at pH 7.5 does not show any aggregation.

Figure 7.6: AFM image of BLC incubated at pH 1.0

AFM Image of amyloid fibrils of liver cystatin adsorbed onglass surface. Image was obtained in tapping mode and sho height variation. This is the 3-D image of the amyloid fibrilsobtained after incubation of BLC (50 μg) with 50mM glycine-HCl buffer and in absence of TFE, for 28 days. AFM height image (1 μm by 1 μm; *z* range, 23.76 nm)

Figure 7.7: AFM image of BLC incubated at pH 1.0 and TFE

AFM Image of amyloid fibrils of liver cystatin adsorbed onglass surface. Image was obtained in tapping mode and showheight variation. This is the 3-D image of the amyloid fibrilsobtained after incubation of BLC (50 μg) with 50mM glycine-HCl buffer and in presence of TFE (20% v/v), for 21 days. AFM height image (2 μm by 2 μm; *z* range, 6 nm)

Figure 7.8: AFM image of BLC incubated at pH 2.0

AFM Image of amyloid fibrils of liver cystatin adsorbed onglass surface. Image was obtained in tapping mode and showheight variation. This is the 3-D image of the amyloid fibrilsobtained after incubation of BLC (50 μg) with 50mM glycine-HCl buffer and in absence of TFE, for 28 days. AFMheightimage (3 μm by 3 μm; *z* range, 10.15 nm)

Figure 7.9: AFM image of BLC incubated at pH 2.0 and TFE

AFM Image of amyloid fibrils of liver cystatin adsorbed onglass surface. Image was obtained in tapping mode and showheight variation. This is the 3-D image of the amyloid fibrils obtained after incubation of BLC (50 μg) with 50mM glycine -HCl buffer and in presence of TFE (20% v/v), for 21 days. AFM height image (2 μm by 2 μm; *z* range, 30.19 nm)

Figure 8.0: AFM image of BLC incubated at pH 3.0 and TFE

AFM Image of amyloid fibrils of liver cystatin adsorbed onglass surface. Image was obtained in tapping mode and showheight variation. This is the 3-D image of the amyloid fibrilsobtained after incubation of BLC (50 μg) with 50mM glycine-HCl buffer and in presence of TFE (20% v/v), for 28 days. AFM height image (2 μm by 2 μm; *z* range, 0.208 μm).

Figure 8.1: AFM image of BLC incubated at pH 3.0

AFM Image of amyloid fibrils of liver cystatin adsorbed onglass surface. Image was obtained in tapping mode and showheight variation. This is the 3-D image of the amyloid fibrilsobtained after incubation of BLC (50 μg) with 50mM glycine-HCl buffer and in absence of TFE, for 28 days. AFM height image (2 μm by 2 μm; *z* range, 2.52 nm).