Chapter -4

High pH induced unfolding of HMGBC and LMGBC and effect of salts on their refolding
4.4 RESULTS

(A) High pH induced modifications of HMGBC and LMGBC

Changes in the functional properties of HMGBC and LMGBC at high pH

High pH induced changes in the functional properties of both the cystatins were studied by loss of their ability to inhibit papain (Fig. 6.8). In case of HMGBC, the inhibitory activity was found to be quite stable from pH 7.0 to pH 9.0 with a maximum at pH 8.0. However, it was found to decrease sharply from pH 10 onwards reaching the complete loss of cystatin activity at pH 14.0, while only 10% of the activity was retained at pH 13.0. Based on the enzyme activity results obtained, the pH of half transition was observed at pH 11.0 (Fig. 6.8).

For LMGBC, 80% inhibitory activity was retained up to pH 10. After pH 10.0, there is a sharp decrease in the inhibitory activity of the cystatin which was completely lost at pH 14.0, retaining 15% activity at pH 13.0. The pH of half transition of inhibitory activity was observed at pH 11.5 (Fig. 6.8).

Changes in the structural properties of HMGBC and LMGBC at high pH

The pH dependent structural modifications of HMGBC and LMGBC were studied by investigating the changes in intrinsic and extrinsic or ANS fluorescence and CD spectra measurements. For LMGBC, the intrinsic fluorescence intensity, extrinsic fluorescence intensity and CD spectra results showed a different pattern of unfolding as compared to HMGBC (Fig. 6.9, 7.0 and 7.1).

For HMGBC, the intrinsic fluorescence at the λmax, 335nm was considered as 100% at pH 8.0 as its native state. Comparative to pH 8.0, the intensity was increased as the pH increased to pH 12.0. At pH 12.0 a 2nm red shift was observed with a sharp increase in the intrinsic fluorescence intensity.
Fig 6.8 Changes in the functional properties of HMGBC (—○—) and LMGBC (—▲—) on high pH induced unfolding

The values reported are relative to that observed at pH 8 for HMGBC and pH 7 for LMGBC. 1μm of HMGBC and LMGBC was dissolved in 50mM sodium phosphate buffer, pH 7.5. The pH of the solution was then adjusted to the desired pH using 1M sodium hydroxide and the samples were then incubated for 3 hrs at room temperature. After this the inhibitory activity of the cystatins was measured by their ability to inhibit caseinolytic activity of papain
Fig 6.9 Intrinsic fluorescence intensity of HMGBC and LMGBC at different pH values

Changes in the intrinsic fluorescence at 335nm of HMGBC (—●—) and LMGBC (—▲—) on alkaline induced unfolding are measured. The values reported are relative to that observed at pH 8 for HMGBC and pH 7 for LMGBC. 1μm of HMGBC and LMGBC was dissolved in 50mM sodium phosphate buffer, pH 7.5. The pH of the solution was then adjusted to the desired pH using 1M sodium hydroxide and the samples were then incubated for 3 hrs at room temperature and then subjected for the measurement of fluorescence.
From pH 12 onwards at pH 13.0 and 14.0, no further significant increase in intrinsic fluorescence intensity was observed (Fig. 6.9). In case of LMGBC, the intrinsic fluorescence intensity of LMGBC at $\lambda_{\text{max}}$ at 335nm was found to increase from pH 7.0 to 10.0. At pH 11.0, the intensity decreased with least at pH 12.0 showing the formation of some compact structure. At pH 13.0 the intensity was further increased reaching the maximum intensity at pH 14.0 (Fig. 6.9).

Fig. 7.0 shows that the ANS fluorescence intensity at 480nm first it increases sharply from pH 8.0 to pH 12.0 in case of HMGBC. This was observed because as the pH increases, the hydrophobic patch or residues of the protein were exposed resulting in the increase of ANS binding. But at pH 13.0 and 14.0, the intensity was again lowered resulting due to the disruption of the hydrophobic patches exposed at pH 12.0. However, the extrinsic fluorescence intensity of LMGBC at 480nm showed increase in the extrinsic fluorescence from pH 7.0 to 11.0 because of the exposure of hydrophobic residues. The intensity of ANS binding decreased at pH 12.0 with least intensity at pH 14.0 (Fig. 7.0).

CD spectral results showed that the maximum secondary structure content was found at pH 8.0 as indicated by the $\theta$ value at 222nm and loss of peak at $\theta_{222}$. It was also observed that there is continuous loss of secondary structure as the pH increases from pH 8.0 to 13.0. The $\theta_{222}$ value at pH 14.0 did not show any further significant increase compared to the value at pH 13 (Fig 7.1). The CD results of LMGBC indicate the formation of compact intermediate structure with significant secondary structure at pH 12.0. The secondary structure was found to be most ordered at pH 7 showing a proper peak at $\theta_{222}$. As the pH increases from pH 7.0 to 11.0 there is loss of secondary structure as shown by the $\theta_{222}$ value. At pH 12.0 the $\theta$ value at 222nm indicated the formation of compact intermediate state (Fig. 7.1).

These above results indicate that prominent or significant structural and functional changes occur with alkaline unfolding of HMGBC and LMGBC.
Fig 7.0 Extrinsic fluorescence intensity of HMGBC and LMGBC at different pH values

Changes in the extrinsic or ANS fluorescence are measured 480nm for HMGBC (—○—) and LMGBC (—▲—) on alkaline induced unfolding. The values reported are relative to that observed at pH 8 for HMGBC and pH 7 for LMGBC. 1μm of HMGBC and LMGBC was dissolved in 50mM sodium phosphate buffer, pH 7.5. The pH of the solution was then adjusted to the desired pH using 1M sodium hydroxide and the samples were then incubated for 3 hrs at room temperature and then subjected for the measurements. The protein: ANS concentration was taken as 1:60.
Fig 7.1 CD measurements at 222nm of HMGBC and LMGBC at different pH values

Changes in the CD measurements are measured at 222nm for HMGBC (–•–) and LMGBC (–▲–) on alkaline induced unfolding. The values reported are relative to that observed at pH 8 for HMGBC and pH 7 for LMGBC. 200μg/ml of HMGBC and LMGBC were dissolved in 50mM sodium phosphate buffer, pH 7.5. The pH of the solution was adjusted to the desired pH using 1M sodium hydroxide and the samples were then incubated for 3 hrs at room temperature and then subjected for the measurements.
Although HMGBC showed the transition of native to denatured state, LMGBC indicated the formation of some intermediate compact state at pH 12.0.

(B) Salt induced refolding of high pH unfolded HMGBC and LMGBC

Effect of salts KCl and Na₂SO₄ on the functional properties of high pH denatured HMGBC and LMGBC

In the absence of salts high pH denatured HMGBC at pH 12.0 showed only 20% recovery on bringing back the pH to 7.5 by dialysis while LMGBC showed a maximum recovery of 28% (Fig. 7.2). In the presence of salts upon dialysis the inhibitory activity recovery was found to be prominently enhanced upto 84% for both HMGBC and 86% for LMGBC in the presence of 1M Na₂SO₄ while in the presence of 3M KCl the HMGBC activity was recovered upto 75% and for LMGBC inhibitory activity was recovered 79% at the same concentration of KCl (Fig. 7.2).

Effect of salts KCl and Na₂SO₄ on the structural properties of high pH denatured HMGBC and LMGBC

The intrinsic fluorescence intensity measurements at 335nm for both the cystatins, HMGBC and LMGBC showed that the fluorescence intensity in the presence of varying concentrations of salts KCl and Na₂SO₄ was recovered as compared to the unfolded state at pH 12.0.

The intrinsic intensity spectral measurements of HMGBC (Fig 7.7) indicate that at pH 12.0, the intensity was much increased as compared to the native state which was decreased in the presence of salts. The 3M KCl and 1M Na₂SO₄ at pH 12.0 showed the spectra much closer to the native state as compared to the unfolded form at pH 12.0 (Fig. 7.3 and 7.5).

The spectral analysis of LMGBC showed that the intensity at pH 12.0 was decreased compared to the native because of the formation of compact
**Fig 7.2 Effect of increasing concentrations of salts on the recovery of inhibitory activity of high pH denatured HMGBC and LMGBC**

The HMGBC and LMGBC solution at pH 12, with the addition of KCl and Na$_2$SO$_4$ was prepared as described in Fig. 6.8. The samples were then dialyzed against 50 volumes of 50mM sodium phosphate buffer, pH 7.5 at 25 ±1°C and then it was monitored for recovery of the enzyme activity by caseinolytic method. Appropriate controls were used. Upon dialysis in the absence of salts HMGBC recovered only 20% of the inhibitory activity while LMGBC recovered 28%of its inhibitory activity as compared to the native. In this figure

(---O---) represents HMGBC with Na$_2$SO$_4$.

(---o---) represents LMGBC with Na$_2$SO$_4$.

(---A---) represents HMGBC with KCl.

(---Δ---) represents LMGBC with KCl.
Fig 7.3 Effect of increasing concentrations of salts on the intrinsic fluorescence intensity of high pH denatured HMGBC and LMGBC

The HMGBC and LMGBC solution at pH 12, with the addition of KCl and Na₂SO₄ was prepared as described in Fig. 6.8. The intrinsic fluorescence intensity at 335nm was measured. In the absence of salts at pH 12 recovery was considered as 0%. In the figure,

(---●---) represents HMGBC with Na₂SO₄,

(---○---) represents LMGBC with Na₂SO₄,

(---▲---) represents HMGBC with KCl

(---△---) represents LMGBC with KCl.
Concentration of salts [M] vs. % Regain of intrinsic fluorescence.
Fig 7.4 Effect of increasing concentrations of salts on the extrinsic fluorescence intensity of high pH denatured HMGBC and LMGBC

The HMGBC and LMGBC solution at pH 12, with the addition of KCl and Na₂SO₄ was prepared as described in Fig. 6.8. The extrinsic fluorescence intensity was measured at 480nm. The protein : ANS concentration was taken as 1:60. In the absence of salts at pH 12 recovery of extrinsic fluorescence intensity was considered as 0%. In this figure,

(—●—) represents HMGBC with Na₂SO₄.

(-----○-----) represents LMGBC with Na₂SO₄.

(—△—) represents HMGBC with KCl.

(-----△-----) represents LMGBC with KCl.
Fig 7.5 Effect of salts on the intrinsic fluorescence of HMGBC

Intrinsic fluorescence of HMGBC was measured after addition of salts Na$_2$SO$_4$ and KCl in the HMGBC mixture incubated at pH 12. The samples were prepared as described in Fig. 4.4.1 and incubated for 3 hrs at room temperature before taking the measurements. In the figure,

a represents HMGBC at pH 8.0

b represents HMGBC at pH 12.0 with 1M Na$_2$SO$_4$

c represents HMGBC at pH 12.0 with 3M KCl

d represents HMGBC at pH 12.0

e represents HMGBC in the presence of 6M GdnHCl.
**Fig 7.6 Effect of salts on the intrinsic fluorescence of LMGBC**

Effect of addition of salts Na$_2$SO$_4$ and KCl on the intrinsic fluorescence of LMGBC is shown in the figure. The samples were prepared as described in Fig. 6.8 and incubated for 3 hrs at room temperature before taking the measurements. In the figure,

a represents LMGBC at pH 7.0,
b represents LMGBC at pH 12.0,
c represents LMGBC at pH 12.0 with 3M KCl,
d represents LMGBC at pH 12.0 with 1M Na$_2$SO$_4$,
e represents LMGBC in the presence of 6M GdnHCl.
Fig 7.7 Effect of salts on the extrinsic fluorescence of HMGBC

Effect of addition of salts Na$_2$SO$_4$ and KCl on the extrinsic or ANS fluorescence of HMGBC is shown. The samples were prepared as described in Fig. 6.8 and incubated for 3 hrs at room temperature before taking the measurements. The protein: ANS concentration was taken as 1:60. In the figure,

a represents HMGBC at pH 8.0,

b represents HMGBC at pH 12.0,

c represents HMGBC at pH 12.0 with 3M KCl,

d represents HMGBC at pH 12.0 with 1M Na$_2$SO$_4$

e represents HMGBC in the presence of 6M GdnHCl
**Fig 7.8 Effect of salts on the extrinsic fluorescence of LMGBC**

In the figure the effect of salts of the extrinsic fluorescence of LMGBC has been shown. The samples were prepared as described in Fig. 6.8 and incubated for 3 hrs at room temperature before taking the measurements. In the figure,

- **a** represents LMGBC at pH 7.0,
- **b** represents LMGBC at pH 12.0,
- **c** represents LMGBC at pH 12.0 with 3M KCl,
- **d** represents LMGBC at pH 12.0 with 1M Na$_2$SO$_4$
- **e** represents LMGBC in the presence of 6M GdnHCl
intermediate state. In presence of 1M Na2SO4 and 3M KCl, the spectra obtained were much closer to the native intrinsic fluorescence spectra at pH 7.0, indicating the refolding of LMGBC in presence of salts (Fig. 7.3 and 7.6).

The extrinsic fluorescence intensity at 480nm, of HMGBC and LMGBC at pH 12.0 was found to be salvaged in the presence of varying concentrations of KCl and Na2SO4. The maximum recovery was observed in the presence of 1M Na2SO4 and 3M KCl at pH 12.0 comparable to the unfolded form at pH 12.0 (Fig. 7.4). The ANS fluorescence was regained back very near to the native state in the presence of 3M KCl and 1M Na2SO4 (Fig. 7.7).

The extrinsic fluorescence intensity at 480nm, for LMGBC at pH 12.0 was found to be recovered maximum in the presence of 1M Na2SO4 and 3M KCl compared to the fluorescence at pH 12.0 (Fig. 7.4). The extrinsic fluorescence of HMGBC was prominently increased at pH 12.0 because of the unfolding of the cystatin leading to the exposure of hydrophobic patches. The extrinsic fluorescence intensity of LMGBC at pH 12.0 was found to be less than the native state at pH 7.0. This may be due to the formation of the compact intermediate state resulting in the burial of hydrophobic patches. The fluorescence intensity was found to be greatly recovered in presence of 3M KCl and 1M Na2SO4 (Fig. 7.8).

From the CD results obtained for HMGBC, it was observed that there is a retention of secondary structure at pH 12.0 in the presence of salts, 3M KCl and 1M Na2SO4 as compared to the HMGBC denatured at pH 12.0 without salts (Fig. 7.9). The CD spectra at pH 12.0 for LMGBC indicate that the secondary structure was more compared to the native. In presence of 3M KCl and 1M Na2SO4 the secondary structure was observed to be closer to the native showing that the secondary structure of the LMGBC at pH 12.0 was reversed back near to native in the presence of salts (Fig. 8.0).

The figures for intrinsic fluorescence, extrinsic fluorescence and CD results of salt induced refolding of HMGBC and LMGBC, also include curve for 6M GdnHCl unfolding to show a comparison with pH induced unfolding.
Fig 7.9 Effect of addition of salts Na$_2$SO$_4$ and KCl on the far UV CD spectra of HMGBC

The samples were prepared as described in Fig. 6.8 and incubated for 3 hrs at room temperature before taking the measurements. 200μg/ml of HMGBC was used for all far UV CD measurements. In this figure.

a represents HMGBC at pH 8.0,
b represents HMGBC at pH 12.0,
c represents HMGBC at pH 12.0 with 3M KCl,
d represents HMGBC at pH 12.0 with 1M Na$_2$SO$_4$,
e represents HMGBC in the presence of 6M GdnHCl
Fig 8.0  Effect of addition of salts Na$_2$SO$_4$ and KCl on the far UV CD spectra of LMGBC

The samples were prepared as described in Fig. 6.8 and incubated for 3 hrs at room temperature before taking the measurements. 200μg/ml of HMGBC was used for all far UV CD measurements. In the figure,

a  represents LMGBC at pH 7.0,
b  represents LMGBC at pH 12.0,
c  represents LMGBC at pH 12.0 with 3M KCl,
d  represents LMGBC at pH 12.0 with 1M Na$_2$SO$_4$
e  represents LMGBC in the presence of 6M GdnHCl
5.4 Discussion

It is a well known fact that the activity of proteins is strongly dependent on its conformational integrity. Folded proteins are usually stable in the thermodynamic sense at ambient temperature and at neutral pH. Although a lot of work has been carried out on alkaline unfolding of multimeric enzymes (Wang et al., 1995; He et al., 1995; Le et al., 1996) but high pH unfolding of monomeric enzymes specifically from mammalian brain has not been explored yet. In the present study the alkaline denaturation of HMGBC and LMGBC was studied to obtain the information about the behaviour of these proteins at high pH. The spectral parameters such as fluorescence intensity are dependent on the dynamic and electronic properties of the chromophore environment, hence fluorescence measurements have been extensively used to obtain information on the structural and dynamic properties of the proteins (Ghisla et al., 1974). The far UV-CD spectra of a protein in the far-UV region gives information about the conformation of polypeptide backbone and the hydrophobic dye ANS which binds to exposed hydrophobic regions of partially folded proteins is used in protein conformational studies to monitor the exposed hydrophobic patches (Johnson and Fersht, 1995, Stryer, 1965).

To see the behaviour of both the cystatins at high pH under alkaline conditions, the cystatins were incubated with buffers at desired pH and then analyzed for their functional and structural modifications. It was observed that the cystatins are functionally stable under a wide range of pH from 7 to 10. But as the pH is further increased there is inactivation of the inhibitors (Fig. 6.8).

For HMGBC, the intrinsic and extrinsic fluorescence and CD results obtained showed that there is no intermediate formation during alkaline unfolding. The intrinsic fluorescence was observed to be maximum at pH 14, indicating the exposure of tryptophan residues because of the unfolding of the inhibitor. As the pH was increased from pH 7 to 12 there is increase in the extrinsic fluorescence of HMGBC while at pH 13 and 14 there is decrease in
the ANS fluorescence, this is because from pH 7 to 12 the hydrophobic residues were exposed leading to the maximum binding of ANS to these patches but as the pH was increased further there is disruption of the hydrophobic patches leading to the decrease of ANS binding. The CD spectra of HMGBC showed that there is steady loss of secondary structural content as the pH increased from 8 to 14. Thus all these results for HMGBC suggest the native state $\rightarrow$ unfolded state transition upon alkaline unfolding.

LMGBC showed the formation of an intermediate state at pH 12.0, the results obtained from intrinsic and extrinsic fluorescence as well as CD spectra (Fig. 6.9, 7.0 and 7.1) showed that upon high pH unfolding firstly there is unfolding of the protein from pH 7 to 10. But at pH 11 and 12, there is decrease in the intrinsic and extrinsic fluorescence intensities indicating the formation of a compact intermediate state. The intrinsic fluorescence was again increased after pH 12. CD results also showed the formation of some compact intermediate state of LMGBC at pH 12 upon high pH induced unfolding. Thus the unfolding of LMGBC can be represented as Native $\rightarrow$ intermediate $\rightarrow$ unfolded state. Formation of compact intermediates upon alkaline denaturation has also been reported earlier (Bai et al., 1998).

In the present study, two salts KCl and Na$_2$SO$_4$ were considered for the refolding and protein stability effects. The results of the activity measurements, intrinsic fluorescence, extrinsic fluorescence and CD spectra indicated that the addition of salts (KCl and Na$_2$SO$_4$) to the protein solution at high pH aids in the refolding of the alkaline unfolded HMGBC and LMGBC. It was observed that the proteins regain their inhibitory activity upon addition of salts. For refolding of the inhibitors by dialysis in the buffer of pH 7.5, in the absence of salts only 20% of HMGBC and 28% of LMGBC inhibitory activity was recovered however in the presence of 3M KCl and 1M Na$_2$SO$_4$ the recovery was significantly increased as a result of the renaturation of both HMGBC and LMGBC in the presence of the salts, KCl and Na$_2$SO$_4$. It also showed that KCl is required at higher concentration (3M) as compared to the
Na₂SO₄ (1M) for refolding of these proteins, suggesting that the latter is more effective in refolding.

The main forces unfolding the protein under extreme conditions of pH are the repulsive forces between charged groups on the protein molecule (Kim and Baldwin, 1990). The folding of protein in the presence of salts occurs by the formation of counter ions as well as the specific effects of the salts. The stabilizing or destabilizing effects of salts on proteins arise either by effects on water structure, hydrophobic interactions or by specific interactions with the charged group. According to the Hoffmeister series (Von Hipple and Wong, 1965), Na₂SO₄ is much more effective than KCl in stabilizing the major hydrophobic interactions needed for protein folding and thus stability of proteins as the results obtained in the study also indicate that Na₂SO₄ is more effective even at lower concentration in protein folding as compared to KCl. Salt dependent conformational changes at alkaline and acidic pH are the general property of proteins. However, the concentration of salt required and the extent of folding depends upon the particular protein and specified conditions.

The present study performed to understand the alkaline unfolding of cystatins from goat brain and their refolding by salts will add to the existing knowledge of unfolding and refolding process of proteins.