BBA - Proteins and Proteomics 1866 (2018) 989–1000

Contents lists available at ScienceDirect

BBA - Proteins and Proteomics

journal homepage: www.elsevier.com/locate/bbapap

Glycation induced conformational transitions in cystatin proceed to form biotoxic aggregates: A multidimensional analysis

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ARTICLE INFO

Keywords:
Aggregation
Biotoxic
Chickpea cystatin
DNA
Glycation
Lymphocytes

ABSTRACT

Hyperglycaemic conditions facilitate the glycation of serum proteins which may have predisposition to aggregation and thus lead to complications. The current study investigates the glycation induced structural and functional modifications of chickpea cystatin (CPC) as well as biological toxicity of the modified protein forms, using CPC-glucose as a model system. Several structural intermediates were formed during the incubation of CPC with glucose (day 4, 8, 12, & 16) as revealed by circular dichroism (CD), altered intrinsic fluorescence, and high ANS binding. Further incubation of CPC with glucose (day 21) formed abundant β structures as revealed by Fourier transform infrared spectroscopy and CD analysis which may be due to the aggregation of protein. High thioflavin T fluorescence intensity and increased Congo red absorbance together with enhanced turbidity and Rayleigh scattering by this modified form confirmed the aggregation. Electron microscopy finally provided the valid physical authentication about the presence of aggregate structures. Functional inactivation of glucose incubated CPC was also observed with time. Single cell electrophoresis of lymphocytes and plasmid nicking assays in the presence of modified CPC showed the DNA damage which confirmed its biological toxicity. Hence, our study suggests that glycation of CPC not only leads to structural and functional alterations in proteins but also to biotoxic AGEs and aggregates.

1. Introduction

The first effect of high glucose concentration in biological fluids and tissues is an important multistep chemical process known as nonenzymatic glycation [1]. In this reaction glucose reacts with the free amino groups of amino acids, proteins, phospholipids and nucleic acids. In case of proteins it involves the reaction of glucose with a number of amino acid side-chains such as the ε-amino groups of lysine residues and the N-terminal group of the protein. This process begins with the conversion of reversible Schiff base adducts to more stable, covalently bound Amadori rearrangement products. Over a course of weeks to months the Amadori products undergo cycles of condensations with additional amines, dehydrations, and oxidative fragmentations to yield a class of heterogeneous chemical compounds collectively referred to as advanced glycation end products (AGEs) [2, 3]. These AGEs can crosslink proteins resulting in the formation of detergent insoluble and protease-resistant aggregates [4]. Glycation and/or aggregation may thus seriously affect protein structure, function and stability.

Recently, much attention has been devoted to the role played by non-enzymatic glycation of proteins in stimulating amyloid aggregation and toxicity. Proteins in amyloid deposits are found often glycated suggesting a direct correlation between protein glycation and amyloidosis [5]. Munch et al., reported that glycation promotes in vitro amyloid aggregation of Aβ peptide, probably because of crosslinking through AGEs formation [6]. Also in the case of β_2-microglobulin, glycation seemed to promote amyloid aggregation which showed significant cytotoxicity to both human SH-SY5Y neuroblastoma and human foreskin fibroblast FS2 cells and induce the formation of intracellular reactive oxygen species [7]. Glycation thus has emerged as a predisposing factor for amyloidosis.

For better understanding of the protein-sugar interactions, we have attempted to study the relationship between the structural and functional properties of chickpea cystatin (CPC) in its native state and in the presence of glucose for different time periods. Cystatins are thiol

Abbreviations: ANS, 8-Anilino-1-naphthalene-sulphonic acid; CPC, chickpea cystatin; AGEs, advance glycation end products; CD, circular dichroism; FTIR, Fourier transform infra-red spectroscopy; ThT, thioflavin T; PBS, phosphate buffer saline; TNBS, trinitrobenzene sulphonate

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https://doi.org/10.1016/j.bbapap.2018.06.006
Received 22 February 2018; Received in revised form 25 May 2018; Accepted 27 June 2018
Available online 28 June 2018

proteinase inhibitors found in many plant as well as animal tissues. These constitute a powerful regulatory system for endogenous cysteine proteinases, which may otherwise cause uncontrolled proteolysis and tissue damage. These inhibitors also serve various other crucial physiological functions. Increased glucose concentration in diabetes mellitus causes glycation of several proteins leading to changes in their properties, formation of AGEs and cross-linking products [8]. As AGEs level rises, these progressively accumulate on tissues and organs developing chronic complications of diabetes - retinopathy, nephropathy, neuropathy and progressive atherosclerosis [9–12]. Moreover as stated above, these AGEs crosslink proteins leading to their amyloid like aggregation that can be cytotoxic thus complicating the diabetes further. In the present study CPC was isolated and purified from chickpea seeds to the stage of homogeneity, which was then used for glycation studies. The structural and functional characteristics of CPC resembled with previously reported phytocystatins and thus aptly places itself in the phytocystatin family or family 4 cystatins [13]. Here the aim was to use CPC-glucose as a model system to mimic in vivo glucose-protein interaction and glycation. A great deal of work has been carried out on glycation of proteins but little has been attempted to show the aftermath of glycation which can proceed to form amyloid like aggregation of proteins and cause cytotoxicity. Here we report the formation of toxic aggregates of CPC upon prolonged glycation in vitro.

2. Materials and methods

2.1. Materials

CPC was purified to homogeneity from chickpea by crystallization method in ammonium sulphate followed by gel filtration on a sephacryl S-100 HR column by method reported earlier [13, 14]. Sugar and other chemicals were obtained from Sigma Chemicals (USA). Other chemicals and reagents used in the study were of analytical grade.

2.2. Methods

2.2.1. Glycation protocol

CPC in 50 mM sodium phosphate buffer, pH 7.4 containing 0.02% sodium azide, to a final protein concentration of 1.5 mg/ml was incubated in sterile conditions in the presence of glucose (0.2 M) as modifier for 4–21 days at 37 °C. CPC without any sugar was incubated under the similar conditions and was used as control. All the solutions were sterilized by filtration through a 0.22 μm pore-size syringe filter (PVDF membrane, Max. pressure 4.5 bars) and then incubated at 37 °C for 21 days under sterile aerobic conditions. After incubation, samples were extensively dialyzed against 50 mM sodium phosphate buffer, pH 7.4 containing 0.5% NaCl and kept frozen at −80 °C until analysis.

2.2.2. Measurement of thiol-proteinase inhibitory activity

The inhibitory activity of cystatin was assessed by its ability to inhibit Caseinolytic activity of papain by the method of Kunitz [15]. The papain inhibitory activity of cystatin without glucose and that incubated with glucose for 4, 8, 12, 16 and 21 days was determined spectrophotometrically using casein as substrate.

2.2.3. UV measurements

The UV absorption measurements of control CPC and those incubated with glucose were obtained by measuring the absorption between 200 and 400 nm on a double beam Shimadzu UV–Vis spectrophotometer using a cuvette of 1.0 cm path length. The protein concentration taken for the analysis was 0.3 mg/ml. Also, fixed wavelength data were obtained at 330, 360 and 400 nm.

2.2.4. Fluorescence measurements

Similar to UV measurements, control and glucose incubated CPC (0.1 mg/ml final concentration) were studied by measuring intrinsic fluorescence at 25 ± 0.2 °C with RF-1501 spectrofluorophotometer (Shimadzu Co., Japan). The samples were excited at 280 nm and emission range was taken at 300–400 nm. Fluorescence measurements (for total AGEs) were also made at 370 nm excitation and an emission range of 390–550 nm. Pentosidine-like fluorescence was also monitored by exciting the samples at 325 nm and recording emission range of 350–550 nm.

2.2.5. Circular dichroism (CD) studies

CD was measured with a JASCO J-815 spectropolarimeter calibrated with ammonium D-10-camphorsulfonate. Cells of path length 0.1 cm were used for scanning between 250 and 190. Each spectrum was the average of three scans. CD analysis was carried out on CPC samples withdrawn from the reaction mixture under incubation for 4–21 days kept at 37 °C. Protein concentrations used were 3.908 μM for far-UV analysis.

2.2.6. Fourier transform infra-red spectroscopy (FTIR)

FTIR spectra were recorded with an Interspec-2020 Fourier transform spectrometer in D_2O. Sample aliquots were placed in a temperature-controlled transmission cell with two BaF_2 windows separated by a 30 μm polyethylene terephthalate spacer. The sample compartment was thoroughly purged with dry nitrogen. Each spectrum was the average of six scans taken with a time gap of 40 s and measured with a resolution of 4 cm^{-1}. Protein concentration of the samples was typically 0.15 mg/ml. The scanning wavenumber was chosen from 1550 to 1700 cm^{-1}.

2.2.7. Determination of free amino groups

The free amino groups present in treated CPC were measured by trinitrobenzene sulphonate (TNBS) method [16]. To the CPC sample aliquot, in a total volume of 1.0 ml of 0.1 M sodium tetraborate buffer, pH 9.3 was added 25 μl of 0.03 M TNBS. The tubes were vortexed instantly to ensure complete mixing of the reactants, and allowed to stand for 30 min at room temperature, 30 °C and absorbance recorded at 420 nm against a blank devoid of any glycated protein.

2.2.8. Determination of Amadori products/fructosamine using NBT

Samples were assayed for Amadori Products using a colorimetric fructosamines assay, as reported by Sattarahmady and co-workers, with slight modification [2]. This assay is based on the nitroblue tetrazolium (NBT) reaction with ketoamines which produces formazan, and which can be detected spectrophotometrically at 530 nm. Briefly, 360 μl of sample protein (0.5 mg/ml) was mixed with 600 μl of 0.5 mM NBT reagent (in 0.2 M carbonate buffer, pH 10.35) in a final reaction volume of 1 ml, and incubated for 1 h at 37 °C. Absorbance was measured at 530 nm on a Shimadzu spectrophotometer model UV-1700. Purified CPC (0.15 mg/ml) was used as control. The deoxymorpholino fructose (DMF) at concentrations between 0 and 1 mM containing 0.15 mg/ml CPC was used for calibration. DMF (1-deoxy-1-morpholino-D-fructose) is an analog of the fructosamine (1-deoxy-1-amino-D-fructose) radical found in glycated proteins.

2.2.9. ANS (8-Anilino-1-naphthalene-sulphonic acid) 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 in the vicinity of 2.3 μM [17].

2.2.10. Thioflavin T (ThT) assay

ThT assay was performed by the method of Bhat et al. [18] with a slight modification. Fluorescence spectra were recorded with a Shimadzu RF 540 spectrofluorophotometer in a 10-mm path length quartz cell. The excitation wavelength was 450 nm and the emission was recorded from 460 to 560 nm. Final concentration of protein in the sample was 2.3 μM while the concentration of Th T was 12 μM in

sodium phosphate buffer 50 mM, pH 7.4. The samples were incubated for 1 h at 37 °C before fluorescence measurements were recorded.

2.2.11. Congo Red (CR) binding assay

Congo red assay (CR) was used for probing the formation of aggregates using UV–Vis spectrophotometer (UV-VIS 1700 Shimadzu, Japan) by scanning the absorption range from 400 to 600 nm. Ideally the protein concentration was kept at 0.1 mg/ml which corresponds to 3.8 μM. The ratio of protein to Congo red concentration was 1:10, i.e. Congo red should be used at a final concentration of 40 μM in the working sample and incubated for half an hour at room temperature [19].

2.2.12. Turbidimetric aggregation analysis

Turbidity assay was used as an indicator to suggest the formation of protein aggregates [20]. Absorbance of control sample and those incubated with glucose was carried out at 450 nm from days 0 to 21 on a UV–Vis spectrophotometer (UV–VIS 1700 Shimadzu, Japan). The concentration of the sample was 0.3 mg/ml.

2.2.13. Rayleigh scattering measurements

Rayleigh scattering measurement was performed on Shimadzu RF-5301 spectrofluorophotometer (Tokyo, Japan) in a 1 cm path length quartz cell. The excitation wavelength was set at 350 nm and emission spectra were recorded from 300 to 400 nm. Fluorescence intensities at 350 nm were plotted. Both excitation and emission slits were 5 nm. The final concentration of CPC was 2.3 μM.

2.2.14. Electron microscopy

To provide the physical evidence about the formation and presence of aggregate structure of CPC incubated for prolonged period of 21 days, electron microscopy (TEM) was performed on a JEM-2100 electron microscope (JEOL, Tokyo, Japan). CPC incubated with and without glucose was assessed by applying 100 μl of each sample on a carbon coated copper grid and left to adsorb for 1 min. After excess fluids were removed from the grid surfaces, the grids were washed with distilled water and stained with 0.3% aqueous uranyl acetate. Excess stain was removed and the samples were dried at room temperature. Images were captured with AMT digital camera system.

2.2.15. Plasmid nicking assay of glycated CPC

To assess the genotoxic effect, pBR322 DNA plasmid was incubated with glycated CPC and the extent of DNA damage was observed. Reaction mixture (30 μl) containing Tris-HCl buffer (pH 7.5, 10 mM), pBR322 DNA plasmid (0.5 μg) and 10 μl of glycated CPC was incubated for 3 h at 37 °C. 10 μl of a solution containing 40 mM EDTA, 0.05% Bromophenol blue (tracking dye) and 50% (v/v) glycerol was added to the treated plasmid after incubation and was then subjected to electrophoresis in a submerged 1% agarose gel. Ethidium bromide stained gel was then viewed and photographed on a UV-transilluminator.

2.2.16. Comet assay (single cell gel electrophoresis) of the glycated CPC

Isolated lymphocytes were exposed to glycated CPC in a total reaction volume of 2.0 ml. Incubation was performed at 37 °C for 1 h. After incubation, the reaction mixture was centrifuged at 716.8*g*, the supernatant was discarded and pelleted lymphocytes were resuspended in 100 μl of PBS and processed further for comet assay. Comet assay of glycated CPC was performed under alkaline conditions by the procedure of Singh et al. [21] with slight modifications. Fully frosted microscopic slides pre-coated with 1.0% normal melting agarose (dissolved in Ca^{2+} and Mg^{2+} free PBS) were used at 50 °C. Around 10,000 cells were mixed with 80 μL of 1.0% low melting point agarose to form a cell suspension and pipetted over the first layer and covered immediately by a cover slip. The slides were placed on a flat tray and kept on ice for 10 min to solidify the agarose. The cover slips were removed and a third layer of 0.5% low melting point agarose (80 μl) was spread. Cover slips were placed over it which was then allowed to solidify on ice for 5 min. The cover slips were removed and the slides were immersed in cold lysis buffer containing 2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10. Triton X-100 (1%) was added before use for a minimum of 1 h at 4 °C. After lysis, DNA was allowed to unwind for 30 min in alkaline electrophoretic solution (pH > 13.0) consisting of 300 mM NaOH and 1 mM EDTA. Electrophoresis was performed at 4 °C in field strength of 0.7 *V*/cm and 300 mA current. The slides were then neutralized with cold 0.4 M Tris, pH 7.5, stained with 75 ml ethidium bromide (20 mg/ml) and covered with a cover slip. They were then placed in a humidified chamber to prevent drying of the gel and analysed on the next day. Slides were scored using an image analysis system (Comet 5.5; Kinetic Imaging, Liverpool, UK) attached to an Olympus (CX41) fluorescent microscope (Olympus Optical Co., Tokyo, Japan) and a COHU 4910-integrated CC camera (equipped with 510–560 nm excitation and 590 nm barrier filters) (COHU, San Diego, CA, USA). Comets were scored at 100× magnification. Images from 50 cells (25 from each replicate slide) were analysed. The parameter taken to assess the lymphocyte DNA damage was the tail length (migration of DNA from their nucleus in μm) and was automatically generated by the Comet 5.5 image analysis system.

2.2.17. Statistics

All experiments were repeated at least four times to document reproducibility and the data has been expressed as mean ± standard error mean (SEM). Significance of difference in mean values was evaluated using one-way analysis of variance (ANOVA) using Origin Software (USA). A probability level of $p < .05$ was selected as indicating statistical significance.

3. Results

3.1. Effect of glycation on the inhibitory activity of CPC

There was a progressive decline in enzyme inhibitory activity of CPC on incubation with glucose for 0, 4, 8, 12, 16, and 21 days as shown in in Fig. 1. CPC (1.5 mg/ml) was incubated with 200 mM of glucose in 50 mM sodium phosphate buffer, pH 7.4 with 0.02% sodium azide at 37 °C for 21 days and its antiprotease activity was examined at

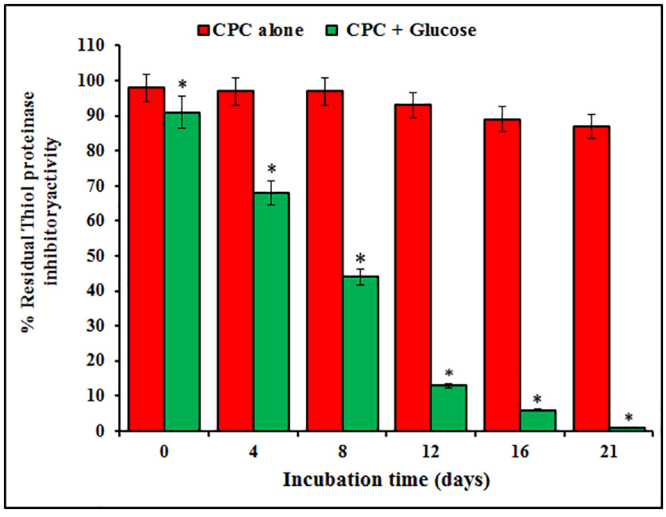

Fig. 1. Bar graph represents the activity of native CPC, and glucose glycated CPC. CPC (1.5 mg/ml) was incubated with Glucose (200 mM) in 50 mM phosphate buffer, pH 7.5 for three weeks under sterile conditions at 37 °C. Aliquots of 100 μl (~6 μM) were removed at appropriate intervals and dialysed to remove excess glucose were then subjected to thiol proteinase inhibitory activity by the method of Kunitz [14]. Error bars indicate the mean ± SEM ($n = 4$). *Significance, $p < .05$ with respect to control.

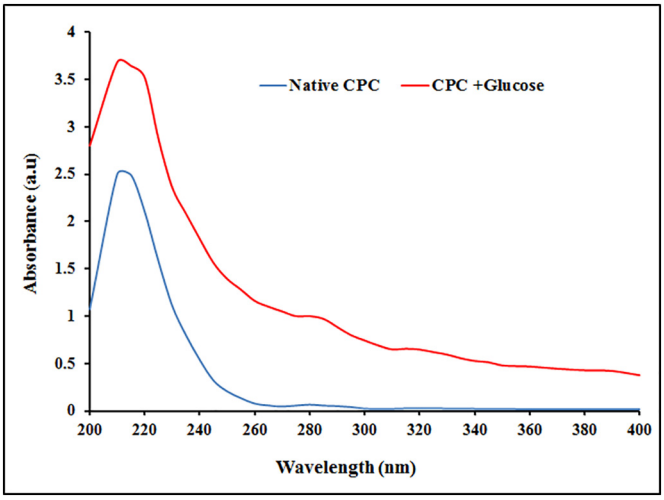

Fig. 2. Ultraviolet spectra of CPC incubated for 21 days with glucose in 0.05 M sodium-phosphate buffer, pH 7.4, under sterile conditions at 37 °C. The absorption spectra of native and glycated CPC were recorded in the range of 200–400 nm on a double beam Shimadzu UV–vis spectrophotometer using a cuvette of path length of 1.0 cm. The protein concentration used was 0.3 mg/ml in a total volume of 1 ml and the spectra were taken at the end of incubation period.

regular intervals. After 21 days, the decrease in the activity from baseline for native CPC was ~11% while as for glucose incubated CPC, it was about 96%. This suggests that that the diminished activity is the resultant of decreased inhibitor concentration in solution due to glycation and aggregation. However, glycation induced functional derangement in part could not be ruled out. This functional inactivation of CPC in the presence of glucose varied as a function of incubation time.

3.2. UV-measurements

The alterations in CPC induced by glucose were also observed with respect to hyperchromicity. The UV spectra of the protein incubated with glucose showed a marked increase in absorbance over the whole spectra after incubation for 21 days as shown in Fig. 2(a). This change in absorbance property of CPC might be attributed to protein unfolding and/or exposure of the chromophoric groups upon incubation with glucose [22]. Furthermore, the strong increase in absorbance of glycated CPC relative to native in the spectral area of 310–400 nm may be due to light scattering or turbidity and thus suggests the presence of aggregates.

Fixed-wavelengths data were recorded at 330, 360 and 400 nm (Fig. S1). It was reported earlier by A. Schmitt and co-workers that the glycation/AGEs lead to absorbance increase at these fixed wavelengths because native protein does not depict any significant absorbance above 300 nm [23]. As depicted in the Fig. S1 glucose-derived CPC-AGEs adducts showed time dependent hike in absorbance at all the three wavelengths when compared to native CPC. Absorbance increase of > 90% was observed at all the three wavelengths on day 21 of incubation of CPC-glucose system. From the absorbance data it can be summarized that absorbance between 300 and 400 nm can be used as benchmark for the onset of AGEs formation of proteins that are derived from the reaction with any of the glycating agent. Furthermore the results also show that of the wavelengths used, the highest sensitivity was obtained at 360 nm especially at lower incubation times.

3.3. Fluorescence measurements

Incubation of CPC with the reducing sugar resulted in the quenching of fluorescence in the protein sugar-system. Fig. 3(a) shows fluorescence spectra after incubating CPC with and without glucose for 4, 8, 12, 16 and 21 days (curve 1–6). The marked decrease in fluorescence intensity upto day 12 could be attributed to the perturbations around the microenvironment of the aromatic residues reflecting the structural changes in the inhibitor during the progression of glycation. Loss of tryptophan fluorescence is ascribed the tryptophan progression to polar solvent or modification of the tryptophan microenvironment leading to the conformational change in CPC and thus suggestive of the formation of some structural intermediates [24, 25]. However, day 16 and 21 incubated sugar-CPC system (curve 5 and 6) showed sharp decrease in fluorescence intensities accompanied with red-shits of 5 nm implying that tertiary structure of the inhibitor molecules was lost and intrinsic fluorophores especially the tryptophan, were exposed to the polar solvent.

Fig. 3(b) shows the glycation specific fluorescence spectra at an excitation wavelength of 325 nm and emission range 350–550 nm for various time intervals. This fluorescence accounts for pentosidine-like and other AGEs produced during the glycation reaction of CPC upon incubation with glucose for 21 days. Fig. 3(c) represents the malondialdehyde-modified protein-like fluorescence or total AGEs fluorescence spectra at excitation of 370 nm and emission recorded in the wavelength range of 390–550 nm. The spectra corresponding to various days of incubation in the presence of glucose showed a marked increase in fluorescence intensity as compared with the control. The formation of AGEs (like pentosidine) was observed from day 4 followed by days 8, 12, 16 and 21 (curve 2–6). With the increase of incubation time, formation of pentosidine-like AGEs and malondialdehyde-modified protein was found to increase. Overall the results showed the marked increase in these of fluorescence types which suggest the formation of various fluorophore AGEs, whose contribution may vary depending upon the extent of glycation of CPC in the presence of glucose which in turn is dependent on the time of incubation. It is noteworthy to mention here that none of the fluorescence measurements showed any significant change for the control CPC over the incubation time points and as such are not therefore presented.

3.4. Circular dichroism (CD) studies

The structural changes at secondary level for glucose incubated CPC were monitored over different time intervals by far-UV CD spectroscopy. Fig. 4 shows the representative CD spectra of CPC both in the absence and presence of glucose for 21 days. It is evident from the Fig. 4, that the CD spectra for CPC (curve 1) shows two minima at 208 and 222 nm, which is a clear signature of the presence of α-helix in the protein under study. There was a progressive decline in negative ellipticity on days 4, 8, 12, 16 and 21 (curve 2, 3, 4, 5 and 6) as compared to native (curve 1). The glycation-induced transition monitored by ellipticity at 208 and 222 nm showed the presence of the residual helical structure even up to day 12 (curve 2, 3, 4), indicating the stability and retention of the protein secondary structure though functionally compromised. This type of change observed in the CD spectra of CPC on day 4, 8, and 12 upon modification by glucose suggests that there may be the formation of several structural intermediates such as partially folding intermediates and molten-globule like states. With further increase in incubation time up to 16 and 21 days (curve 5 and 6), there was a remarkable loss in α-helical structure as indicated by more decrease in ellipticity at 208/222 nm (inset), and the appearance of minima peak at 217 nm which is indicative of formation of β-sheet conformation. The secondary structural content during different points of incubation has been summarized in Supplementary Table ST1. However for the control (non-glycated CPC) the CD spectrum didn't show any significant change in shape at any point of time during incubation suggesting its stability (data not shown). Thus our results show that in addition to the classical steps, Schiff's base generation, Amadori rearrangement and AGE formation, formation of molten globule-like state, partially folded state (conformational transitions), and

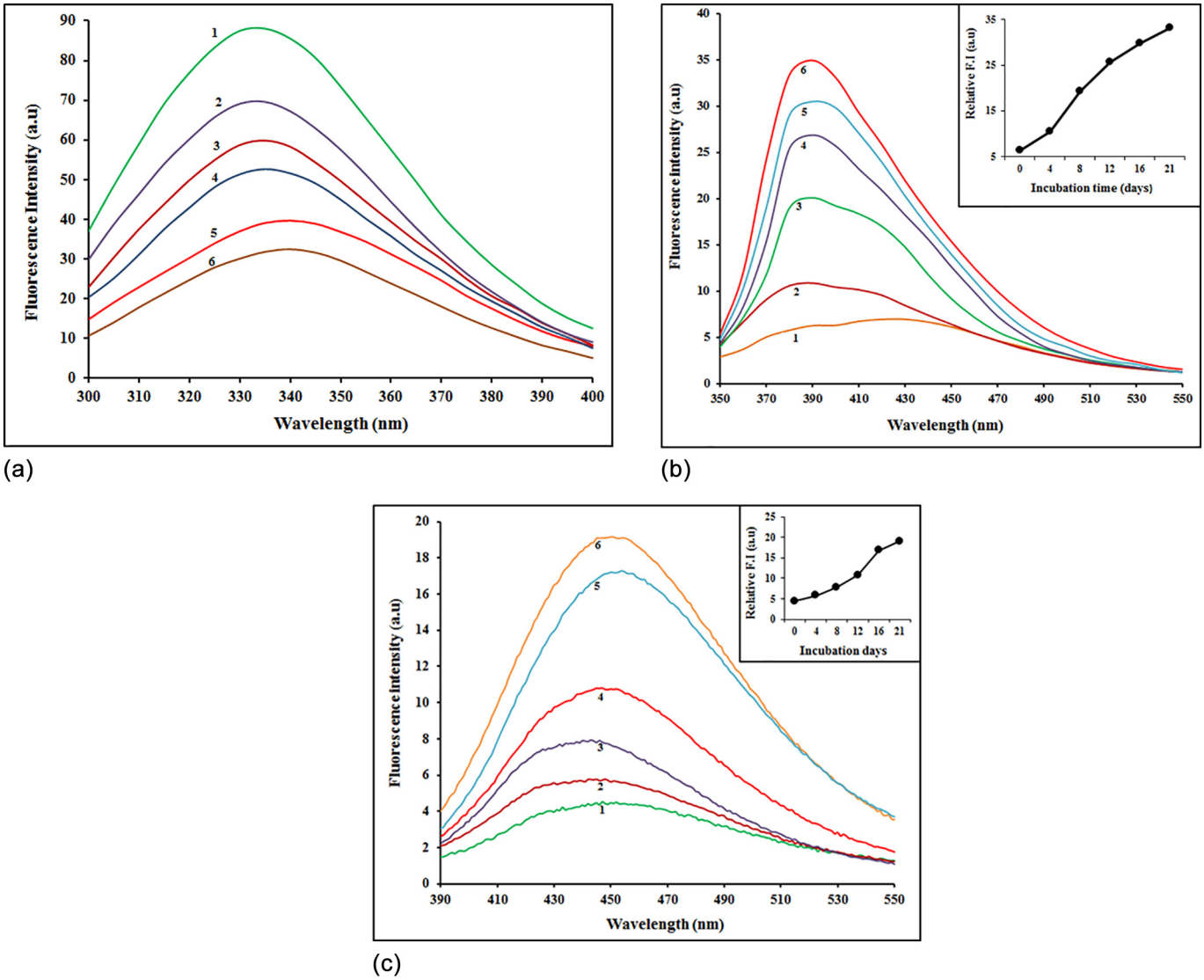

Fig. 3. (a): Intrinsic fluorescence spectra of native CPC and glucose incubated CPC for 4, 8, 12, 16, and 21 days represented by curves 2–6. Control represents CPC incubated without sugar for the same duration. The fluorescence was monitored at an excitation wavelength of 280 nm and spectra recorded from 300 to 400 nm, with a slit width of 5 nm. The CPC concentration was 0.1 mg/ml in a sample volume of 1 ml.
(b): The glycation specific fluorescence corresponding to AGEs like pentosidine (excitation: 325 nm; emission: 350–550 nm) of native and modified CPC incubated with glucose. Peaks achieved around 395 nm on respective days are plotted in the inset showing the relative extent of glycation of CPC time dependently. Experimental conditions remain same as that of Fig. 3(a). All spectra were normalized to the spectrum of the CPC control.
(c): Figure represents malondialdehyde-modified protein-like fluorescence spectra which is another glycation specific fluorescence, developed during the incubation of CPC with glucose for 4–21 days (curves 2–6). Curve 1 corresponds to the control or native CPC. Peaks obtained at around 440–450 nm on respective days are plotted against the time of incubation in the inset, reflecting the relative extent of glycation with time. The experimental conditions remain the same as that of Fig. 3(a). The fluorescence was monitored at an excitation wavelength of 370 nm and spectra recorded from 390 to 550 nm with a slit width of 5 nm.

aggregates also take place during the progression of glycation reactions.

3.5. Fourier transform infra-red spectroscopy (FTIR) analysis

As a complimentary measure to analyse the secondary structure of CPC in the absence and presence of glucose over a period of 21 days, FTIR spectroscopy was also done. CPC (1.5 mg/ml) was incubated with 200 mM glucose in 50 mM phosphate buffer, pH 7.4 containing 0.02% sodium azide, at 37 °C for 4, 8, 12, 16, and 21 days and investigated for conformational transitions at secondary structure level. In the IR spectra of proteins, the secondary structure is most clearly reflected by the amide I and amide II bands, particularly the former [26–28], the amide I band which absorbs at around 1657 cm^{-1} (mainly C═O stretch), and the amide II band absorbs at 1542 cm^{-1} (C—N stretching coupled with N—H bending modes). It has also been reported that, for a native protein the amide I component which corresponds to the α-helical structure is located at 1656 ± 4 cm^{-1}, while as the band components for beta sheet structure should locate between 1622 and 1642 cm^{-1} (lower wave number beta sheet bands) and between 1690 and 1698 cm^{-1} (higher wavenumber beta sheet bands) [26–29]. Fig. 5 shows the original spectra of native as well as glucose incubated CPC over different time periods (in days). As can be seen from the figure native state of CPC shows peak around wave number 1652 cm^{-1} (curve 1) which indicates the presence of abundant α-helical conformation. With the increase in time of incubation of CPC with glucose there was a marked decrease in absorbance on day 4, 8, and 12 (curves 2, 3, and 4) which suggests the decrease in secondary structural content especially α-helices. Thus the states obtained on days 4, 8, and 12 showed retention of α-helical content suggesting the formation and existence of some structural intermediates which could be some partially folding

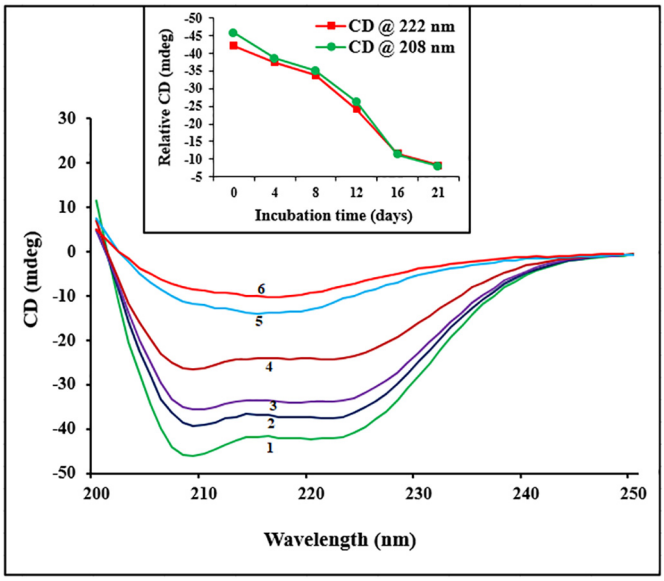

Fig. 4. Far-UV CD spectra of native CPC (curve 1) and glucose glycated CPC for 4 days (curve 2), 8 days (curve 3), 12 days (curve 4), 16 days (curve 5), 21 days (curve 6) showing changes in secondary structure during the progress of glycation reaction. CPC concentration was 3.908 μM and path length was 0.1 cm. Inset of the Fig. shows time dependent relative decrease in α-helical content as indicated by decrease in CD signal at 208 and 222 nm.

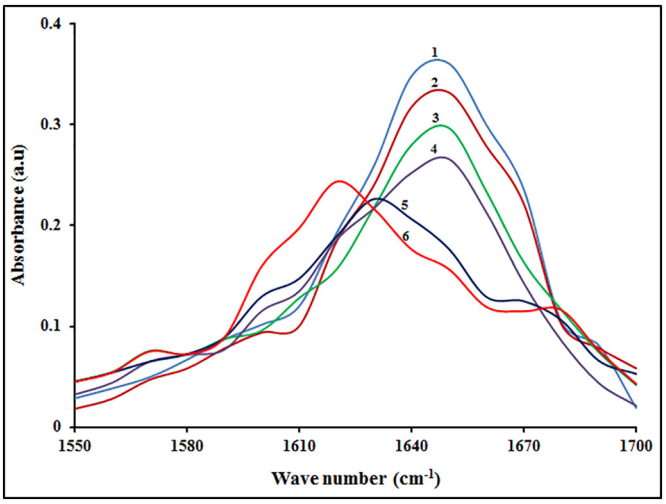

Fig. 5. FTIR spectra of native CPC (curve 1) and glucose glycated CPC for 4 days (curve 2), 8 days (curve 3), 12 days (curve 4), 16 days (curve 5) and 21 days (curve 6) showing changes in secondary structure during the progress of glycation reaction. CPC concentration used was 0.15/ml.

intermediates and molten globule like states which reflect the conformational transitions of CPC during the progression of glycation reaction. Thus, states observed upto day 12 have secondary structure similar to native but having altered tertiary structure to render them functionally inactive. On the other hand, day 16 and day 21 showed decrease of peak at wave number 1652 cm^{-1}, and simultaneous appearance of shoulder peaks at around 1620–1630 cm^{-1} (curve 5, 6) which may be due to alteration from α-helix to β-sheet conformation. The increase in β-sheet content as revealed by the shift of peak to around 1630 cm^{-1} and emergence of a small shoulder peak at around 1680 cm^{-1}, both of which correspond to β-sheet structures of protein. After 21 days of incubation CPC showed a profound effect on the overall shape of the spectrum compared to native. The emergence of two pronounced peaks, one at 1620 cm^{-1} and the other at 1680 cm^{-1} signifies the abundant amyloid aggregate specific β-sheet structures.

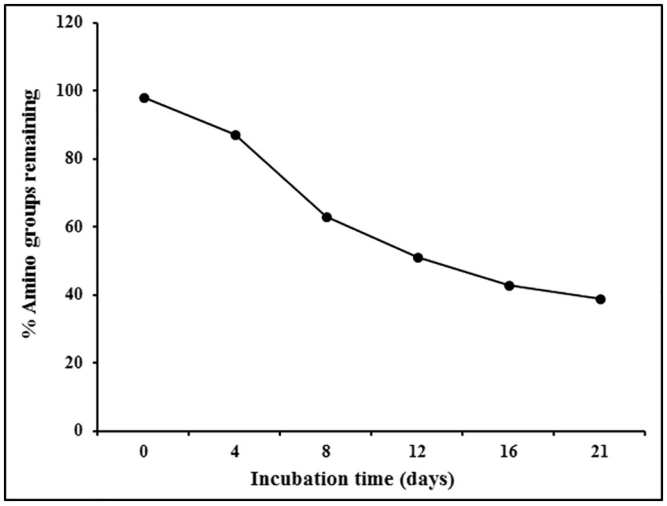

Fig. 6. Free amino group availability in CPC during different incubation periods with glucose. CPC (1.5 mg/ml) was incubated with Glucose (200 mM) in 50 mM phosphate buffer, pH 7.5 for three weeks under sterile conditions at 37 °C. Aliquots from each sample were removed during respective days and analysed for free amino group content. The results are presented as percent decrease in free amino group content with respect to native CPC or control.

3.6. Determination of free amino groups in glycated CPC

Glycation involves the reaction of free amino groups such as ε-NH_2 groups of lysine in proteins with the carbonyl groups of reducing sugars leading to the loss of free amino groups. CPC incubated in the absence and presence of glucose was examined for its free amino group content over a time period of 21 days by TNBS method. The native CPC showed highest reading suggesting that the exposed amino groups may be playing an important role in determining the susceptibility to glycation by sugars. As the incubation time was increased the content of free amino groups in glycated CPC significantly decreased from day 4 up to day 21 (Fig. 6) reflecting the remarkable lysine side chain modifications as well as the extent of glycation on the respective days. Nagy et al. [30] have also reported decrease in free amino groups upon glycation.

3.7. Amadori products/fructosamines determination

Amadori products or fructosamines are stable ketoamines formed during the intermediate stage of a glycation reaction. The formation of these intermediate products in the present case determining the degree of glycation of CPC has been detected by using a colorimetric test using NBT. The test relies on the ability of Amadori compounds to reduce nitroblue tetrazolium (NBT) to the tetrazinolyl radical NBT which disproportionates to yield a highly coloured formazan dye with absorption maximum at 530 nm. Fig. 7 shows the formation of formazan dye in glycated CPC in the presence of 200 mM glucose during different days of incubation depicting the generation of Amadori products indicating the extent of glycation. Results showed that the extent of Amadori product increased gradually during the incubation period of CPC-glucose system.

3.8. ANS (8-Anilino-1-naphthalene-sulphonic acid) fluorescence measurements

8-anilinonaphthalene-1-sulfonate (ANS) is a small organic compound which is fluorescent only when associated with hydrophobic materials and as such can be used to probe for the accessibility of hydrophobic domains in proteins [31]. It is also widely used for the

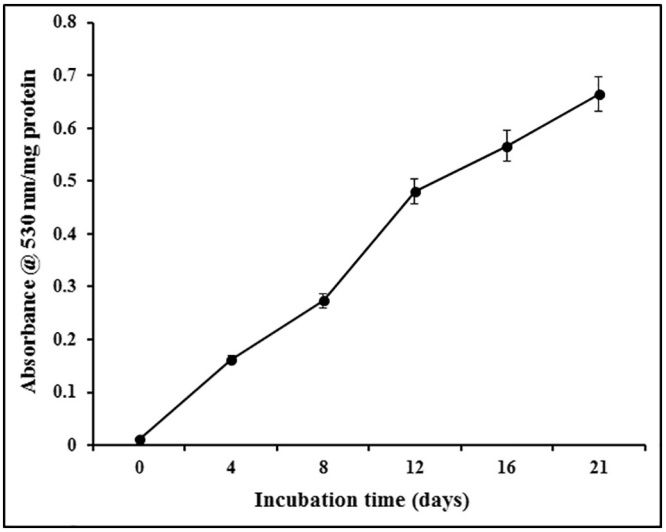

Fig. 7. Amount of Amadori products formed per milligram of CPC incubated with 200 mM glucose in 50 mM sodium-phosphate buffer, pH 7.4 at 37 °C, during different days of incubation. Each point represents absorbance ± SEM, of three independent assays.

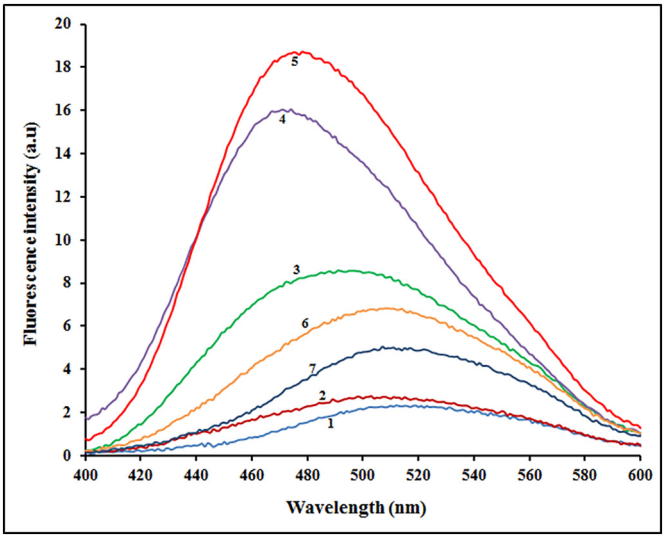

Fig. 8. ANS fluorescence spectra of native CPC (curve 2) CPC incubated with glucose for days 4, 8, 12, 16, and 21 (curves 3–7). Curve 1 represents the spectrum of ANS only. The CPC concentration was 2.3 μM and the path length was 1 cm. The fluorescence was monitored at an excitation wavelength of 380 nm and emission recorded from 400 to 600 nm, with a slit width of 5 nm.

characterization of molten globule and other folding intermediate states of proteins [16]. As depicted in the Fig. 8, there was a continuous increase in fluorescence intensity of CPC until day 12 of its incubation with glucose (curves 3, 4, and 5). With further increase of incubation time there was a slight decrease in ANS binding on day 16 (curve 6) and day 21 (curve 7) as compared to day 12 (curve 5) but was still higher when compared to native CPC (curve 2) which may be due to the cross-linking of CPC molecules leading to burial of some portions of hydrophobic patches and consequently reducing the intensity. Moreover on day 12, the maximum ANS binding was observed suggesting the formation of partially unfolded state or pre-molten globule state. This may be due to the fact that glycation exposes hydrophobic patches of the protein to the surface that otherwise remain buried making them available for ANS binding. This may be attributed to unfolding and other structural alterations of CPC due to glycation giving rise to some

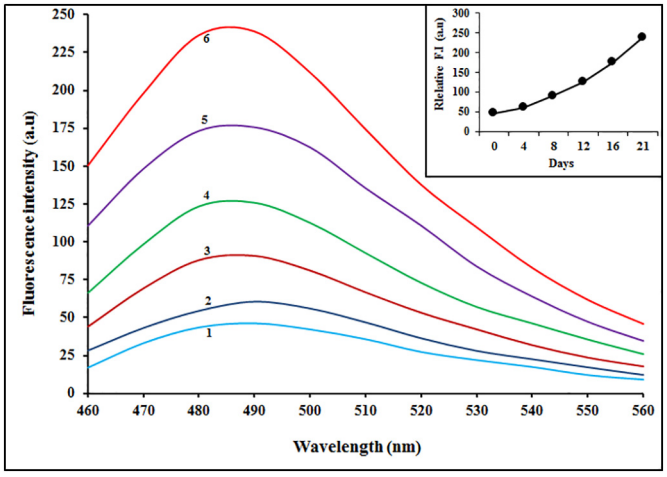

Fig. 9. ThT fluorescence of native CPC (curve 1), and CPC-glucose system incubated for 4, 8, 12, 16, and 21 days (curves 2–6). The CPC concentration was 2.3 μM while ThT concentration was 12 μM and the path length was 1 cm. The fluorescence was monitored at an excitation wavelength of 450 nm with slit width of 5 nm. Inset shows the relative ThT fluorescence of glucose incubated CPC samples.

structural intermediates like those of partially folding intermediate, molten globule like state or pre-molten globule state during the course of glycation reaction which further proceed to undergo cross-linking and form aggregated structures on day 21.

3.9. Thioflavin T (ThT) assay

ThT is an amyloid/aggregate specific dye used widely to probe for the presence of aggregated form of proteins. ThT fluorescence intensity was recorded for CPC samples (1.5 mg/ml) incubated without and with reducing sugar (200 mM glucose) at an excitation of 450 nm and emission recorded from 460 to 560 nm, as depicted in the Fig. 9. The inset of the figure shows ThT fluorescence signal as a function of time which corresponds to the extent of formation of CPC aggregates as a function of incubation time (days). Negligible aggregates were detected in native as indicated by very low ThT signal (curve 1). There was an increase in ThT fluorescence with the increase in incubation period of CPC-glucose system relative to native (curve 2–6). There was 3.8-fold hike in ThT signal on day 16 (curve 5) while as 5.2-fold increase on day 21 (curve 6) relative to native. This suggests that partially folded states (day 4–16) of CPC induced by glycation are highly hydrophobic and prone to precipitation which may proceed to form protein aggregates (day 16–21) via cross-linking.

3.10. Congo Red binding assay

Congo red binding assay was used as an additional measure to probe the formation of β-sheet structures, which are a prime feature of protein aggregates. As is quite evident from Fig. 10, the native state of protein (curve 2) shows an absorbance peak at around 500 nm. Day 4 and 8 also show absorption spectra with no significant shift in λ_{max} (data not shown). However, on day 12 there was a considerable increase in absorbance, while as on day 16 (curve 3) increases in absorbance was accompanied by a small shoulder peak corresponding to about 535 nm relative to native CPC. On day 21 (curve 4) there appeared a prominent red shifted shoulder peak of about 40 nm and λ_{max} corresponding to 540 nm, this appearance of a shoulder peak at around 540 nm on day 21 suggested the emergence of a β-sheet rich structure indicating the aggregation of CPC molecules and [32]. The inset shows the relative absorbance at 540 nm of CPC-glucose system incubated for different times as an index of the extent of CPC aggregate formation with time. Thus

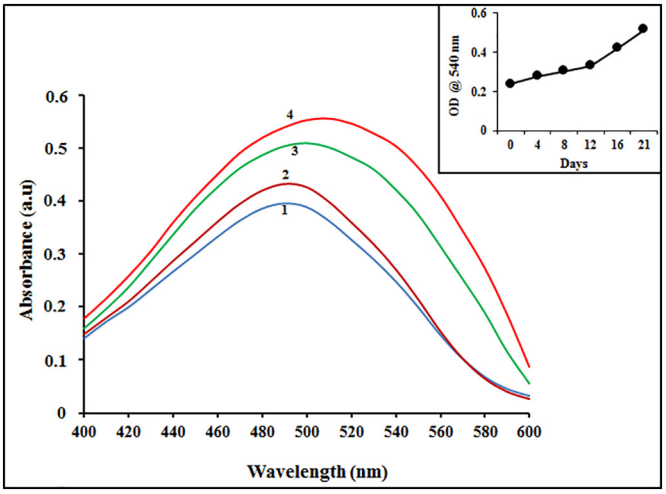

Fig. 10. Congo red assay demonstrating aggregate formation. Absorbance spectra of the Congo red alone (curve 1), native CPC (curve 2), and glucose incubated CPC on day 16 (curve 3), and day 21 (curve 4). Absorbance was measured from 400 to 600 nm. The protein concentration was 0.1 mg/ml. Inset shows the relative absorbance of incubated CPC.

day 16 of incubation of CPC with glucose marks the initiation of CPC aggregation. These results are indicative of the formation a partially unfolded state and other intermediate states on days 4–12 that proceed to form aggregates from day 16–21.

3.11. Turbidity measurements

The turbidity measurements were performed by recording the absorbance of CPC sample solutions at 450 nm as an index of the extent of protein aggregation to further support the above results. As depicted in Fig. 11, the absorbance of CPC increased markedly during the course of glycation reaction especially from 12 day onwards of the incubation of CPC-glucose system which reflects the progression of aggregation. Our turbidity measurements suggested that the prolonged incubation of CPC with glucose results into the formation of larger CPC species (day 16–21) probably due to the cross-linking and coalescing of partially unfolded states (day 4–12).

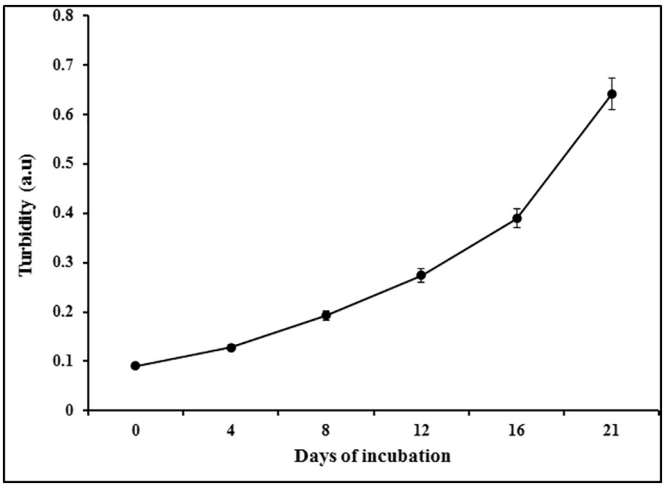

Fig. 11. Absorbance at 450 nm depicting turbidity as a measure of protein aggregation for the glucose (200 mM) incubated CPC over a period of 0–21 days. The protein concentration was kept at 0.3 mg/ml. Each point represents absorbance ± SEM, (n = 3).

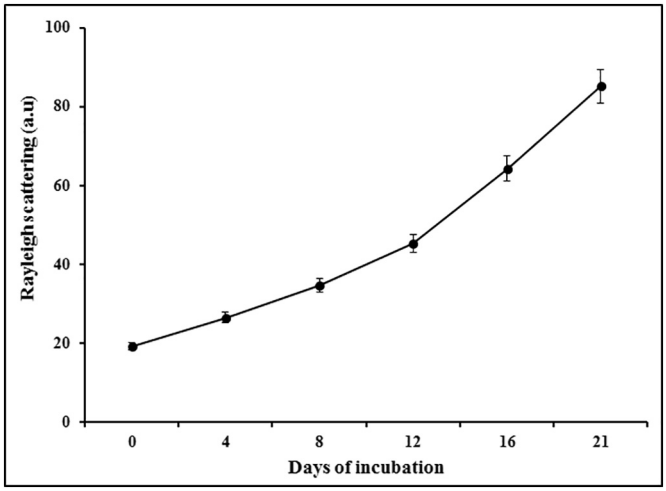

Fig. 12. Rayleigh scattering measurements of CPC incubated with 200 mM glucose over a period of 4–21 days, as an index of protein aggregation. The excitation wavelength was 350 nm and emission spectra were recorded from 300 to 400 nm. Protein concentration was 2.3 μM.

3.12. Rayleigh scattering analysis

Rayleigh scattering proves to be a valid indicator of the formation of protein aggregates. As can be seen in Fig. 12, glucose incubated CPC showed profound increase of about 9-fold increase in fluorescence intensity on day 21 relative to native, indicating the formation of aggregates. From day 12 of incubation with sugar, there was considerable increase in relative scattering which suggests that molten globule-like partially unfolded states of CPC formed on day 12 might be paving way for the aggregate formation as is evident from day 16 to day 21.

3.13. Electron microscopy study

Electron microscopy was performed to add authenticity to our results about the formation of aggregated structures of CPC on day 21 of incubation with glucose. The morphological characteristics of glycation induced aggregated structure of CPC were also examined by using TEM. As depicted in the electron micrographs, bundles of unbranched as well as amorphous aggregates of CPC were observed after 21 days of glycation period (Fig. 13, panel b). CPC incubated without glucose was devoid of any aggregate structure, as shown in the Fig. 13, panel (a).

3.14. Plasmid nicking assay of pBR 322 with glucose glycated CPC

Plasmid nicking assay was performed as an index of genotoxic potential of AGEs, aggregates, and other CPC species generated upon incubation of CPC-glucose system over a period of 21 days. The damaging effect of glucose glycated CPC species on the plasmid DNA was observed as shown in Fig. 14. The fig. shows a difference in the mobility of plasmid treated by CPC incubated with glucose for 4–21 days (lane T1-T5) compared with the negative control (lane C1 and C2). The results show the formation of open circular form of plasmid DNA after being treated with glucose incubated CPC. Lane C3 represents positive control of methyl methane sulfonate (25 mg/ml) treated plasmid which results in conversion of supercoiled plasmid into open circular and/or linear form. The extent of plasmid nicking varied with the CPC species showing highest for the day 21 incubated sample. This shows that AGEs and aggregates of protein are highly destructive to DNA. As the nicking of plasmid has taken place, it can be concluded that glycated products have genotoxic potential.

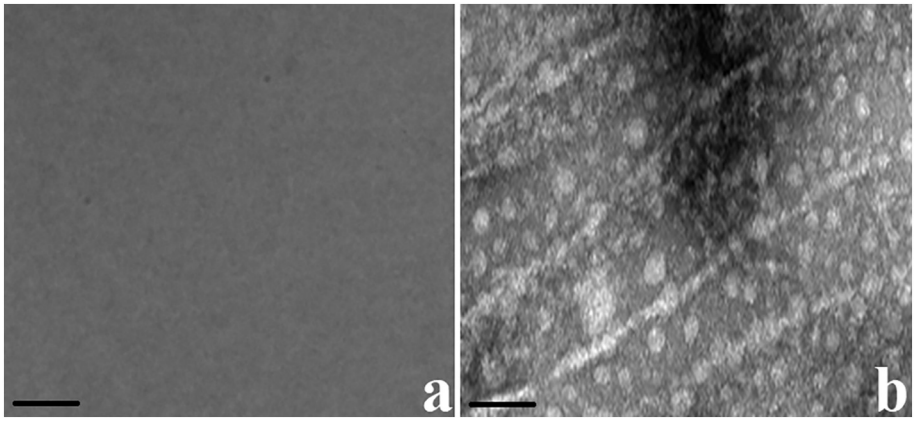

Fig. 13. TEM images of CPC depicting the fibrillar morphology on day 21 (panel b) of incubation with 200 mM glucose showing bundles of fibrillar as well as amorphous aggregates, as compared with the native (panel a) where such structures are absent.

3.15. Cytotoxicity assessment of glycated CPC using comet assay (single cell gel electrophoresis)

After assessment of genotoxicity the different CPC samples (native CPC as well as glucose incubated ones for different time periods) were investigated for their ability to incur DNA damage in human lymphocytes as an index of cytotoxicity by carrying out single cell electrophoresis. The effect of different treatments on lymphocytes is depicted in Fig. 15. In the Fig. S2, NC represents the negative control (native CPC treatment) image of lymphocyte, while as panels i–v show images of lymphocytes treated with CPC-glucose samples incubated for days 4, 8, 12, 16, and 21 respectively. Nuclear DNA damage in lymphocytes by glucose incubated CPC over a period of 21 days clearly demonstrated that glycated CPC species cause a significant damage and have a genotoxic effect on lymphocytes in vitro. In Fig. S2, PC as positive control represents effect of methyl methane sulfonate on nuclei of lymphocytes, showing maximum damage. Moreover these glucose incubated CPC samples caused nuclear DNA breakage ranging from 10 to 18 μm tail lengths in human lymphocytes when compared to around 27 μm-long tails seen in the nuclei of lymphocytes due to the damaging effect of positive control as depicted in Fig. 15. DNA damage in case of lymphocyte treated with glucose incubated CPC samples for different time periods may be attributed to the fact that the cell membrane and nuclear pore complex are permeable to small molecules such as ROS and AGEs which are also products of glycation process [33].

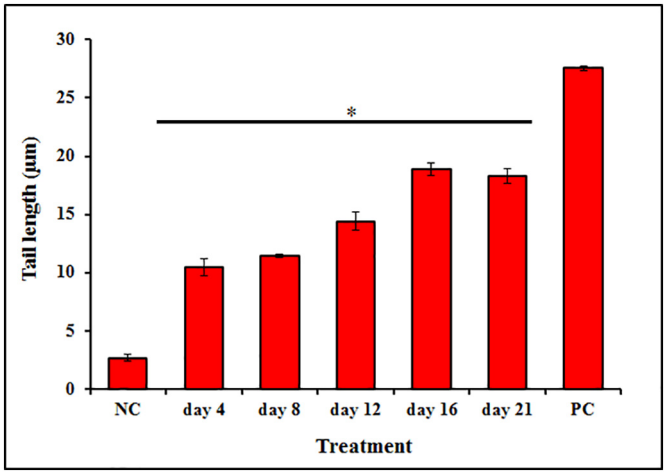

Fig. 15. Nuclear tail length as a measure of the extent of lymphocyte DNA breakage in negative control (NC), treated by day 4- day 21 samples of glucose glycated CPC, treated by 5 μl of methyl methane sulfonate (25 μg/ml) as positive control (PC). Error bars indicate the mean ± SEM ($n = 4$).
*Significantly different from normal control (NC) at $p < .05$ by one way-ANOVA.

4. Discussion

Glycation and AGEs are known to be involved in the pathogenesis of several diseases and therefore effects of AGEs on cells and cell components are the objective of numerous investigations. In the present study we adopted a multi-technique approach to study the sequence of structural changes associated with in vitro glycation of CPC at non-physiological glucose concentration. The nonphysiological glucose level (100–500 mM) is suggested as a model for long-term entrapment of plasma proteins in ageing tissue [34]. Similarly, CPC was used as model protein owing to its evolutionary relation and significance with animal cystatins. Cystatins are a superfamily of evolutionary, structurally and

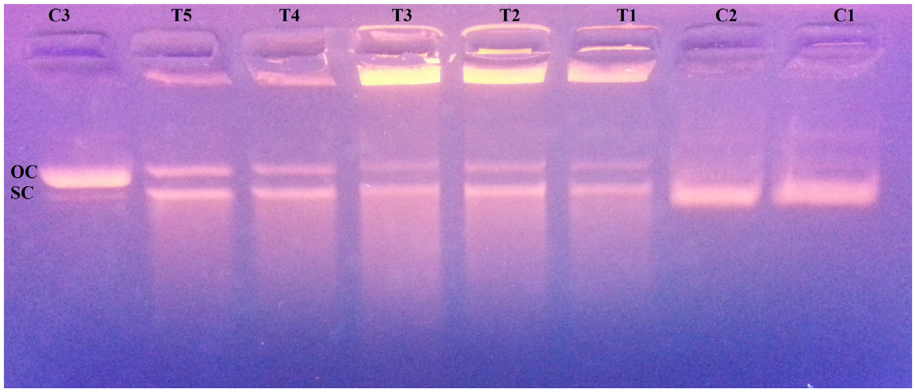

Fig. 14. Plasmid nicking assay to examine genotoxicity of glucose incubated CPC. Plasmid DNA breakage in negative control (C1: plasmid only, C2: plasmid + native CPC), treated by day 4, 8, 12, 16 and 21 sample of glucose incubated CPC (T1, T2, T3, T4, and T5 respectively) and positive control of plasmid treated with 3 μl of methyl methane sulfonate (25 mg/ml) (C3). OC denotes open circular and SC denotes supercoiled form of plasmid DNA.

functionally related cysteine protease inhibitors, and phytocystatins or plant cystatins are the plant homologs of animal cysteine proteinase inhibitors of this superfamily [35]. Sequence alignment of phytocystatins to the animal cystatins especially humans cystatins such as human cystatin F, human stefin A, and human stefin B revealed significant homology as well as identical points of interactions with proteases [35]. However, there are several dissimilarities between CPC and animal cystatins like in case of presence of carbohydrate content, size, hydrodynamic properties, secondary structure (α-helix) content, kinetic parameters etc. [13]. Nevertheless we envisioned that CPC being related to human cystatins could be used as model protein in the present study.

As is depicted in Fig. 1, there is a progressive loss in inhibitory activity of CPC upon increasing incubation period with glucose up to 16 days and beyond which there was not any residual activity left (21 days). Modification of lysine and arginine residues by glucose glycation may bring about alteration at the binding site of the CPC which results in its functional inactivation. Furthermore, glycation with glucose might be involved in the modification of active functional groups of the inhibitor leading to its compromised inhibitory activity. Also, in the later stages of incubation the diminished activity in part could be resultant of decreased inhibitor concentration in solution due to glycation and aggregation. UV–Visible absorption spectra of sugar modified CPC along with native CPC (Control) as depicted in Fig. 2a, shows a strong increase of absorbance over the whole range of spectrum. Increase in absorbance at 280 nm is attributed to protein unfolding and/or exposure of the chromophoric groups [22]. Thus glycation of CPC by incubation with glucose incurred structural changes upon the inhibitor molecules upto the extent of cross-linking and aggregation as indicated by the hyperchromicity over the spectral area from 300 to 400 nm in which native protein hardly shows any absorbance. Fig. S1 shows the fixed wave length data recorded at 330 nm, 360 nm and 400 nm. As can be seen the absorbance of CPC-glucose system increased time dependently at all these wavelengths in comparison to the native. Detection of absorbance at these wave lengths was found to be very useful to make a rough estimation of the total degree of modification with regard to lysine and arginine modifications and thus, is a simple and convincing method to estimate the onset of protein modification during incubation with sugars or sugar degradation products.

Intrinsic fluorescence is an interesting tool to follow AGE formation. The intrinsic fluorophores or aromatic amino acid residues especially the tryptophan, are highly sensitive to the surrounding microenvironment, and thus intrinsic fluorescence is an excellent parameter to monitor the tertiary structural changes in the protein [36]. The dip in fluorescence may be due to the energy transfer from tryptophan to flavin groups or may be due to internalization of exposed residues at different positions [37], thus reflecting the global structural changes in the inhibitor upon incubation with the sugar. Incubation of biomolecules with sugars or sugar degradation products results in the formation of glycophores or advanced glycation products (AGEs). Some of these glycophores like pentosidine, argpyrimidine, etc. are fluorophores and have a characteristic fluorescence emission also referred to as Maillard fluorescence or glycation specific fluorescence which has been used for detection or monitoring the onset of the formation of advanced glycation end products [38]. Increase in such fluorescence intensity with time, upon incubation of CPC with glucose indicated AGEs formation. Prolonged incubation of CPC with glucose led to the formation of higher level of pentosidine-like AGE fluorescence and malondialdehyde-modified protein like fluorescence as shown in Fig. 3.

Secondary structural changes in the glucose modified CPC have been analysed by CD and FTIR studies over the incubation time period of 4–21 days. The CD spectrum of native CPC in the far-UV region showed double minima at 208 and 22 nm, and negative ellipticity at 217 nm, which is typical for a protein containing α-helices and β-sheet, specifying the native conformation of CPC [39]. The CD spectra of CPC-glucose recorded at different time periods show upward shift till day 12 which clearly indicates the considerable changes in the protein secondary structure, and a decrease of the α-helical content in protein (Fig. 4). On day 16 and 21 the loss of CD signals at 208 and 222 nm indicated the conformational transition from α-helix to β-sheet as depicted by the retention of CD signal around 217 nm (Table ST1). The conformational changes in CPC upon incubating it with glucose for different time period were also monitored in the amide I band region of FTIR spectra (Fig. 5). Structural intermediate states of CPC attained on day 4–12 of incubation with glucose gave spectral peaks at around 1654 cm^{-1} in FTIR spectra similar to the native, however, with relatively less absorbance confirming that there is some loss in secondary structure. Shift in wavenumber demonstrates that structural transitions taking place in CPC molecules upon incubation with glucose for prolonged time periods. Disappearance of peak at 1656 ± 4 cm^{-1} and appearance only at around 1630 cm^{-1} in state attained on day 16 and 21 of incubation of CPC in the FTIR analysis confirm that there is loss in α-helix and gain in β-sheet conformation. As it is a well-known fact that protein aggregates have extensive amount of β-sheet structure, it can be concluded that CPC states on day 16 and 21 may be aggregated ones. These findings provide the evidence of α-helix to β-sheet conformational transition of protein [40]. Overall the structural analysis of CPC incubated with glucose for 21 days suggests the formation of structural intermediates such as partially unfolded states, molten globule like states or pre-molten globule states from day 4–12, as indicated by the retention of significant secondary structures but loss of tertiary contacts, which upon prolonged incubation lead to the formation of aggregates as proved by the loss of α-helical content and subsequent gain in β-sheet content. Moreover, day 16 of incubation of CPC with glucose may be the initiation point of aggregation.

Loss of free amino groups and formation of amadori products or fructosamines in a protein upon incubation with reducing sugars also indicates the extent of glycation. The side chain free amino groups and N-terminal amino groups of proteins react with sugars via Schiff base to start the glycation process. Our results show that free amino groups of glucose incubated CPC samples decreased as compared to the native CPC (control). Results also show that prolonged incubation of CPC with glucose enhances the extent of glycation as loss in free amino group is more in case of 21 day as compared with others (Fig. 6). Nagy et al. [30] have also reported decrease in free amino groups upon glycation. The gradual increase of the presence of Amadori products in the CPC-glucose system as a function of incubation time signifies again the extent of glycation (Fig. 7).

ANS, a fluorescent hydrophobic probe, has higher affinity for the molten globule (MG) intermediate of protein than for the proteins in the native or fully unfolded state [41]. Moreover it is reported that incubation of α-crystallin with chaotropes exposes more hydrophobic regions of the protein to the surface making them available for ANS [42]. Similarly our results also showed the increased binding of ANS to glycated CPC samples with respect to native as shown in Fig. 8. The highest ANS binding of CPC-glucose system was observed on day 12 followed by day 8, and then day 4 and so these states can be characterised as differently unfolded states or structural intermediate states with variable surface hydrophobicity. With further increase in incubation time, decrease in ANS fluorescence intensity on day 16 to day 21 relative to day 12 may be attributed to the burial of ANS binding sites due to cross-linking and aggregation of glucose glycated CPC molecules. Hence it may be postulated that CPC structural states on day 4, 8, 12 and 16 represent some folding intermediates between native (day 0 or control) and aggregated forms (day 21).

Aggregational analyses of CPC-glucose system were also performed to validate and authenticate the results of CD and FT-IR studies by using the assays such as ThT, Congo red, turbidity, and Rayleigh scattering. All these assays established the formation of aggregates on day 16–21 of incubation of CPC with glucose. Partial denaturation of proteins caused by sugars often results in formation of folding intermediates such as MG state that contain almost the same level of the secondary structure as

the native protein, but decreased number of the tertiary contacts [40, 42], unpaired domains [43], or incorrectly formed disulfide bonds [44]. As a result of which such intermediates are highly hydrophobic and possess propensity to precipitate and/or aggregate. From the results of far-UV CD, intrinsic fluorescence, ANS binding and ThT binding, Congo red absorbance, turbidity, and Rayleigh scattering, we propose that glycation induces some structural intermediates such as partially unfolded intermediate/molten globule like state/pre-molten state of CPC on day 4–12 of incubation with glucose which proceed to form aggregates with further increase in incubation time (day 16–21). The aggregated state of CPC was further confirmed by a sensitive technique of electron microscopy which showed the matured stage of aggregated CPC, thus providing the physical evidence about the formation and presence of the same (Fig. 13).

AGEs produced during the glycation reaction of proteins have a role in the pathophysiology of ageing and diabetic complications [45]. The glycation reaction also produces some reactive species especially reactive dicarbonyls such as glyoxal and methylglyoxal which further complicate the ageing and diabetic processes. Glucose although a key cellular fuel in the body can also cause damage in elevated levels by glycating the proteins resulting in loss of their shapes and ultimately making them insoluble or unstable. For e.g. cataracts is a result of glycation of lens proteins which lowers their solubility leading to loss of transparency in the lens and blurred vision. Another example is the functional inactivation of some anti-oxidant enzymes (SOD, catalase etc.) upon glycation in diabetic patients contributing to the overall complications of diabetes and ageing [46]. Glycated products have also been implicated in DNA damage in specific diabetic cells causing both, base modification, and apurinic/apyrimidinic sites in DNA, as well as strand breaks [47]. Moreover cytotoxic effects of AGEs have also been reported in diabetic patients [48]. Our study shows that glycated CPC and AGEs formed as results of CPC-glucose incubation are genotoxic in nature. Plasmid nicking assay and comet assay were performed to check the toxicity of glycated CPC to DNA or whole cell both of which showed significant results thus establishing the biotoxicity of CPC species formed during prolonged incubation with glucose. Moreover the bigger the area of the halo corresponds to the greater the level of nuclear DNA fragmentation (Fig. S2), thus allowing a comparative and quantitative determination of the nuclear injury corresponding to tail lengths (Fig. 15). The results of nuclear DNA injury revealed in lymphocytic cells by the alkaline-comet assay are consistent with the plasmid nicking assay.

5. Conclusion

The elevated glucose concentration as a result of diabetes and other physiological stresses can trigger glycation reaction which has implications in ageing and diabetic complications. Keeping in view the multiple routes to the onset of these glycation related complications, the present study investigated the effect of glucose on protein in terms of glycation and aggregation, taking CPC-glucose as a model system. From our studies, it can be concluded that glucose glycated CPC can induce the formation of several structural intermediates from day 4–12 of incubation as revealed by intrinsic fluorescence, far-UV CD, FT-IR studies and ANS fluorescence. Upon prolonged incubation i.e. beyond 12 days (day 16 and 21) glucose was observed to induce aggregation of CPC as evidenced by far-UV CD, FTIR, ANS, Congo red, turbidity, Rayleigh scattering and Th T fluorescence, and electron microscopy. Further studies examined the glucose incubated CPC products formed over different incubation periods for their genotoxic effects all of which showed significant damage and toxicity. This works thus adds to the spectrum of knowledge that reducing sugars cause glycation of proteins leading to formation of AGEs, folding intermediates, and aggregates, which can have cytotoxic potential, and thus complicating further the diabetic conditions.

Acknowledgements

The authors extend their appreciation to the Deanship of Scientific Research at KSU for funding this work through research group project number RGP-215.

Conflict of interest

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

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbapap.2018.06.006.

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