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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.
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1. Introduction

Crystallin proteins are made up of monomeric subunits with the molecular masses between 20 and 28 kDa [1]. These β-sheet rich and highly stable proteins are accounted for >90% 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, βγ-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 βγ-crystallins is four calcium binding Greek key motifs organizing in two similar crystallin domains [11]. Therefore, βγ-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, ageing 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.

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Therefore, the aim of this study was to investigate the effect of increased level of calcium on structure and aggregation propensity of glycated and non-glycated lens crystallins in a comparative manner.

2. Materials and methods

2.1. Materials

Sephacryl S-300 HR, *o*-phthaldehyde (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% NaN_3, and 10 mM β-mercaptoethanol (β-ME). After centrifugation of the cell lysate with 10,000 *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 μg 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 (1 mM) was prepared in acetonitrile. The incubated protein samples (25 μl containing 1 mg/ml of protein) were mixed with 500 ml of sodium phosphate buffer (50 mM). At the end, 250 μl 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 and 700 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 glycated and non-glycated 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 10,000 *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 and 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 and 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 and 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 h. 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 λ_{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:

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

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. $\Delta G^0{}_1$ indicates

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