Chapter – 3

Glycation of Cystatin: Interaction of reducing sugars with purified Buffalo liver cystatin

4.3 RESULTS

4.3 EFFECT OF REDUCING SUGARS ON LIVER CYSTATIN

Non-enzymatic glycosylation or glycation, the reaction of sugars with proteins occurs through the formation of schiff's base between the aldehyde (or keto groups) of the sugar and amino groups of the protein chain. In vitro glycation of liver cystatin was studied using 0.5 M glucose, fructose and ribose as glycating agent. The changes in liver cystatin upon glycation were measured in terms of change in the inhibitory activity. The spectral and conformation changes induced upon glycation were analyzed by fluorescence, UV, circular dichroism and fourier infrared spectroscopy respectively.

4.3.1 EFFECT OF SUGARS ON THE INHIBITORY ACTIVITY OF CYSTATIN

Buffalo liver cystatin was incubated with reducing sugars (glucose, fructose and ribose) at 37°C for a period of 3, 7, 14, 21 and 28 days. Figure 4.9 shows that among the sugars investigated, ribose was found to be the most effective in causing the loss in activity, after 7 days of incubation and after 14 days of incubation almost complete loss of activity occurred.

Functional inactivation of liver cystatin was also observed for glucose and fructose but to a lesser extent and it was found that 7 days incubation at 37 °C resulted in 33% and 44% loss in inhibitory activity, respectively. However, after 14 days of incubation with glucose and fructose resulted in 75% and 88% loss of inhibitory activity. Incubation with these three sugars for 21 days resulted in 95% loss in inhibitory activity for glucose and almost complete loss of inhibitory activity for fructose and ribose. In case of incubation for 28 days there was no activity present for all the sugars (glucose, fructose and ribose. Among the three sugars investigated ribose was found to be significantly reactive.

4.3.2 EFFECT ON THE SPECTRAL PROPERTIES OF LIVER CYSTATIN ON INCUBATION WITH SUGARS

3.3.2.1 UV absorption studies

The alterations in buffalo liver cystatin induced by three sugars (glucose, fructose and ribose) were also observed with respect to hyperchromicity. The UV spectra of BLC in the presence of three sugars (glucose, fructose and ribose) at 37°C showed marked increase in absorbance at 280 nm after incubation for varying time intervals. Figure 5.0 shows the UV spectra of liver cystatin incubated with sugars (glucose, fructose and ribose) for 21 days. The magnitude of alteration was maximum in case of ribose followed by fructose and glucose. Figure 5.2 shows the percent change in absorbance at 280 nm incubation for 3, 7, 14, 21 and 28 days.

4.3.2. Fluorescence spectroscopy studies

The spectral parameters of fluorescence emission spectra such as position, shape and intensity are dependent on the electronic and dynamic properties of chromophore environment, hence fluorescence studies have been extensively used to obtain information on the structural and dynamic properties of a protein (Prajapati et al., 1998). 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 (λmax) as a function of time interval for incubation at 37°C.

Incubation of buffalo liver cystatin with the reducing sugars resulted in the quenching of fluorescence in all the BLC samples (glycated protein samples) studied. Figure 5.1 shows the magnitude of quenching for 3 days incubated samples, maximum quenching was observed in case of ribose treated with liver cystatin followed by fructose upon incubation. Minimum quenching was seen in glucose protein system. Figure 5.2 shows the percent decrease in fluorescence intensity at 337 nm after incubating buffalo liver cystatin for 3, 7, 14, 21 and 28 days. Figure 5.3 shows the fluorescence spectra of BLC with reducing sugars (glucose, fructose and ribose) after 7 days of incubation while Figure 5.4, figure 5.5 and figure 5.6 depicts

Figure 4.9: Activity of liver cystatin in the presence of sugars

Incubation with sugars and its effect on thiol proteinases inhibitory activity for varying time intervals (3, 7, 14, 21 and 28 days). Liver cystatin (2μM) was incubated with 0.5M sugars (glucose, fructose and ribose) in 0.05M sodium phosphate buffer, pH 7.5 under sterile conditions at 37°C. Aliquots were removed at appropriate intervals, followed by dialysis to remove excess sugars and the activity was measured by the method of Kunitz (1947). Each value represents the average of three independent experiments performed in duplicates. Activity in the absence of sugars was taken as 100%. Data is expressed as mean ± SEM for four experiments.

Figure 5.0: UV absorption spectrum of buffalo liver cystatin in the presence and absence of reducing sugars

Ultraviolet spectra of buffalo liver cystatin taken after incubation for 21 days with reducing sugars (glucose, fructose and ribose) followed by dialysis to remove excess sugar. Liver cystatin (2 μM) was incubated with 0.1M sugars (glucose, fructose and ribose) in 0.05M sodium phosphate buffer under sterile conditions at 37°C. The absorption spectra of native BLC in the presence of sugars was recorded in the range of 200-300 nm on a double beam Shimadzu UV-vis spectrophotometer.

Figure 5.1: Intrinsic fluorescence spectrum of liver cystatin in the presence and absence of reducing sugars

Fluorescence spectra for incubation of buffalo liver cystatin with sugars (glucose, fructose and ribose) on the fluorescence intensity incubated for 3 days followed by dialysis to remove excess sugar. Protein concentration was 2 μM, concentration of sugars was 0.1 M. Each spectrum is the average of three individual scans. 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 the 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 5.2: Changes in fluorescence intensity of sugar-cystatin complex for varying time intervals (Data calculated from fluorescence spectrum figure 5.1)

Change in fluorescence at 280 nm upon incubating buffalo liver cystatin (2μM) with sugars (glucose, fructose and ribose at 0.5 M) for 3, 7, 14, 21 and 28 days followed by dialysis to remove excess sugars. Percent decrease in fluorescence has been calculated taking control as 100. Control represents BLC incubated without sugars for the same duration. Data are expressed as mean ± SEM for four experiments.

Figure 5.3: Intrinsic fluorescence spectrum of liver cystatin in the presence and absence of reducing sugars (7 days)

Effect of incubation of buffalo liver cystatin with sugars (glucose, fructose and ribose) on the fluorescence intensity incubated for 7 days followed by dialysis to remove excess sugar. Protein concentration was 2 μM, concentration of sugars was 0.1 M. Each spectrum is the average of three individual scans. 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 the 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 5.4: Intrinsic fluorescence spectrum of liver cystatin in the presence and absence of reducing sugars (14 days)

Changes induced by the incubation of buffalo liver cystatin with sugars (glucose, fructose and ribose) on the fluorescence intensity for 14 days followed by dialysis to remove excess sugar. Protein concentration was 2 μM, concentration of sugars was 0.5 M. Each spectrum is the average of three individual scans. 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 the 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 5.5: Intrinsic fluorescence spectrum of liver cystatin in the presence and absence of reducing sugars (21 days)

Buffalo liver cystatin incubated with sugars (glucose, fructose and ribose) on the fluorescence intensity incubated for 21 days followed by dialysis to remove excess sugar. Protein concentration was 2 μM, concentration of sugars was 0.1 M. Each spectrum is the average of three individual scans. 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 the 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 5.6: Intrinsic fluorescence spectrum of liver cystatin in the presence and absence of reducing sugars (28 days)

Effect of incubation of cystatin with sugars (glucose, fructose and ribose) on the fluorescence intensity incubated for 28 days followed by dialysis to remove excess sugar. Protein concentration was 2 μM, concentration of sugars was 0.1 M. Each spectrum is the average of three individual scans. 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 the 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.

the change in the fluorescence intensity (spectra) after 14, 21 and 28 days of incubation with glucose, fructose and ribose respectively. In all the figures fluorescence intensity decreases most for ribose followed by fructose and glucose suggesting ribose is a potent and highly reactive towards BLC.

4.3.3 CD measurements of BLC in presence of reducing sugars

Secondary structure measurements of BLC were carried out in the presence of 0.5M sugar solutions (glucose, fructose and ribose) incubated for 7, 14, 21 and 28 days. Far UV-CD studies on glucose, fructose and ribose induced glycation of cystatin were carried out to investigate the effect of these reducing sugars on the secondary structure of the protein. In the far UV-region the CD spectrum of native BLC shows presence of 30.89% α-helical content determined by the method of Chen. et.al 1975 and significant amount of β structure.

Fig 5.7, fig 5.8 and 5.9 shows effect of glucose, fructose and ribose on the ellipticity values on day 7, 14, 21 and 28 respectively. Figure 5.7 shows the respective transistion curves after day 7, 14, 21 and 28 in terms of ellipticity values at 222 nm (θ_{222} nm). There is a loss in the α-helix content of BLC with increasing time of incubation. On day 7 there is a slight decrease in ellipticity around 6.1% whereas on day 28 there is a huge change in the value ofα-helix after glycation with glucose and almost a loss of 44.2% α-helix is observed. Figure 5.8 shows the effect of fructose on the ellipticity values with increasing time of incubation after day 7, 14, 21 and 28. In case of fructose there is a profound effect on the ellipticity values at 222 nm on day 7, 14, 21 and 28 compared to glucose. Fructose causes decrease in the α-helix content of around 11% on day 7 and 46% after 28 days of incubation with BLC compared with native which is taken as 100%. Ribose causes almost 15% loss in the α-helix content compared with the native (BLC) protein on day 7 whereas on day 28 the loss in the α-helix content is almost 63.38% compared to native protein.

Figure 5.7: Secondary structure analysis of BLC in the presence of glucose

The figure shows changes in far-UV CD spectra of BLC.Buffalo liver cystatin was incubated with 0.5 M glucose for different time intervals in days (7, 14, 21 and 28 days) and the samples were dialyzed before taking CD spectra.each spectrum is the average of four scans. 0.2mg/ml of BLC was taken in 50mm sodium phosphate buffer, pH 7.5. CD spectra was recorded in the range of 200-250 nm.

Figure 5.8: Secondary structure analysis of BLC in presence of fructose

The figure shows changes in far-UV CD spectra of BLC.Buffalo liver cystatin was incubated with 0.5 M fructose for different time intervals in days (7, 14, 21 and 28 days) and the samples were dialyzed before taking CD spectra.each spectrum is the average of four scans. 0.2mg/ml of BLC was taken in 50mm sodium phosphate buffer, pH 7.5. CD spectra was recorded in the range of 200-250 nm.

Figure 5.9: Secondary structure analysis of BLC in presence of ribose

The figure shows changes in far-UV CD spectra of BLC.Buffalo liver cystatin was incubated with 0.5 M ribose for different time intervals in days (7, 14, 21 and 28 days) and the samples were dialyzed before taking CD spectra.each spectrum is the average of four scans. 0.2mg/ml of BLC was taken in 50mm sodium phosphate buffer, pH 7.5. CD spectra was recorded in the range of 200-250 nm

Figure 6.0: Transition curve of BLC glycated with glucose, fructose and ribose

Transistion curve of BLC determined in terms of MRE values at wavelength of 222nm. BLC concentration was 0.2mg/ml and the concentration for glucose, fructose and ribose was 0.5M respectively.

Figure 6.1: % ellipticity value for transition curve of BLC glycated with glucose, fructose and ribose

Transition curve of BLC denaturation in glucose, fructose and ribose determined in terms of % ellipticity values at wavelength of 222nm (with θ_{222} nm of native BLC taken as 100 %). θ_{222} nm% values of curves corresponding to glucose (0.5 M) fructose (0.5 M) and ribose (0.5 M) were taken at an interval of 7, 14, 21 and 28 days.BLC concentration was 0.2mg/ml.

4.3.4 ADVANCED GLYCATION END PRODUCT (AGEs) SPECIFIC FLUORESCENCE

The sugar protein system that were incubated for 28 days revealed an increase in AGE specific fluorescence (435 nm em) upon excitation at 370 nm. As can be seen in Figure 6.2, the development of AGE fluorescence was maximum in case of ribose treated followed by fructose and glucose when present in complex with cystatin respectively. BLC without sugar which was incubated for same duration showed a peak around 435 nm upon excitation at 370 nm. Fluorescence spectra is shown only for 21 days of incubation. Figure 6.3 shows the percent increase in AGE fluorescence upon incubating Buffalo liver cystatin for 7, 14, 21 and 28 days.

Figure 6.2: Extrinsic fluorescence of liver cystatin in the presence of sugars

Effect of incubation of sugars (glucose, fructose and ribose) on the fluorescence intensity at 435 nm of liver cystatin incubated for 28 days followed by dialysis to remove excess sugar. Protein concentration was 2 μM, concentration of sugars 0.1M. Each spectrum is the average of three individual scans. 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 the wavelength region 400-500 nm after exciting the protein solution at 370 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 6.3: Change in extrinsic fluorescence of sugar-cystatin complex for varying time intervals. (Data calculated from fluorescence spectrum figure previous).

Buffalo liver cystatin was incubated with sugars (glucose, fructose and ribose) for 3, 7, 14, 21 and 28 days and the samples were dialyzed before recording fluorescence. Percent increase in AGE fluorescence has been calculated. Native BLC without sugar was taken as control. Data are expressed as mean ± SEM for four different experiments.