The structural alteration and aggregation propensity of glycated lens crystallins in the presence of calcium; importance of lens calcium homeostasis in development of diabetic cataracts.

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Abstract

The imbalance of the calcium homeostasis in the lenticular tissues of diabetic patients is an important risk factor for development of cataract diseases. In the current study, the impact of elevated levels of calcium ions were investigated on structure and aggregation propensity of glycated lens crystallins using gel electrophoresis and spectroscopic assessments. The glycated proteins indicated significant resistance against calcium-induced structural insults and aggregation. While, glycated crystallins revealed an increased conformational stability; a slight instability was observed for these proteins upon interaction with calcium ions. Also, in the presence of calcium, the proteolytic pattern of native crystallins was altered and that of glycated protein counterparts remained almost unchanged. According to results of this study it is suggested that the structural alteration of lens crystallins upon glycation may significantly reduce their calcium buffering capacity in eye lenses. Therefore, under chronic hyperglycemia accumulation of this cataractogenic metal ion in the lenticular tissues may subsequently culminate in activation of different pathogenic pathways, leading to development of lens opacity and cataract diseases.

Keywords: Calcium, Lens crystallins, Aggregation, Cataract, Diabetes.

1. Introduction

Crystallin proteins are made up of monomeric subunits with the molecular masses between 20-28 kDa [1]. These β -sheet rich and highly stable proteins are accounted for more than ninety percent of the total soluble lens proteins [2]. In solution, while α - and β -crystallins are respectively found as large oligomers (800 kDa) and dimers to octamers (50-250 kDa); γ -crystallins are existed in the monomeric forms [3]. As a molecular chaperone, α -crystallin performs a key role in keeping other proteins in their native states and thereby preventing their aggregation in the lenticular tissues [1, 4]. Also, $\beta\gamma$ -crystallins are thought to play mainly structural function and indicate significant calcium binding capacity [5]. The short range spatial order and close packing at high concentration of these intrinsically stable proteins play a significant role in the achievement of lens transparency [6]. Additionally, these β -sheet rich proteins have dissimilar distribution in cortex and nucleus of eye lens [7]. Moreover, α -crystallins are predominantly found in lens cortex and γ -crystallins mostly identified in the lens nucleus [1].

Due to their remarkable longevity, human lens crystallins undergo a number of age-related post-translational modifications including non-enzymatic glycation which results in generation of heterogeneous protein-sugar cross linking adducts, collectively known as advanced glycation end products (AGEs) [8, 9]. These pathological reactions are accelerated in diabetic patients, possessing markedly higher level of blood glucose. Additionally, eye lenses do not need insulin for the glucose influx and the glucose concentration of the lens interior reflects the blood sugar level. Under this condition lens crystallins, as long-lived proteins, are highly prone to the non-enzymatic glycation [10].

The common feature shared by $\beta\gamma$ -crystallins is four calcium binding Greek key motifs organizing in two similar crystallin domains [11]. Therefore, $\beta\gamma$ -crystallins are involved in the maintaining calcium homeostasis in eye lens which appears important in the terms of their abilities to prevent cataract development [12]. While, the physiological range of calcium concentration is necessary for the maintenance of normal lens transparency, its elevated levels are related to numerous processes contributing in alterations of lens molecular structure culminating in light-scattering [13]. For instance, calcium alters the structural stability of α -crystallin, resulting in impaired chaperone activity and thereby a lower protective ability towards aggregation of other lens proteins [14]. A significantly increased calcium level has been already reported in eye ball during cataract, aging and chronic hyperglycemia [15]. Additionally, the impaired calcium homeostasis has been indicated in lenses from diabetic rats and human [16, 17]. The imbalance of the calcium homeostasis in eye lens, particularly in diabetic patients, seems to be an important risk factor for cataract development. Therefore, the aim of this study was to investigate the effect of increased level of calcium on structure and aggregation propensity of glycosylated and non-glycosylated lens crystallins in a comparative manner.

2. Materials and methods

2.1. Materials

Sephacryl S-300 HR, o-phthalaldehyde (OPA), fluorescamine, 1-anilino-8-naphthalene sulfonate (ANS), and thioflavin T (ThT) were purchased from Sigma-Aldrich Company. Other reagents were provided by Merck Chemical Company.

2.2. Methods

2.2.1. Preparation of total soluble lens proteins

Preparation of total soluble lens proteins (LPs) was carried out according to our previous publication [18]. Briefly, the bovine eye lenses were dissected from the eyeballs obtained from a local slaughterhouse. A 10% homogenate of eye lenses was prepared in 25 mM Tris buffer pH 8.0, containing 100 mM NaCl, 0.5 mM EDTA, 0.01 % NaN₃, and 10 mM β -mercaptoethanol (β -ME). After centrifugation of the cell lysate with 10000 g for 30 min at 4°C, the supernatant was collected as total soluble LPs. The protein concentration was measured by the Bradford assay [19]. Also, the protein samples were stored at -20°C until use.

2.2.2. Non-enzymatic glycation of LPs

The non-enzymatic glycation of LPs was carried out according to previous report [20]. These proteins (10 mg/ml) were incubated with D-glucose (500 mM) in 200 mM sodium phosphate buffer, pH 7.4, containing 50 μ g of penicillin/streptomycin mixture and 0.01% sodium azide in Buffer A for 1 month. The experiment was done at 37°C in the dark and under sterile condition. Also, protein samples were incubated in the absence of D-glucose under similar condition to serve as controls. At the end of incubation, glycated lens proteins (gLPs) were dialyzed against 20 mM sodium phosphate buffer pH 7.4, to remove unreacted sugars using an appropriate dialysis tube (cutoff =10 kDa).

2.2.3. Evaluation of lens protein glycation

2.2.3.1. Gel electrophoresis

The formation of high molecular weight aggregates due to glycation and its subsequent protein cross-linking was monitored by SDS-PAGE under non-reducing and reducing conditions, on a 12% polyacrylamide gel. Lens proteins (15 µg in each well) were loaded on the gel and the protein bands were visualized using an appropriate Coomassie Brilliant Blue (CBB) staining protocol [21].

2.2.3.2. OPA assay

The OPA assay was applied to determine the amount of free amino groups in lens proteins after non-enzymatic glycation [22]. The OPA reagent was prepared by combining 25 ml sodium borate (100 mM), 2.5 ml SDS (20% solution), 100 µl β-ME and 40 mg of OPA powder, which had previously been dissolved in 1 ml methanol. The final volume was adjusted to 50 ml with double distilled water. To assay lysine availability, a 50 µl aliquot of lens proteins (2 mg/ml) was added to 1 ml of OPA reagent in a 1 ml cuvette. This solution was incubated for 2 min at room temperature and the absorbance was read at 340 nm against a blank containing OPA reagent [23].

2.2.3.3. Fluorescamine assay

The non-enzymatic glycation of lens proteins was confirmed using fluorescamine assay [23]. A stock solution of fluorescamine (1mM) was prepared in acetonitrile. The incubated

protein samples (25 μ l containing 1mg/ml of protein) were mixed with 500 μ l of sodium phosphate buffer (50 mM). At the end, 250 μ l fluorescamine (stock solution) was added to a reaction mixture which incubated for 10 min in the dark. The fluorescence emission spectra of protein samples were recorded between 400-600 nm and the excitation wavelength was fixed at 390 nm.

2.2.4. Aggregation study

The lens proteins (1 mg/ml) were incubated in 25 mM Tris buffer, pH 8.0 (Buffer B) under sterile conditions in the absence and presence of calcium chloride (0-20 mM) at 60°C for 50 min (short-term incubation) and at 37°C for one week (long-term incubation). The change in absorbance profile and aggregation of the protein samples were recorded on a T90⁺ UV-Vis spectrophotometer instrument (PG Instrument Ltd., UK) equipped with Peltier Temperature Controller (Model PCT-2). Moreover, the kinetic study of aggregation for both glycosylated and non-glycosylated lens proteins was performed by recording their optical density changes at 360 nm for 30 min at 60°C [24]. Prior to the incubation, the samples were centrifuged at 10000 g for 30 min to remove protein aggregates. To assess the aggregation propensity in a kinetic fashion, lens proteins were incubated individually with calcium for 30 min and their optical density was measured at 360 nm as a function of time. All the experiments were carried out in Buffer B.

2.2.5. Fluorescence analysis

Both control sample and glycated lens proteins (2 mg/ml) were incubated in the presence of calcium chloride at 37°C, for one week. Then, the fluorescence spectra were collected in Buffer B with a Cary-Eclipse spectrofluorimeter (model Cary-100, Australia). The Trp fluorescence of lens proteins (0.15 mg/ml) was recorded between 300-500 nm at excitation wavelength of 295 nm [24]. The fluorescence emission of protein samples (0.15 mg/ml) which incubated with 100 µM ANS in the dark condition, at room temperature, was recorded between 400-600 nm. Also an excitation wavelength at 365 nm and the slit band widths of 10 nm were chosen in both channels [25]. The lens proteins (2 mg/ml) which incubated with different concentrations of calcium for one week at 37°C were used for ThT fluorescence analysis. The protein samples were diluted to 0.15 mg/ml and incubated with 20 µM of freshly prepared ThT for 5 min in the dark, at room temperature. To measure the ThT fluorescence, the protein samples were excited at 440 nm and the emission spectra were recorded between 450-600 nm. Both excitation and emission widths were fixed at 10 nm [26]. All the experiments were performed in Buffer B.

2.2.6. The equilibrium chemical protein denaturation analysis

The stability of glycated and non-glycated lens proteins was measured in the absence and presence of 5 mM calcium ion. The experiments were carried out by equilibrium chemical unfolding in the presence of urea as a denaturant agent. Protein samples (0.1 mg/ml) were incubated with increasing concentrations of urea (0-8 M) at room temperature for 18 hours. Trp fluorescence was recorded between 300 and 500 nm using an excitation wavelength of 295 nm.

The equilibrium unfolding profile was fitted to a three-state model [27, 28]. The ratio of intensities (I_{395}/I_{335}) was plotted against the increasing urea concentrations, where I_{395} and I_{335} are $\lambda_{\max}$ intensities of the completely unfolded and completely folded states, respectively. Denaturation profiles were extracted from the sigmoidal unfolding curve. In order to quantify the stability parameters, all profiles were analyzed with the aid of a global three-state fitting procedure, according to the following equation:

$$F = \frac{F_N + \frac{F_I \exp(-\Delta G_1^0 + m_1[\text{Urea}])}{RT} + \frac{F_U \exp(-\Delta G_2^0 + m_2[\text{Urea}])}{RT}}{1 + \frac{\exp(-\Delta G_1^0 + m_1[\text{Urea}])}{RT} + \frac{\exp(-\Delta G_2^0 + m_2[\text{Urea}])}{RT}} \quad (1)$$

where F_N , F_I and F_U indicate the fluorescence ratios (I_{395}/I_{335}) for folded, intermediate and unfolded states of protein, respectively. ΔG_1^0 indicates the standard free energy changes between native and intermediate states, while ΔG_2^0 shows the standard free energy changes between intermediate and unfolded states. ΔG^0 (the sum of ΔG_1^0 and ΔG_2^0) shows the free energy changes between folded and unfolded states (free energy changes of unfolding) in the absence of urea.

2.2.7. Dynamic light scattering (DLS) experiment

Native and glycated lens proteins (2 mg/ml) were incubated with and without 5 mM calcium chloride at 37°C for one week. Then, DLS experiments were carried out in a Nanotracer Wave (Microtrac) dynamic light scattering instrument at 25°C using a laser wavelength of 780 nm and a scattering angle of 90°. DLS data were analyzed by Microtrac FLEX operating software [29].

2.2.8. Circular dichroism (CD) spectroscopy

Both native and glycated lens proteins (2 mg/ml) were incubated in the presence and absence of 5 mM calcium chloride at 37°C, for one week. Then different protein samples were dialyzed against buffer B and Far UV-CD spectra of control and calcium-incubated proteins were recorded in buffer B at 25°C with a JASCO 810 spectropolarimeter instrument. Protein concentration was fixed at 0.2 mg/ml and path length was 0.1 cm. All measured data were expressed as the millidegree unit [30].

2.2.9. Enzymatic digestion analysis with α -chymotrypsin

The proteolysis of lens proteins (LPs and gLPs) was examined in the presence of 5 mM calcium according to our previous study with a little modification [31]. Briefly, the protein sample (1 mg/ml) was treated with α -chymotrypsin at a ratio of 50:1 (w/w) in Tris buffer (25 mM, pH 8) at 37°C for 3- and 15 hours. The enzymatic reactions were stopped after boiling of the reaction mixture at 100°C for 10 min. The protein hydrolysates (12.5 μ g in each well) were analyzed by SDS-PAGE under reducing conditions on a 12% polyacrylamide gel which followed by CBB staining protocol [32].

2.2.10. Estimation of calcium-binding capacity of lens proteins

Inductively coupled plasma atomic emission spectrometry (ICP-AES, Varian, VISTA-MPX) was used to quantify the calcium content of LPs and gLPs. The lens proteins were incubated with 5 mM EDTA, in buffer B, to prepare the calcium depleted protein samples. Then, the protein samples were extensively dialyzed against buffer B to remove EDTA. After that, 5 ml

of each protein sample (2 mg/ml) was incubated with 300 μ M calcium chloride for 4 hours. Then, the protein samples were individually dialyzed against 50 ml buffer B for 24 hours. At the end, the concentration of free calcium ions in dialysates was measured using ICP-AES analysis. The condition of ICP-AES analysis was as follow: radio frequency power, 1.15 kW; plasma gas flow rate (Ar), 15 l/min; nebulizer gas flow rate (Ar), 0.75 l/min and auxiliary gas flow rate (Ar), 1.5 l/min. The concentration of bound calcium was calculated according to following equation [33]:

$$C_b = C_t - C_f \times d \quad (2)$$

where C_b , C_t and C_f are corresponding to bound, total and free Ca^{2+} concentrations, respectively. Also in this equation, d stands for the dilution factor.

2.2.11. Protein assay

The protein concentration was measured using the UV absorption on a T90⁺ UV-Vis spectrophotometer instrument. The concentration of lens proteins was determined with a standard Bradford protocol [19].

3. Results

3.1. Glycation promotes formation of HMW protein crosslinks

Non-enzymatic glycation of lens proteins was determined by gel mobility shift analysis, OPA and fluorescamine assays. According to results of SDS-PAGE (**Fig. 1A**), glycation of lens

crystallins results in formation of significant amount of covalently cross-linked proteins with lower electrophoretic motilities. Applying reducing condition, the pattern/intensity of HMW protein bands, corresponding to the cross-linked proteins, remained largely unchanged. This finding suggests the formation of non-disulfide covalent cross-linking upon glycation of these proteins. To quantify the extent of protein glycation, both OPA test and fluorescamine assay were carried out. The reaction with amine group leads to an important increase in the absorbance of OPA at 340 nm and a significant enhancement in the fluorescence emission of fluorescamine.

◀Figure 1 to be inserted here▶

A marked reduction in the OPA absorbance (**Fig. 1B**) and a significant decrease in fluorescamine emission (**Fig. 1C**) were also observed upon glycation of lens proteins. It can be concluded that reaction of glucose with amino groups reduces their availability for the subsequent reaction with either OPA or fluorescamine. Overall, our results suggest the extensive glycation of these proteins after long-term incubation with D-glucose.

3.2. Trp and ANS fluorescence assessments of lens crystallins in the presence of calcium

The Trp fluorescence emission was collected after incubation of lens proteins with calcium chloride at different concentrations (5, 10, and 20 mM). The protein samples were excited at 295 nm, as the emission spectra were collected between 300-500 nm. As a consequence of incubation with calcium, a reduction in the Trp emission of both non-glycated (**Fig. 2A**) and glycated (**Fig. 2B**) LPs was observed. However, for the non-glycated lens proteins, a dose response fluorescence reduction was seen in the presence of increasing concentration of

calcium ions. Our results suggest the movement of Trp residues to a more polar environment upon interaction of these proteins with calcium ions.

◀Figure 2 to be inserted here▶

The obtained results can be further explained with the protein structural alteration and/or changes in the pattern of fine interactions among lens proteins. In order to assess the solvent exposed hydrophobic surfaces in the presence of calcium ions, ANS fluorescence analysis was performed for both glycosylated and non-glycosylated lens crystallins. Upon binding of this fluorescent dye to the low polarity regions in proteins, an increase in the fluorescent intensity of ANS was observed [23]. As seen in **Figs. 2C&2D**, non-enzymatic glycation results in the significant movement of hydrophobic sites of lens proteins to the aqueous solvent. With the increase in the calcium concentration, the ANS emission in both glycosylated and non-glycosylated proteins were increased. Therefore, calcium can augment the surface hydrophobicity of LPs and the changes were more significant in the case of non-glycosylated protein counterparts.

3.3. Far UV-CD analysis of native and glycosylated lens crystallins in the presence of calcium

In the current study, far UV-CD analysis was applied to further investigate the structural properties of LPs and gLPs upon incubation with calcium ions (**Fig. 3**). As seen in **Fig. 3A**, the far UV-CD spectrum of LPs indicates a wavelength minimum centered at 217 nm which is characteristic of β -sheet structure. Upon treatment with Ca^{2+} , the intensity of this minimum peak was significantly reduced suggesting important changes in the secondary structural contents and conversion of β -sheet to the other structures.

◀Figure 3 to be inserted here▶

According to both position and intensity of the wavelength minima in LPs and gLPs, a significant structural alteration is occurred upon glycation of lens proteins. Also, in the presence of calcium ions the CD profile of gLPs indicates no significant changes compared to the control sample. Overall, CD analysis data suggest that native lens proteins display significant structural changes upon treatment with calcium ions while their glycosylated counterparts resist against this structural alteration.

3.4. Evaluation of calcium induced aggregation of lens crystallins upon glycation

The aggregation propensity of glycosylated and non-glycosylated crystallins, incubated for both short and long periods, was investigated in the presence of different concentrations of calcium ions. In the short-term incubation system, the proteins were treated with 5, 10, 20 mM calcium ions at 60°C for 30 min. Also, the optical density at 360 nm, which is an important indication of the protein aggregation, was obtained (insets). As shown in **Figs. 4A&4B**, upon incubation with increasing concentrations of calcium ions, the increment of absorbance in the visible region of glycosylated proteins are significantly lower than that of non-glycosylated protein counterparts.

◀Figure 4 to be inserted here▶

Our results suggest that upon glycation lens crystallins partially resist against calcium-induced aggregation. This assumption was further examined with the incubation of these proteins with calcium ions at 37°C, for one week. At the end of incubation, the protein samples were diluted to 0.5 mg/ml and their optical densities were obtained at 360 nm (**Fig. 4C**). According to results in

Fig. 4C, glycation confers significant resistance to the calcium-induced aggregation of lens crystallins. The calcium-induced aggregation of lens crystallins was also investigated in a kinetic fashion (**Fig. 4D**). The experiments were done in the presence of 5 and 10 mM calcium at 60°C. When optical density at 360 nm was followed as function of time, gLPs indicated significantly lower aggregation propensity than LPs. The slope of the initial phase of aggregation curves can be used to compare the rate of protein aggregation [34]. According to data in **Fig. 4D**, upon glycation, the rate of calcium-induced aggregation of lens proteins was significantly reduced.

The aggregation of lens proteins was further assessed using DLS instrument. The hydrodynamic diameter of different protein samples was measured. As shown in **Fig. 5A**, native LPs indicate the average oligomeric size distribution of 29.05 ± 1.34 nm. Upon incubation with calcium ions for one week, LPs demonstrate two main populations with the average sizes of 45.30 ± 1.70 nm (89%) and 333 ± 2.16 nm (11%) (**Fig. 5B**).

◀Figure 5 to be inserted here▶

The hydrodynamic diameters of gLPs in the absence and presence of calcium were 26.55 ± 0.85 nm (**Fig. 5C**) and 28.33 ± 1.63 nm (**Fig. 5D**), respectively. Therefore, incubation with calcium ions did not show significant effect on the oligomeric size distribution of gLPs. The obtained results from DLS experiment are in good agreement with those of aggregation analyses (**Fig. 4**). Overall, as a result of non-enzymatic glycation, lens proteins scientifically resist against calcium-induced aggregation.

3.5. The ThT fluorescence assessment of LPs and gLPs in the presence of calcium ions

One of the pathological events of cataract is the formation of amyloid fibrils by lens crystallins [26]. The ThT assay was carried out to assess the amyloidogenic nature of glycosylated and non-glycosylated LPs at 50°C in the presence of calcium ions. Binding of ThT to beta-sheet structures of amyloid fibrils results in enhancement of the fluorescent intensity. As shown in **Figs. 6A&B**, the fluorescence emission intensities were enhanced for both LPs and gLPs after incubation under the above mentioned condition.

◀Figure 6 to be inserted here▶

Our results suggested possible formation of protein amyloid fibrils in both samples; however, the extent of fibril formation in non-glycosylated LPs was larger than glycosylated protein counterparts. This finding suggests that upon glycation, lens crystallins likely resist against fibril formation.

3.6. Conformational stability of LPs and gLPs upon treatment with Ca^{2+}

To determine the effect of glycation and treatment with Ca^{2+} (5 mM) on the structural stability of lens proteins, chemical unfolding method was applied. The equilibrium unfolding was performed by monitoring the intrinsic Trp fluorescence intensity of protein samples at various concentrations of urea [27, 28]. The ratio of the fluorescence emission intensities at 395 nm and 335 nm (I_{395}/I_{335}) was plotted against the increasing urea concentrations (**Fig. 6C**) with λ_{395} and λ_{335} being the emission maxima of fluorescence intensity in the entirely unfolded and completely folded states. Also, denaturation parameters were obtained from the sigmoidal

unfolding curves (**Fig. 6C**). In order to quantify the stability parameters, all profiles were analyzed with the aid of a three-state fitting procedure (eq.1) which has been discussed in the materials and methods [27, 28].

► Table 1 to be inserted here ◄

According to equilibrium unfolding data (**Table 1**), the transition midpoint values ($C_{1/2}$) for the control/glycated samples, before and after treatment with Ca^{2+} , were 3.42/4.17 and 3.13/3.80 M, respectively. While glycation increases stability of lens proteins, the melting temperatures of both glycated and non-glycated proteins indicate slight reduction in the presence of calcium. Also, the ΔG° values for the control/glycated samples, before and after treatment with Ca^{2+} were 17.64/19.23 and 17.12/18.48 kJ/mol, respectively. These data suggest that gLPs reveal more conformational stability. Also, upon treatment with 5 mM Ca^{2+} both LPs and gLPs indicated a slight reduction in their conformational stability compared to the untreated protein samples.

3.7. Evaluation of calcium-binding capacity of LPs and gLPs

In order to evaluate the calcium-binding capacities of soluble lens proteins and their glycated protein counterparts, these proteins were subjected to IPC-AES analysis. Among different fractions of lens proteins, β - and γ -crystallins play a prominent role in maintaining the calcium concentration in eye lenses at a homeostatic level [5, 35]. β - and γ -crystallins comprise 38.2% and 12% of total soluble bovine lens proteins with the average molecular weights of 26 and 20 kDa, respectively [36, 37]. Considering both percentages and average molecular masses of these proteins, the concentrations of β - and γ -crystallins, when 2 mg of lens proteins are

dissolved in one milliliter of buffer B, were estimated to be 29.4- and 12 μM , respectively. Since each subunit of β - and γ -crystallin can bind four calcium ions [5], according to our theoretical calculation, 1 mg of LPs can totally trap 82.8 μM of this metal ion. Based on IPC-AES analysis and using equation 2, concentration of the protein-bound calcium was calculated and presented in **Fig. 6D**. As shown, calcium-binding capacity value of LPs (1 mg) was estimated to be 66.8 ± 9.4 μM which is almost comparable with the obtained value from the theoretical calculation. Also, gLPs (1 mg) were capable to trap only 10.6 ± 2.2 μM of this metal ion. Our results suggest that gLPs were markedly failed to sequester calcium ions in comparison to the native protein counterparts. Therefore, non-enzymatic glycation significantly reduces the calcium buffering capacity of these proteins.

3.8. The proteolytic stability of lens proteins upon incubation with calcium ions

Previous study suggested an important association between partial degradation of lens crystallins and development of lens opacity and cataract disorders [38].

◀Fig.7 to be inserted here▶

In order to assess proteolytic stability of control and glycated lens crystallins (1mg/ml), these proteins were incubated individually with α -chymotrypsin at a ratio of 50:1 (w/w) in Tris buffer (25 mM, pH 8.0) for 3 and 15 h at 37°C. At the end of incubation, the digestibility of each protein sample was estimated by SDS-PAGE analysis (**Fig. 7**). As shown in **Fig. 7A** upon incubation with Ca^{2+} , non-glycated protein sample indicates a fully digestible protein band with an approximate mass of 37 kDa in the presence of α -chymotrypsin. However, in the absence of calcium, this protein band was fully resisted against chymotrypsinolysis. The incubation of

glycated lens proteins with Ca^{2+} (**Fig. 7B**) had no significant impact on their degradation by α -chymotrypsin.

4. Discussion

The insulin-independent glucose influx into eye lenses causes the accumulation of relatively large amount of this reducing sugar in the lenticular tissues, accelerating the rate of crystallin glycation during hyperglycemia [39]. Other important factors contributing in glycation of these proteins are their extremely high longevity and concentration (>300 mg/ml) in the cytoplasm of lens fiber cells. In addition, hyperglycemia-associated oxidative stress catalyzes the pathway of AGEs formation [40]. The oxidative stress in lenticular tissues and formation of AGEs by lens proteins may also alter the homeostasis of calcium in the lenticular tissues which is extremely important to the clarity of human lens [17]. Under this condition, the proper structure and function of proteins contributing in calcium transport or intracellular calcium sequestration are critical to keep concentration of this metal ion in a rather narrow physiological range, necessary for the maintaining normal lens transparency. In Eye lens, the inward passive diffusion of calcium occurs through a non-specific cation channel which is countered by the action of plasma, mitochondrial and endoplasmic reticular calcium ATPase pumps and sodium-calcium exchanger (**Scheme 1**) [13].

► **Scheme 1 to be inserted here** ◀

The impaired calcium homeostasis occurs in lenses and many other tissues with diabetes [41, 42]. The altered membrane permeability and inefficient Na-K pump activity in diabetic cataracts result in elevated calcium and sodium levels in eye lenses [41]. In addition, calcium-ATPase pump is sensitive to lipid order, which changes with age and cataract (**Scheme 1**) [42-45]. In diabetes and during ageing, membrane defects lead to significant reduction in calcium ATPase activity and increased calcium inward leak [13]. According to previous report, calcium levels in normal human lenses vary between 0.5 and 3 mM and in some cataractous human lenses, its level increases as much as 13 times of the mean value of its concentration in the healthy human subject [15]. This is one of the highest concentrations of this element in any tissue except bone. Also, in the lens fiber cells, only a very trace fraction of free calcium is seen due to sequestration of this metal ion by proteins and phospholipids which act as calcium buffers, and because of its active transport into intra cellular organelles such as ER and mitochondrion (**Scheme 2**) [43].

► **Scheme 2 to be inserted here** ◄

According to results of the current study, glycation of lens crystallins confers these proteins a significant resistance against structural alteration and aggregation/fibrillation induced by calcium ions (see **Figs. 2-6**). Although these observations are quite the opposite of cataractogenic nature of calcium ions in diabetic lenses, following explanations may clarify the specific role of calcium in development of diabetic cataracts. As mentioned before, β and γ -crystallins indicate important potential reservoirs for calcium sequestration. The extensive modification of these proteins in diabetic lens either by reducing sugars or via associated oxidative stress may diminish their capacity for intra cellular calcium sequestration. This assumption supports with the marked reduction in capability of gLPs for calcium binding (**Fig.**

6D). In addition, chronic hyperglycemia in diabetes could impair the proper membrane influx and efflux of calcium ions (**Scheme 1**), resulting in significant intracellular accumulation of this cataractogenic metal ion. Under this condition, the increased level of free calcium in eye lens is potentially an important causative player activating the following pathological pathways which at end culminate in structural and functional insults of lens crystallins (**Scheme 2**). Calcium elevation in eye lens may result in activation of calpain which subsequently participates in partial hydrolysis of lens crystallins, leading to development of lens opacification [46]. An increase in lens calcium concentration seems to trigger the activity of transglutaminase (TGase) which catalyzes the formation of glutamyl-lysyl protein crosslinks, leading to lens crystallin aggregation and cataract development [47]. Prolonged elevation of calcium results in activation of protein kinase C and subsequent increment of lenticular ROS level which may cause significant biomolecular damages. Additionally, calcium induces aggregation of α -Cry and markedly attenuates its chaperone activity which is essential for the maintenance of lens transparency (**Scheme 2**) [47]. During chronic hyperglycemia in diabetic patients, all of the above mentioned pathological events may contribute in development of lens opacity and cataract diseases. Overall, according to results of the current study we suggest that glycation of lens proteins in diabetic patients may participate in alteration of calcium homeostasis in lenticular tissues, which subsequently plays a significant role in development of diabetic cataract.

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Conflict of interest

The authors certify that there is no conflict of interest relevant to this article to declare.

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Table 1: ΔG^0 and $C_{1/2}$ values of lens proteins obtained from equilibrium Urea unfolding study.

$C_{1/2}$ (M)	ΔG^0 (kJ/mol)	Ca^{2+}	Protein sample
3.42	17.64	-	LPs
3.13	17.12	+	LPs
4.17	19.23	-	gLPs
3.80	18.48	+	gLPs

Figure legends

Figure 1. The assessment of lens proteins glycation

(A) The SDS-polyacrylamide gel electrophoresis of LPs and gLPs. LPs (10 mg/ml) incubated in 100 mM phosphate buffer, pH 7.4, in the absence and presence of 500 mM glucose at 37°C for 1 month. Then LP and gLP proteins subjected to SDS-PAGE analysis. The 15 µg of each protein sample was loaded on to the SDS-PAGE well. Also, electrophoresis was performed on a 12% SDS-PAGE gel. 1 and 2 are corresponding to non-reducing and reducing gel electrophoresis conditions respectively. (B) The control and glycated LPs (2 mg/ml) was incubated with 0.1 M OPA for 2 min and absorption was obtained at 340 nm. The bars indicate $\pm$ SD of three independent assays. (C) Both control and LPs (1mg/ml) were incubated with 1 mM fluorescamine. While, the excitation wavelength was 390 nm and the emission spectra were collected between 400 to 700 nm.

Figure 2. Trp and ANS fluorescence study of different LPs samples

The control (A, C) and glycated LPs (B, D) samples (1 mg/ml) were incubated in the presence of 5, 10 and 20 mM calcium chloride at 37°C for 2 weeks. Intrinsic Trp fluorescence analysis (A, B) of different samples at excitation wavelength of 295 were measured and the emission spectra were collected between 300-500 nm. The excitation wavelength for ANS binding (C, D) was 380 nm, and the emission spectra were obtained between 400-600 nm. Also, the excitation and emission widths were fixed at 10 and 10 nm, respectively. All the experiments

performed in 25 mM Tris buffer, pH 8.0 and the protein samples were diluted to a final concentration of 0.15 mg/ml prior measurements.

Figure 3. Far UV-CD assessment of LPs and gLPs in the presence of calcium ions

Far UV-CD spectra of LPs (**A**) and gLPs (**B**) in the absence and presence of calcium ions were recorded in buffer B. The protein concentration was fixed at 0.2 mg/ml. The measurements were done using a cuvette with 0.1 cm path length.

Figure 4. Effect of calcium on aggregation of control and glycated LPs and kinetic Study

(**A**) The LPs and (**B**) gLPs (2 mg/ml) were incubated with different concentrations of calcium chloride at 37°C for 1 week (long term incubation) and UV-Vis absorption spectra were recorded between 200-700 nm. The experiments performed in 25 mM Tris buffer, pH 8.0. Prior to the incubations, all the samples were centrifuged at 10000 g for 30 min to remove any existed protein aggregate. (**C**) The LPs and gLPs (2 mg/ml) were incubated individually with different concentrations of calcium chloride from 0 to 80 mM, at 60°C for 50 min (short term incubation). Aggregation propensity was evaluated by measuring the absorbance at 360 nm. (**D**) Heat-induced aggregation of LPs and gLPs was kinetically assessed by recording the optical density changes at 360 nm, for 30 min in the presence of 5 and 10 calcium mM. The experiments performed in 25 mM Tris buffer, pH 8.0.

Figure 5. DLS analysis of LPs and gLPs in the presence of calcium ions

The LPs and gLPs (2 mg/ml) were incubated with 5 mM of calcium chloride at 37°C for one week. The oligomeric size distribution of different protein samples, based on their relative

volumes, was estimated using DLS instrument. (A) and (B) indicate the oligomeric size distribution of LPs in the absence and presence of calcium ions, respectively. Also (C) and (D) display the oligomeric size distribution of gLPs without and with calcium ions, respectively.

Figure 6. The ThT fluorescence study of different LPs samples.

The control (A) and glycosylated LPs (B) samples (2 mg/ml) were incubated at 37°C for one week in 25 mM Tris buffer, pH 8.0, in the presence of different concentrations of calcium (0-20 mM). At the end, the protein samples were diluted to a final concentration of 0.15 mg/ml in the same buffer for the incubation with ThT and fluorescence was measured in the presence of 20 μ M ThT. Also, the excitation and emission widths were fixed at 10 and 10 nm, respectively.

(C) Equilibrium urea unfolding profile of different LPs samples was monitored at different Urea concentrations. The intrinsic fluorescence ratio (I_{395}/I_{335}) was plotted against urea concentration and plot was fitted according to a three state model. Protein samples with a concentration of 0.1 mg/ml were prepared in 25 mM Tris buffer pH 8.0 and incubated individually with different concentrations of Urea. (D) LPs and gLPs (2 mg/ml) were incubated with 300 μ M CaCl_2 and then dialyzed against buffer B. The dialysates of LPs and gLPs were subjected to ICP-AES analysis. Calcium-binding capacity of LPs and gLPs was estimated as indicated in the material and methods. The data are average of three independent determinations.

Figure 7. Chymotryptic digestion of different LPs samples

Control (A) and glycosylated (B) LPs incubated with and without Ca^{2+} (5 mM) with a concentration of 1mg/ml were incubated with α -chymotrypsin at ratio of 50:1 (w/w), at 37°C for

different times. Time course of proteolysis was set at 3 and 15 h as indicated in the bottom of gels.

Scheme 1. The role of various plasma membrane transporters in the calcium homeostasis of eye lenses.

This scheme also indicates how diabetes mellitus (DM) and ageing can participate in disturbance of calcium homeostasis in eye lenses.

Scheme 2. The suggested mechanisms by which hypercalcemia could participate in development of cataract disorders.

Fig.1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig.6

Fig. 7

Graphical abstract

The structural alteration and aggregation propensity of glycated lens crystallins in the presence of calcium; importance of lens calcium homeostasis in development of diabetic cataracts.

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During chronic hyperglycemia, the reduction in calcium binding capacity of glycated lens crystallins may participate in the pathogenesis of diabetic cataracts.