J Fluoresc (2014) 24:1107–1117
DOI 10.1007/s10895-014-1391-2

ORIGINAL PAPER

Effect of Non-Enzymatic Glycation on Cystatin: A Spectroscopic Study

Sheraz Ahmad Bhat · Aamir Sohail · Azad Alam Siddiqui · Bilqees Bano

Received: 30 January 2014 / Accepted: 7 April 2014 / Published online: 2 May 2014

Abstract The study shows the effect of nonenzymatic glycation on conformation and inhibitory activity of chick pea cystatin (CPC). CPC was incubated with different reducing sugars, pentose (D-Ribose), hexoses (D-Glucose, D-Fructose) at 37 °C for 5 weeks. To evaluate the modification of CPC by these different sugars during the glycation process the extent of the Maillard reaction, conformational, structural and functional changes were investigated. The behaviour of glycated CPC was monitored by the techniques of UV and fluorescence spectroscopies. Specific fluorescence was employed to characterise the glycation and AGEs. The anti-papain activity of glycated CPC was found to be significantly lower as compared to its non-glycated form. Glycation with ribose led to maximum loss in inhibitory activity. It was found that the incubation of CPC with all the mentioned sugars led to a parallel increase in tryptophan fluorescence as well as in Maillard and other AGEs specific fluorescence values and hyperchromicity in the UV-region. Among the sugars studied comparatively ribose was found to be the most active in inducing structural and conformational alterations in the protein suggesting its high reactivity with protein amino groups.

Keywords Maillard reaction · AGEs · Anti-papain · Chick pea cystatin · Glycation

Abbreviations

AGEs	Advanced glycated end products
ANS	8-Anilino-1-Naphthalene-Sulphonic acid
TNBS	Trinitrobenzene sulphonate
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
HSA	Human serum albumin
GOD	Glucose Oxidase

S. A. Bhat · A. Sohail · A. A. Siddiqui · B. Bano (✉)
Department of Biochemistry, Faculty of Life Sciences, Aligarh Muslim University, Aligarh 202002, India
e-mail: bilqeesbanos@gmail.com

Introduction

Maillard reaction is the non-enzymatic glycated adduct formation between amino groups (predominantly the ε-amino group of lysine and the guanidine group of arginine) [1, 2] and carbonyl groups of reducing sugars or other carbonyl compounds. This reaction is subdivided into three main stages: early, intermediate, and late. In the early stage, glucose (or other reducing sugars such as fructose, pentoses, galactose, mannose and xylulose) reacts with a free amino group of biological amines to form an unstable aldimine compound, the Shiff base. Then through acid–base catalysis, this labile compound undergoes a rearrangement to a more stable early glycation product known as Amadori product [3]. In the intermediate stage, via dehydratation, oxidation and other chemical reactions, the Amadori product degrades to a variety of reactive dicarbonyl compounds such as glyoxal, methylglyoxal, and deoxyglucosones which, being much more reactive than the initial sugars, act as propagators of the reaction, again reacting with free amino groups of biomolecules. In the late stage of the glycation process through oxidation, dehydratation and cyclization reactions, irreversible compounds called AGEs are formed.

The AGEs are yellow-brown, often fluorescent and insoluble adducts that accumulate on long-lived proteins thus compromising their physiological functions [4]. Sometimes also called as glycophores or fluorophores these AGEs are formed upon the reaction of sugars with lysine, arginine and free NH_2 of N-terminus of proteins. Glycation of proteins can

interfere with their normal functions by disrupting molecular conformation, altering enzymatic activity, reducing degradation capacity, and interfering with receptor recognition [5]. AGE-modified proteins lose their specific functions and undergo accelerated degradation to free AGEs such as pentosidine, N-ε-carboxy-methyl-lysine (CML), argpyrimidine, malondialdehyde-modified protein and others. Some of these (pentosidine, argpyrimidine etc.) are intrinsic fluorophores having characteristic fluorescence emission that can be used to detect the onset of AGEs [6]. Moreover, AGEs can also act as cross-linkers between proteins, resulting in the production of proteinase-resistant aggregates [7]. Besides accumulation during healthy aging, AGEs are formed at accelerated rates in diabetes [8]. They are markers and also important causative factors for the pathogenesis of diabetes [9], cataracts [10], atherosclerosis [11], diabetic nephropathy [12] and neurodegenerative diseases, including Alzheimer's disease [13].

In the present study, glycation of chick pea cystatin was carried out to examine the influence of various sugars on the proteinase inhibitory activity of cystatin and to determine effect on conformational and biochemical properties of the protein. The characterisation of AGEs was also done. Cystatins are thiol proteinase inhibitors that are found in many plant tissues as well as animal tissues. They constitute a powerful regulatory system for endogenous cysteine proteinases, which may otherwise cause uncontrolled proteolysis and tissue damage. Cystatins also have role in storage proteins [14], as regulators of endogenous proteolytic activity [15] and as participants in the mechanism of programmed plant cell death [16] and in prevention of certain types of cancer [17]. Cystatins have been purified from plants, animals and microorganisms. In the present study it has been for the first time isolated from a new plant source chick pea (*Cicer arietinum*) and has been used as a model protein to study glycation by subjecting to modification with different sugars through Maillard reaction. Comparative studies were done to evaluate the effects of these different sugars on the structural and functional properties of CPC by means of spectral analysis and its anti-papain activity. The glycation and AGEs formed were also characterised by spectroscopic techniques to shed some more light in the myriad of knowledge of glycation. The spectral analysis employed included intrinsic fluorescence, glycation specific fluorescence, and AGEs specific fluorescence, and UV spectroscopy. Glycation specific fluorescence monitored intensities at excitation wavelengths of 325 and 485 nm and emission wavelengths of 395 and 530 nm respectively. AGEs were determined by "total AGE fluorescence," measuring fluorescence at excitation and emission wavelengths of 370 and 440 nm respectively [18].

This communication for the first time reports the in vitro glycation of chick pea cystatin (CPC) in the presence of various sugars (pentose, hexoses) and the characterisation of AGEs formed.

Materials and Methods

Materials

Papain, Sephacryl-S100HR, casein, acrylamide, ethylene diamine tetra acetic acid (EDTA), acetone, commassie brilliant blue R-250, L-cysteine, sodium azide were purchased from Sigma Chemical company, St. Louis, USA. Sugars and other chemicals were obtained from SRL Chemicals (India). All other chemicals and reagents used in the study were of analytical grade.

Methods

Purification of CPC

The purification of cystatin was done by modification of the method of sharma et al. [19]. 100 g of chick pea was soaked overnight and then homogenized in homogenization buffer (50 mM sodium-phosphate, 3 mM EDTA, 0.15 M NaCl, pH 7.5) in a homogenizer. The crude extract was centrifuged at 5,000 rpm for 20 min (4 °C) in a Sigma cooling centrifuge (3 K30 model, Germany). The supernatant was collected and subjected to ammonium sulphate saturation (40–60 %). The precipitate was then collected by centrifugation at 10,000 rpm for 30 min (4 °C) and dissolved in minimum amount of buffer (50 mM sodium phosphate, pH 7.5) and then dialyzed against the same buffer containing 0.15 M NaCl to remove ammonium sulphate. A column of sephacryl S100-HR was packed and equilibrated with sodium-phosphate buffer (50 mM, pH 7.5) at room temperature (25 °C). The dialyzed sample was subjected to gel filtration chromatography on this column (70×1.8 cm). The flow rate of the column was 15 ml h^{-1}. A single protein peak with papain inhibitory activity was obtained which was named as CPC. Its homogeneity was checked by native as well as SDS- PAGE.

Protein Estimation

Protein concentration was estimated by the method of Lowry et al. [20].

Glycation of CPC

AGEs formation was carried out by incubating CPC (2.3 μM) in 50 mM sodium phosphate buffer (pH 7.4), sodium azide

(0.02 %) using D-ribose (25 mM), D-glucose (20 mM), D-Fructose (20 mM) as modifiers for 5 weeks at 37 °C. CPC without any sugar was incubated under the similar conditions and was used as control. All solutions were filtered through 0.22 μm syringe filters (sterilized, max. pressure 4.5 bars) under aseptic conditions before their use. After incubation all the sugar treated samples were dialysed against the same phosphate buffer and NaCl (0.15) then stored at 4 °C till further analysis.

Assay of Anti-Papain Activity

The anti-papain activity of native as well as glycated CPC was measured according to Kunitz [21]. The inhibitory activity of cystatin was assessed by its ability to inhibit Caseinolytic activity of papain. The activity was measured at different time intervals (days) and the untreated CPC activity was taken as 100 %.

UV Spectroscopic Studies

The UV absorption measurements of native CPC and that incubated with ribose, glucose and fructose were obtained by measuring the absorption spectra between 250 and 350 nm in a Shimadzu spectrophotometer using a cuvette of 1.0 cm length. Fixed wavelength data were obtained at 330, 360 and 400 nm.

Fluorescence Studies

The fluorescence data were recorded with a RF-1501 spectrofluorophotometer (Shimadzu Co. Japan). For the total protein fluorescence, excitation wavelength for the samples was 280 nm while the emission spectra were recorded in the wavelength range of 300–400 nm. The path length of samples was 1 cm while the slit width was set at 5 nm both for excitation as well as emission. The protein concentration used for fluorescence studies was 2.3 μM. The fluorescence was recorded after regular intervals of day 1, 4, 7, 14, 21, 28 and finally day 34 for these sugar treated samples. Glycation specific fluorescence intensities were also monitored by exciting the samples at 325 nm and 485 nm at emission wave length of 395 nm and 530 nm respectively. Total AGE fluorescence was taken by exciting the samples at 370 nm and recording the emission spectrum in the range of 390–550 nm [22].

Determination of Free Amino Groups

The free amino groups present in treated CPC were measured by trinitrobenzene sulphonate (TNBS) method as given by Haynes et al. in 1967 [22] with slight modification. The absorbance was taken at 420 nm against a blank devoid of any glycated protein. After that the calculation for the free amino groups was done by the following equation

$$\frac{\text{standard O.D} \times \text{Sample O.D}}{\text{Sample Volume} \times \text{protein concentration}\left(\frac{mg}{ml}\right)}$$

Where,

Standard 1 O.D was calculated from standard glycine plot under similar conditions.

Sample O.D is the absorbance of different samples taken at 420 nm,

Sample volume is the volume of different samples used for measurements and,

Protein concentration is the protein amount in the samples in mg/ml.

ANS Fluorescence Measurements

ANS binding was measured by fluorescence emission spectra with excitation at 380 nm and emission in the range of 400–600 nm. ANS concentration was taken 100 molar excess of protein concentration while protein concentration was taken 2.3 μM as proposed by Matulis et al. [23].

Statistical Analysis

Data have been expressed as mean ± standard deviation (n= 3). Statistical analysis was performed with one-way ANOVA software. Data were considered significant at $p<0.05$.

Results

Purification of CPC

The two step purification of cystatin included ammonium sulphate precipitation and gel filtration chromatography. The precipitate obtained after 40–60 % ammonium sulphate saturation was chromatographed on sephacryl S-100 HR column and single peak giving significant papain inhibition was obtained as shown in Fig. 1a. The fractions corresponding to the inhibitory peak were pooled and used for further studies. The homogeneity of this purified CPC was further proved by gel electrophoresis under non-denaturing conditions. The electrophoretic pattern is shown in Fig. 1b, lane d. The inhibitor moved as a single band in native PAGE. In SDS-PAGE, CPC again migrated as a single band under both reducing as well as non-reducing conditions exhibiting the monomeric nature of the protein Fig. 1c. By this method we achieved 20.2 % yield and 400-fold purification of CPC.

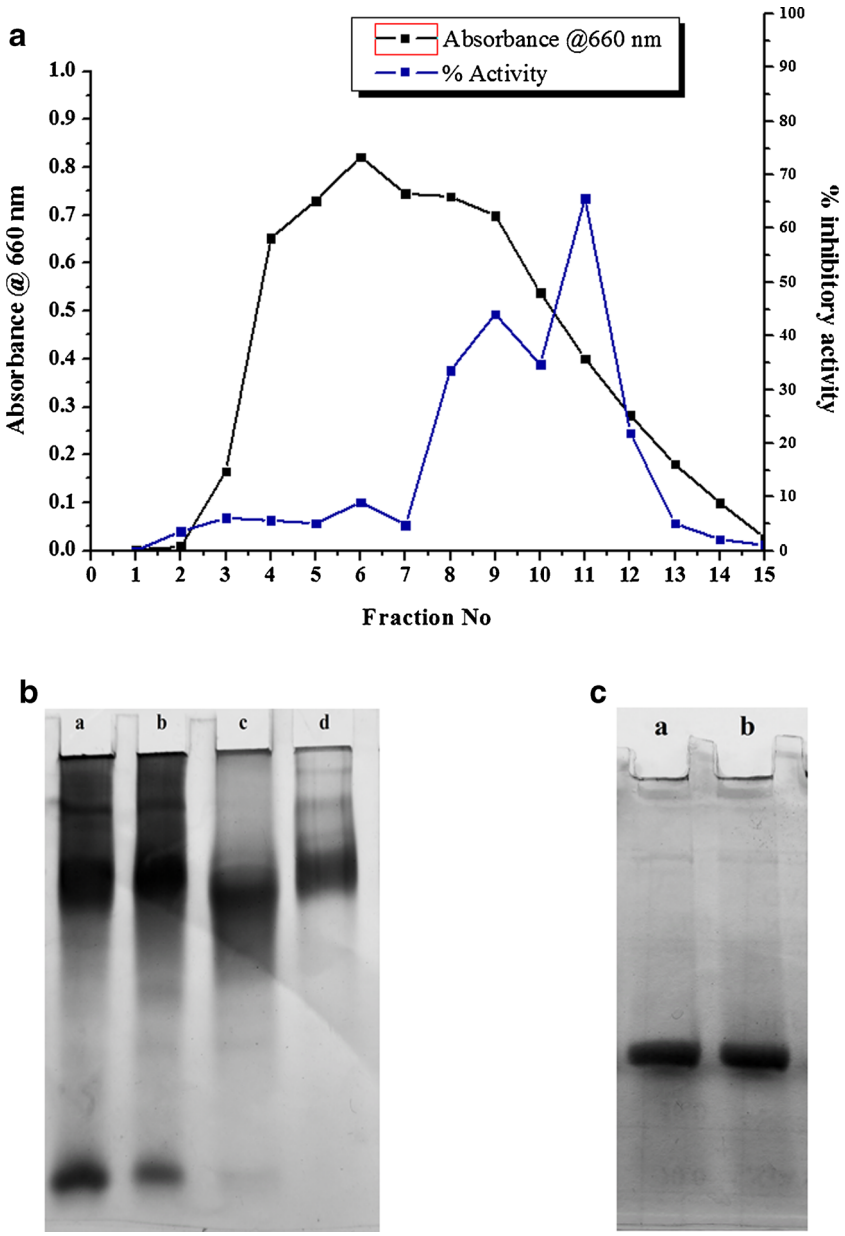


Fig. 1 **a** The dialysed fraction obtained after (40–60 %) ammonium sulphate precipitation was further purified by gel permeation chromatography on a sephacryl S-100 HR column (70× 1.8 cm) equilibrated with sodium-phosphate buffer (50 mM, pH 7.5) at room temperature (25 °C). Elution was performed in 5 ml fractions at a flow rate of 15 ml. h^{-1}. Each fraction was assayed for thiol proteinase inhibitory activity and protein concentration. **b** Native PAGE of aliquots from cystatin shows purification scheme that was performed on 7.5 % non-denaturing gel. *Lane a*: Crude extract after homogenization, *Lane b*: Dialyzed sample after ammonium sulphate precipitation, *Lane c and d*: Purified CPC. **c** SDS-PAGE (12.5 % denaturing gel) of CPC under both reducing (*lane b*) and non-reducing (*lane a*) conditions indicating its homogeneity as well as monomeric nature

Effect of Sugars on the Anti-Papain Activity of CPC

CPC was incubated with different sugars [D-ribose (25 mM), D-glucose (20 mM), D-Fructose (20 mM)], separately in 50 mM sodium phosphate buffer (pH 7.4), sodium azide (0.02 %) at 37 °C for a period of 1, 4, 7, 14, 21, 28 and 34 days. Figure 2 shows the progressive decline in the anti-papain activity on increasing incubation days. At the end of incubation period (34 days), the decrease in the activity from baseline for native CPC was found to be ~15 %, while as for the sugar incubated with CPC, almost complete loss of activity occurred. Amongst the sugars investigated, ribose was found to be the most effective in causing the loss in activity followed by fructose and glucose. Functional inactivation of CPC was observed completely in presence of ribose after 14th day while as glucose and fructose incubated CPC showed some inhibitory activity till 21st day. After 14 days of incubation ribose incurred full inactivation of inhibitory activity in comparison to fructose 90 %, glucose 76 % approximately. However glucose and fructose incubated CPC did not show any activity beyond 21st day.

UV Absorption Studies

The alterations in CPC structure and conformation induced by different sugars were also observed with respect to hyperchromicity. Figure 3a shows the UV spectra of CPC and sugars incubated CPC samples. The UV spectra of various sugars incubated CPC showed marked increase in absorbance at 280 nm at the end of incubation period. The

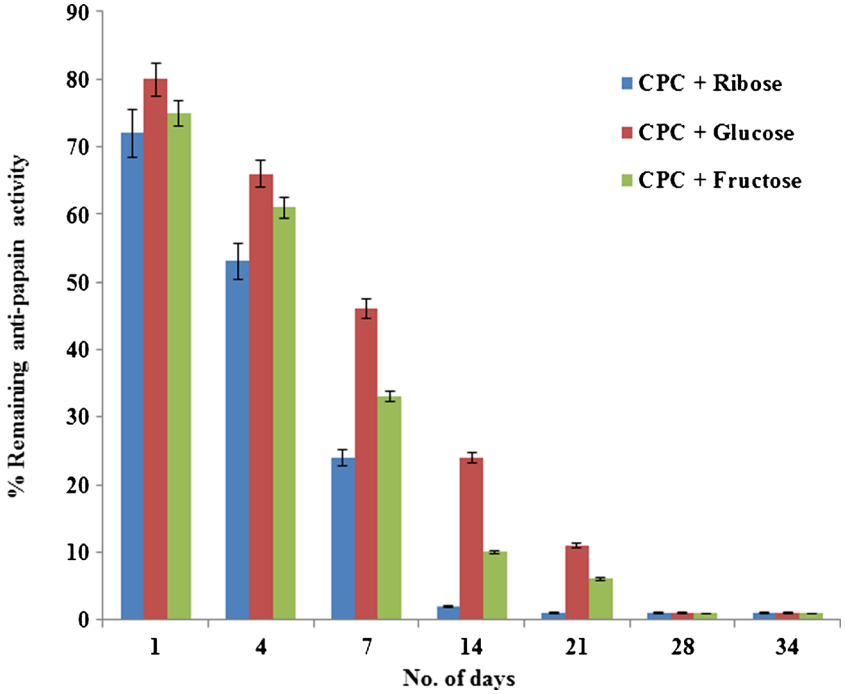


Fig 2 Effect of incubation with sugars on thiol proteinases inhibitory activity of CPC for varying time intervals (1, 4, 7, 14, 21, 28 and 34 days). CPC (2 μM) was incubated with 25 mM D-ribose, 20 mM D-glucose and D-Fructose separately in 0.05 M sodium-phosphate buffer under sterile conditions at 37 °C. Aliquots were removed at appropriate intervals, followed by dialysis to remove excess sugars and the activity was measured by the method of Kunitz [25]. Each value represents the average of three independent experiments performed in duplicates. Activity in the absence of sugars was taken as 100 %. Data are expressed as mean ± SEM for three experiments

maximum magnitude of alteration was in case of ribose followed by fructose and glucose.

Fixed-wavelengths data were recorded at 330, 360 and 400 nm (Fig. 3b). Due to the dependence of absorbance on concentration and optical path length, the measured absorbance results have to be normalized in order to allow comparison with absorbance obtained in different experimental setups. Because native protein does not depict any absorbance above 300 nm it was not possible to normalize data in the same way as was done for the fluorescence data. It was reported earlier by A. Schmitt et al. [24] that the AGEs absorbance increases at these fixed wavelengths. Ribose-

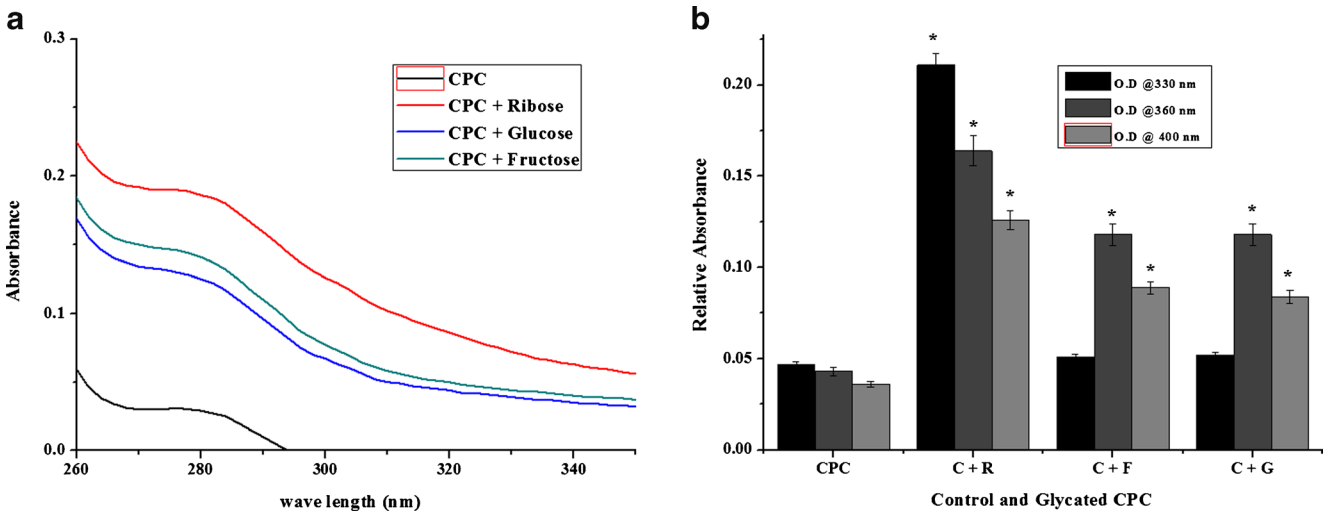


Fig. 3 **a** Ultraviolet spectra of CPC (2.3 μM) incubated for 34 days with sugars (ribose, glucose, fructose) in 0.05 M sodium-phosphate buffer under sterile conditions at 37 °C. The absorption spectra of native and glycated CPC were recorded in the range of 200–300 nm on a double beam Shimadzu UV–vis spectrophotometer. **b** Relative absorbance of native and sugar incubated CPC at fixed wave lengths 330 nm, 360 nm and 400 nm to estimate the onset of glycation and AGEs. CPC (2.3 μM) was incubated separately with different sugars as explained in the Methods section. The data was taken at the end of incubation. Results are expressed as mean ± SEM for five experiments. * Significantly different from control at $p<0.05$ by one way ANOVA. [C: CPC Control, C + R: CPC + Ribose, C + G: CPC + Glucose, C + F: CPC + Fructose]

derived CPC-AGEs adducts show the highest absorbance at all three wavelengths. From the absorbance data it can be concluded that absorbance between 300 and 400 nm can be used to judge the onset of AGEs formation of proteins that are derived from the reaction with any of the glycating agent. From the wavelengths used in our experiments the highest sensitivity was obtained at 360 nm.

Fluorescence Spectroscopy Studies

Figure 4b shows the relative increase in fluorescence intensity from native CPC (control) as the function of varying time incubation time of CPC with different sugars. When 2.3 μM CPC was incubated with the sugars as mentioned above, separately in 50 mM phosphate buffer with 0.02 % sodium azide, pH 7.4 and 37 °C for 34 days the intrinsic fluorescence of glycated CPC was much higher compared to that of native CPC. A widely accepted assumption is that the higher the fluorescence intensity, the higher the degree of modification. These data support the increased absorbance in 280 nm region in absorption spectroscopic study. The increase in intrinsic fluorescence of glycated protein has also been reported earlier by Roy et al. [25].

Fluorescence spectra for glycated CPC and controls were also recorded at emission wavelengths of 395, 440 and 530 nm with excitation wavelengths of 325, 370 and 485 nm respectively.

Figure 4c shows glycation specific fluorescence that records fluorescence intensity in the range 350–550 nm after being excited at 325 nm. For this emission at 395 nm maximum excitation was found between 325 and 335 nm, which is in agreement with data from Westwood and Thornalley [26], who reported an excitation maximum of 335 nm for emission at 406 nm for Glc-modifed HSA. This fluorescence accounts for argpyrimidine and pentosidine, both well-known AGE structures derived from arginine [27].

For emission at 440 and 485 nm the excitation maximum was found between 360 and 370 nm. The fluorescence at excitation/emission wavelength of 360/430 nm was first reported for AGE-modifed lens proteins by Monnier and Cerami [28] and was found to be a useful tool to determine the total AGE fluorescence [29] Fig. 4d. A third AGE-specific fluorescence was detected in emission scans with excitation at 485 nm and maximum emission at 530 nm. Spectra obtained from emission scans are shown in Fig. 4e. This fluorescence accounts for arginine derived AGEs as reported earlier by A. Schmitt et al. [24]. Therefore this fluorescence should be derived exclusively from arginine modifications and thus is a very useful tool to characterize AGEs. Thus our results show

Fig. 4 **a** Effect of incubation of CPC with sugars (ribose, glucose, and fructose) on the fluorescence emission spectra incubated for 28 days followed by dialysis to remove excess sugar. Protein concentration was 2.3 μM, concentration of sugars is explained in the Methods section. 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 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. **b** Relative fluorescence of native CPC and sugar (ribose, glucose, and fructose) incubated CPC for 1, 4, 7, 14 and 21, 28, 34 days followed by dialysis to remove excess sugars. Control represents CPC incubated without sugars for the same duration. The fluorescence was monitored at an excitation wavelength of 280 nm (Peaks achieved on respective days are plotted) with a slit width of 5 nm. Data are expressed as mean ± SEM for three experiments Data was generated from intrinsic fluorescence spectra taken at different time intervals (cf figure (**a**). **c** The glycation specific fluorescence (excitation: 325 nm; emission: 350–550 nm) of modified CPC incubated in presence of different sugars. Experimental conditions remain same as that of figure (**a**). Spectra were taken at the end of incubation period. All spectra were normalized to the spectrum of the CPC control. **d** The total AGE fluorescence (excitation: 370 nm; emission: 390–550 nm) of modified CPC incubated in presence of different sugars. Experimental conditions remain same as that of figure (**a**). Spectra were taken at the end of incubation period. All spectra were normalized to the spectrum of the CPC control. **e** The glycation specific fluorescence (excitation: 485 nm; emission: 500–550 nm) of modified CPC incubated in presence of different sugars. Experimental conditions remain same as that of figure (**a**). Spectra were taken at the end of incubation period. All spectra were normalized to the spectrum of the CPC control

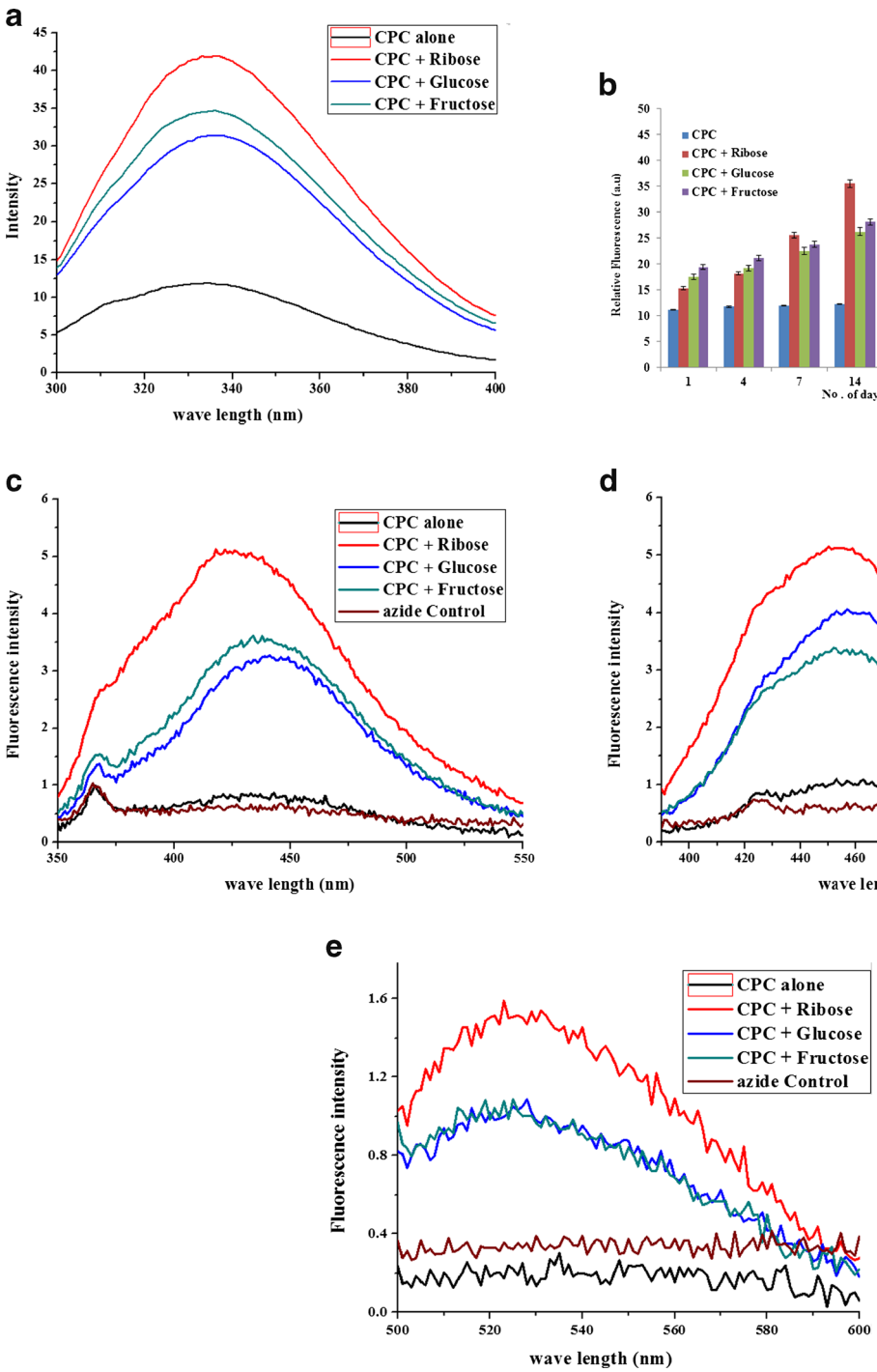
that ribose is more reactive and potent glycating sugar among the three sugars investigated.

Determination of Free Amino Groups in Glycated CPC

At an early stage of Maillard reaction, the protein containing free amino groups such as ε-NH_2 groups of lysine reacts with the carbonyl groups leading to the loss of free amino groups. CPC when incubated with different sugars as explained in Methods section in 50 mM phosphate buffer with 0.02 % sodium azide, pH 7.4 and 37 °C for 1–34 days shows loss in free amino groups in its glycated form. For this, CPC was subjected to TNBS method. The result demonstrates that the content of free amino groups in glycated CPC significantly decreases upon glycation (Table 1).

ANS Fluorescence

In order to further assess the changes in the structure of CPC, binding of ANS to the modified and unmodified CPC samples was studied. As shown in Fig. 5, the ANS fluorescence increased considerably for CPC treated with ribose and glucose after the incubation period. This is indicative of the solvent exposure of hydrophobic patches of the protein. ANS binding to CPC and glycated CPC was studied to



determine the role of glycation on surface hydrophobicity of CPC. ANS is strongly fluorescent when bound to the protein and essentially non-fluorescent when surrounded by water. Enhancement of its fluorescence and the shift of the emission maximum when surrounded by nonpolar amino acid residues of proteins [30] are useful in studying surface hydrophobicity of a protein. The fluorescence intensity of glycated CPC was found to be higher than CPC when bound to ANS and the emission maxima of control and treated CPC showed considerable shift of emission maxima. Figure (5) depicts the extent of ANS binding with different sugar treated CPC and CPC alone, as indicated by fluorescence emission when excited at 380 nm. The fluorescence intensities of ANS (100 molar excess) with different glycated CPC were found to be higher

Table 1 Free amino group content in native and glycated CPC

CPC samples	Free amino groups
CPC alone (control)	1.365±0.44
CPC + Ribose	0.645±0.06*
CPC + Glucose	1.035±0.11*
CPC + Fructose	0.870±0.09*

Results are mean ± SEM of three different experiments

Free amino groups are in millimoles/mg of protein

CPC Chick pea cystatin

*Significantly different from control at $p<0.05$ by one way ANOVA

when compared with native CPC, suggesting the increased surface hydrophobicity of glycated CPC than native CPC.

Discussion

The non-enzymatic reaction of reducing sugars with amino groups of amino acids, peptides and proteins is called as non-enzymatic glycosylation or glycation. The non-enzymatic reaction of the amino groups of the amino acids, peptides and proteins with reducing sugars, ultimately resulting in the formation of complex brown pigments and protein-protein crosslinks, was first studied under defined conditions by L. C. Maillard in the early 1900s [31]. Mimicking the in vivo conditions are valuable approaches for studying the conformational changes occurring in proteins due to physiological stress which in turn will be helpful in understanding the pathological conditions responsible for disease. Glycation affects protein structure and function thus makes it important for characterization [32]. Extensive reports have appeared on characterizing, quantifying and for monitoring the structural changes of glycated proteins [33, 34, 24]. Of these, fluorescence spectroscopy is widely used technique to study the same. It is one of the reliable and sensitive method to study structural changes in biomolecules especially proteins.

Exposure of proteins in vitro to high sugar concentration is considered as a relevant model for studying the structural alterations occurring in glycation. After its purification (Fig. 1) from a new source when cystatin (CPC) was incubated with the sugars (Ribose, Glucose and Fructose), it led to the glycation as a result of which its functional characteristics were compromised to the extent that complete loss of antpapain activity was observed after incubation as depicted in Fig. 2. This loss in papain inhibitory activity varied with incubation time which directly correlates to the extent of glycation of CPC as glycation and AGEs formation increase with the incubation period of CPC with different sugars. These results were consistent with UV results. As seen in Fig. 3a, UV spectra of sugar modified CPC along with native CPC shows a strong increase of absorbance over the whole range of spectrum. Increase in absorbance at 280 nm is attributed to protein unfolding and exposure of the chromophoric groups [35]. The maximum absorbance at 280 nm was observed for ribose. To detect further conformational alterations, UV spectra were recorded at fixed wave length data recorded at 330, 360 and 400 nm as shown in Fig. 3b. The UV-spectra that were recorded from all treated samples showed that wavelength between 300 and 400 nm is the effective range

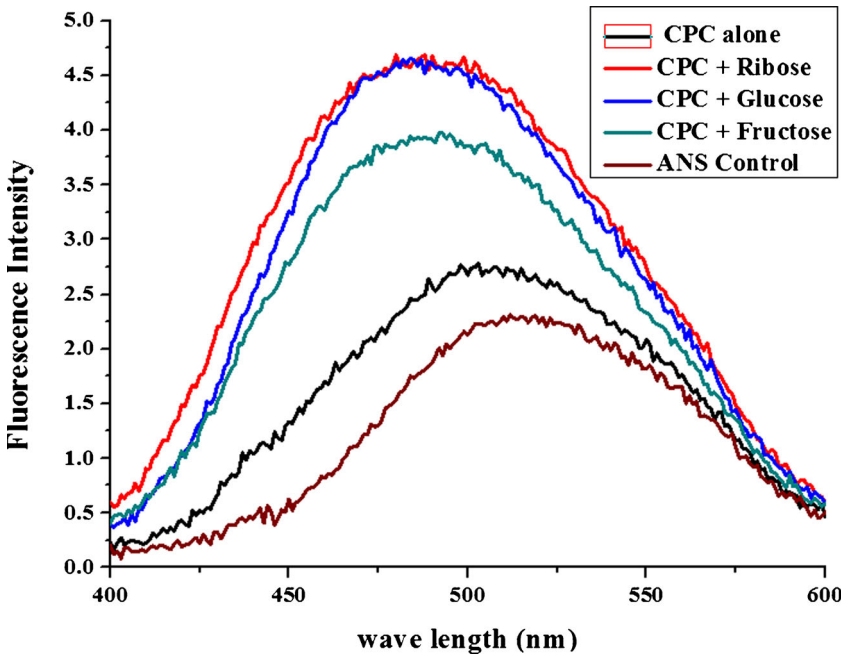


Fig. 5 Fluorescence spectra of ANS binding at excitation wavelength 380 nm: native CPC (control) and CPC incubated with different sugars. Protein concentration was 2.3 μM, concentration of sugars is explained in the Methods section. Each spectrum is the average of three individual scans. Fluorescence 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 wavelength region 400–600 nm after exciting the protein solution at 380 nm. The slits were set at 5 nm for excitation and emission. The path length of the sample was 1 cm. Spectra were taken at the end of incubation period

for detection of modifications with the highest sensitivity at the shorter wavelengths. Detection of absorbance was found to be very useful to make a rough estimation of the total degree of modification with regard to lysine and arginine modifications. Therefore taking absorbance at 330, 360 and 400 nm is a simple and convincing method to estimate the onset of protein modification during incubation with sugars or sugar degradation products. Our results showed that ribose incubated CPC was modified more as compared to glucose and fructose incubated CPC.

Intrinsic fluorescence is a promising technique to follow AGEs formation. The time-course of the changes in the fluorescence intensity are shown in Fig. 4b. The increase in fluorescence intensity of sugar incubated CPC might be due to some structural unfolding of CPC leading to exposure of more aromatic amino acid residues towards the surface. When excited at 280 nm, protein fluorescence is enhanced which may be correlated with increased absorbance of the modified protein around 280 nm. Similar results were obtained by Roy et al. [25] who reported the enhanced mobility of the fluorophores in increased native state volume corresponds to increased fluorescence. Therefore, hyper intrinsic fluorescence of glycated CPC may be due to the enhanced fluorophore (tryptophan) mobility in an increased native state volume caused by glycation.

Figure 4c–e shows the AGE formation over incubation time as studied by non-tryptophan fluorescence spectroscopy. Formation of different AGEs upon incubation of CPC with different sugars was analysed using the excitation wavelengths of 325, 370 and 485 nm as described by Schmitt et al. [24]. Excitation at 325 nm and emission at 395 nm has also been reported for glucose modified HSA [26]. This fluorescence accounts for argpyrimidine and pentosidine, both well-known AGEs' structures derived from arginine [31]. Fig. 4c shows the fluorescence spectra recorded at an excitation wavelength of 325 nm. The results show that ribose was more reactive towards the production of arginine derived AGEs. It was followed by fructose and then glucose. For emission at 440 nm the excitation maxima has been reported to be 370 nm. The fluorescence at excitation/emission wavelength of 370/440 nm also known as total AGE fluorescence or Maillard fluorescence, has been reported for AGEs-modified lens proteins by Monnier and Cerami [28] and was found to be a useful tool to determine the total AGE fluorescence [33]. Fig. 4d illustrates the AGE production with respective sugars investigated. This fluorescence also shows that ribose incubations of CPC led to increased production of AGEs, followed by glucose and then fructose. Upon excitation at 485 nm the maximum emission was observed at around 530 nm. The intensity around 530 nm varied with different sugar incubated CPC samples as shown in Fig. 4e. Ribose incubated CPC showed highest fluorescence intensity. Glucose and Fructose incubated CPC showed almost the same intensity. The changes in fluorescence emission profiles with changes of the excitation wavelengths indicate that multiple fluorophores with overlapping absorbance and emission spectra exist in the samples. The alteration in all these glycation specific fluorescence measurements towards increasing side is attributed to the production of different fluorescent AGEs or fluorophores upon glycation. The said non intrinsic fluorescence measurements also varied in a time dependent manner indicating extent of glycation and formation of AGEs increase with time of incubation. Furthermore fluorescence studies indicated that ribose is the most reactive in terms of modifying and glycating CPC among the three sugars used. Control CPC did not show any significant fluorescence under these conditions.

To further detect the extent of glycation, free amino groups were determined as shown in Table 1. The side chain free amino groups and N-terminal amino groups of proteins react with sugars via Schiff's base to start the glycation process. Our findings show that free amino groups of sugar incubated CPC samples decreased as compared to the native CPC (control). Results also show that ribose is more active sugar than rest of the sugars investigated as loss in free amino group is more in case of ribose as compared with others. However fructose was more reactive than glucose. Iram et al. [36] have also reported a decrease in free amino groups upon glycation.

Glycation of GOD exposes more hydrophobic regions of the protein to the surface making them available for ANS binding [37]. This might be attributed to the unfolding of the proteins during glycation. Our findings also go well with the above, and possibly is the reason for the increased UV absorbance of glycated CPC at 280 nm (Fig. 3a) and also for the enhanced intrinsic fluorescence of glycated CPC (Fig. 4b). Fig. 5 shows the ANS binding spectra of native as well as sugar incubated CPC.

To summarize the results, glycation studies of CPC in the presence of pentose (ribose) and hexose sugars (glucose and fructose) were performed to present CPC as a model system for observing glycation related conformational alterations of protein on incubation with sugars for long time period. The study deals with the fluorescence and absorbance properties of AGEs and their possible application for AGE characterization. Various compounds, like methylglyoxal, ribose etc. are present in human body that can act as glycating agents and can readily react with amino groups of proteins to produce AGEs, which have a role in the pathophysiology of ageing and diabetic complications [38]. Glucose, a key cellular fuel in the body, can also cause damage to the body by glycating the proteins resulting in loss of their shape and ultimately making them insoluble or unstable. This is because on incubation for longer time period aldehyde group forms Schiff's base between protein and sugar moiety subsequently disrupting the native conformation of proteins. A good example of this is cataracts which occur when protein glycation takes place.

Similar is the case with fructose. The process of glycation lowers the level of solubility of the lens proteins which in turn leads to a loss of transparency in the lens. DNA damage has also been reported in specific diabetic cells when DNA interacts with glycated products. These glycated products produce both base modification and apurinic/apyrimidinic sites in DNA, in addition to the strand breaks [39]. The inactivation of catalase and SOD in diabetic patients is a consequence of glycation, where sugar combining with catalase and SOD could lead to their inactivation and thus to accumulating peroxide and superoxide, which might contribute to the overall complications of diabetes and aging [40]. Cytotoxic effects of AGEs have also been reported in diabetic patients [41].

Conclusion

Glycation is implicated in variety of disorders like diabetes, atherosclerosis and various micropathies. The interaction of sugars with CPC was carried out in vitro under conditions that more or less mimic the in vivo reaction with various sugars. The formation of advanced glycation end products (AGEs) and their characterization through various fluorescence measurements has a significant importance in diseased states. It might be useful tool to estimate the effectiveness of inhibitors of AGEs formation. The sugar induced alterations in the CPC could shed some light on the role of glycation reaction in various diseases.

Acknowledgments The authors are highly thankful for the facilities obtained at AMU Aligarh. Financial support to SAB as a Senior Research Fellow of the University Grants Commission (UGC), New Delhi, India is acknowledged. We are grateful to SAP-DRS and UGC-FIST programmes for their generous research support.

Conflict of Interest The authors confirm that this article content has no conflicts of interest.

References

1. Baynes JW, Watkins NG, Fisher CI, Hull CJ, Patrick JS, Ahmed MU, Dunn JA, Thorpe SR (1989) The Amadori product on protein: structure and reactions. Prog Clin Biol Res 304:43–67
2. Bry L, Chen PC, Sacks DB (2001) Effects of hemoglobin variants and chemically modified derivatives on assays for glycohemoglobin. Clin Chem 47:153–163
3. Neglia CI, Cohen HJ, Garber AR, Ellis PD, Thorpe SR, Baynes JW (1983) ^{13}C NMR investigation of nonenzymatic glucosylation of protein. Model studies using RNAase A. J Biol Chem 258:14279–14283
4. Lapolla A, Traldi P, Fedele D (2005) Importance of measuring products of nonenzymatic glycation of protein. Clin Biochem 38: 103–115
5. Hsieh CL, Yang MH, Chyau CC, Chiu CH, Wang HE, Lin YC, Chiu WT, Peng RY (2007) Kinetic analysis on the sensitivity of glucose- or glyoxal-induced LDL glycation to the inhibitory effect of Psidiumguajava extract in a physiomimic system. Bio Systems 88: 92–100
6. Vetter SW, Indurthi VS (2011) Moderate glycation of serum albumin affects folding, stability, and ligand binding. Clin Chim Acta 412: 2105–2116
7. Kikuchi S, Shinpo K, Takeuchi M, Yamagishi S, Makita Z, Sasaki N, Tashiro K (2003) Glycation-a sweet temper for neuronal death. Brain Res Rev 41:306–323
8. Reddy VP, Beyaz A (2006) Inhibitors of the Maillard reaction and AGE breakers as therapeutics for multiple diseases. Drug Discov Today 11:646–654
9. Ahmed N (2005) Advanced glycation endproducts-role in pathology of diabetic complications. Diabetes Res Clin Pract 67:3–21
10. Stitt AW (2005) The Maillard reaction in eye diseases. Ann N Y Acad Sci 1043:582–597
11. Forbes JM, Yee LT, Thallas V, Lassila M, Candido R, Jandeleit-Dahm KA, Thomas MC, Burns WC, Deemer EK, Thorpe SR, Cooper ME, Allen TJ (2004) Advanced glycation end product interventions reduce diabetes-accelerated atherosclerosis. Diabetes 53:1813–1823
12. Yamamoto Y, Doi T, Kato I, Shinohara H, Sakurai S, Yonekura H, Watanabe T, Myint KM, Harashima A, Takeuchi M, Takasawa S, Okamoto H, Hashimoto N, Asano M, Yamamoto H (2005) Receptor for advanced glycation end products is a promising target of diabetic nephropathy. Ann N Y Acad Sci 1043:562–566
13. Jono T, Kimura T, Takamatsu J, Nagai R, Miyazaki K, Yuzuriha T, Kitamura T, Horiuchi S (2002) Accumulation of imidazolone, pentosidine and N (epsilon)- (carboxymethyl) lysine in hippocampal CA4 pyramidal neurons of aged human brain. Pathol Int 52:563–571
14. Boušová I, Vukasović D, Juretić D, Palička V, Dršata J (2005) Enzyme activity and AGE formation in a model of AST glycoxidation by D-fructose in vitro. Acta Pharma 55:107–114
15. Ryan CA (1989) Proteinase inhibitor gene families: strategies for transformation to improve plant defenses against herbivores. Bioessays 10:20–24
16. Solommon M, Belenghi B, Delledonne M, Menachen E, Levine A (1999) The involvement of cysteine proteinase and proteinases inhibitor genes in the regulation of programmed cell death inplants. Plant Cell 11:431–444
17. Schelp FP, Pongpaew P (1988) Protection against cancer through nutritionally induced increase of endogenous thiol proteinases inhibitor- a hypothesis. Int J Epidemiol 17:287–292
18. Sebekova K, Podracka L, Blazicek P, Syrova D, Heidland A, Schinzel R (2001) Plasma levels of advanced glycation end products in children with renal disease. Pediatr Nephrol 16:1105–1112
19. Sharma S, Rashid F, Bano B (2006) Unfolding during urea denaturation of a low molecular weight phytocystatin (thiol protease inhibitor) purified from Phaseolusmungo (Urd). Protein Pept Lett 13:323–329
20. Lowry OH, Rosenberg NJ, Randall AL (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275
21. Kunitz M (1947) Crystalline soya bean trypsin inhibitor, general properties. J Physiol 30:291–310
22. Haynes R, Osuga DT, Feeney RE (1967) Modification of amino groups in inhibitors of proteolytic enzymes. Biochemistry 6:541–547
23. Matulis D, Baumann CG, Bloomfield UA, Lovrien UA (1999) 1-Anilino-8 naphthalene sulfonate as a protein conformational tightening agent. Biopolymers 49(6):451–458
24. Schmitt A, Schmittb J, Münchc G, Gasic-Milencovica J (2005) Characterization of advanced glycation end products for biochemical studies: side chain modifications and fluorescence characteristics. Anal Biochem 338:201–215
25. Roy A, Sil R, Chakraborti AS (2010) Non-enzymatic glycation induces structural modificationsof myoglobin. Mol Cell Biochem 338:105–114
26. Westwood ME, Thornalley PJ (1995) Molecular characteristics of methylglyoxal-modified bovine and human serum albumins.

Comparison with glucose-derived advanced glycation end product-modified serum albumins. J Protein Chem 14:359–372
27. Ahmed N, Argirov OK, Minhas HS, Cordeiro CA, Thornalley PJ (2002) Assay of advanced glycation endproducts (AGEs): surveying AGEs by chromatographic assay with derivatization by 6-aminoquinolyl-N-hydroxysuccinimidyl-carbamate and application to Nepsilon-carboxymethyl-lysine- and Nepsilon-(1-carboxyethyl)lysine-modified albumin. Biochem J 364:1–14
28. Monnier VM, Cerami A (1981) Nonenzymatic browning in vivo: possible process for aging of long-lived proteins. Science 211:491–493
29. Sharma SD, Pandey BN, Mishra KP, Sivakami S (2002) Amadori product and AGE formation during nonenzymatic glycosylation of bovine serum albumin in vitro. J Biochem Mol Biol Biophys 6:233–242
30. Bismuto E, Gratton E, Lamb DC (2001) Dynamics of ANS binding to tuna apomyoglobin measured with fluorescence correlation spectroscopy. Biophys J 81:3510–3521
31. Millard LC, Gautier MA, Seances CR (1912) Maillard browning reaction. Acad Sci III 154:66–68
32. Nakajou K, Wantanabe H, Kragh-Hansen U, Maruyama T, Otagiri M (2003) The effect of glycation on the structure, function and biological fate of human serum albumin as revealed by recombinant mutants. Biochem Biophys Acta 1623:88–97
33. De Sa PG, Treubig JM, Brown PR, Dain JA (2001) The use of capillary electrophoresis to monitor Maillard Reaction Products (MRP) by glyceraldehyde and the epsilon amino group of lysine. Food Chem 72:379–384
34. Dutta U, Cohenford MA, Dain JA (2006) Monitoring the effect of glyceraldehyde glycation on the secondary structure human serum albumin and immunoglobulin G: an analysis based on circular dichroism, thermal melting profiles and UV-fluorescence spectroscopy. Anal Chim Acta 558:187–194
35. Traverso N, Menini S, Cottalaso D, Odetti P, Marinari UM, Pronzato MA (1997) Mutual interaction between glycation and oxidation during non-enzymatic protein modification. Biochim Biophys Acta 1336:409–418
36. Iram A, Alam T, Khan JM, Khan TA, Khan RH, Naeem A (2013) Molten globule of haemoglobin proceeds into aggrgates and advanced glycated end products. PLoS ONE 8:e72075
37. Amani S, Naeem A (2011) Acotonitrile can promote formation of different structural intermediate states on aggregation pathway of immunoglobin G from human and bovine. Int J Biol Macromol 49:71–78
38. Thornalley PJ (2008) Protein and nucleotide damage by glyoxal and methylglyoxal in physiological systems–role in ageing and disease. Drug Metabol Drug Interact 23(125):50
39. Mullokandov EA, Franklin WA, Brownlee M (1994) DNA damage by the glycation products of glyceraldehyde 3-phosphate and lysine. Diabetologia 37:145–149
40. Yan H, Harding JJ (1997) Glycation-induced inactivation and loss of antigenicity of catalase and superoxide dismutase. Biochem J 328:599–605
41. Boyanova M, Huppertz B (2009) Cytotoxic effect of advanced glycation end products. Biotechnol Biotechnol Equip 23:1072–1078