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Full Length Research

Effect of the tannoid enriched fraction of *Emblica officinalis* on α-crystallin chaperone activity under hyperglycemic conditions in lens organ culture

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Chaperone-like activity (CLA) of α-crystallin is known to be compromised in diabetic conditions and associated with cataract formation. Protecting α-crystallin CLA may help in delaying and/or preventing cataracts. In this study, we employed a lens organ culture model to study the effect of hyperglycemia on the CLA of α-crystallin and investigated the protective effect the tannoids of *Emblica officinalis* had on the CLA of α-crystallin. Goat lenses were treated with 30 mM glucose with or without an aqueous extract of *E. officinalis* tannoids (25 or 50 µg/ml) for 12 days. Cataract development due to hyperglycemia was monitored and a lens soluble protein profile was analyzed using HPLC. α-crystallin fractions from cultured lenses were isolated by gel filtration; CLA, hydrophobicity and structural confirmation of α-crystallin were assessed using light scattering methods. Culturing the lenses with 30 mM glucose resulted in the development of cortical cataracts and the formation of high molecular weight aggregates. α-crystallin isolated from lens incubated in hyperglycemic conditions displayed a significant decrease in CLA. Co-culturing lenses with glucose and tannoids normalized the altered crystallin profile, preserved α-crystallin CLA and prevented cataract formation. This suggests that tannoids may mitigate hyperglycemia mediated manifestations to α-crystallin thereby preventing cataract formation. Tannoids of *E. officinalis* prevented the loss of α-crystallin CLA and cataract formation in lens organ culture. Thus, lens organ culture can be employed to investigate the pharmacological potential of compounds that modulate α-crystallin CLA and consequently delay or prevent cataractogenesis.

Key words: α-Crystallin, chaperone-like activity, hyperglycemia, lens organ culture, tannoids.

INTRODUCTION

Cataracts, which result from the loss of transparency of normally crystalline eye lens is mainly due to the disruption of the micro architecture of the lens. Human cataract formation is a debilitating eye disease that afflicts millions worldwide. Cataracts account for an estimated 16 million cases of blindness worldwide, with approximately half of all cases originating from Africa and Asia (Brian and Taylor, 2001; Congdon et al., 2003). Besides aging, diabetes mellitus (DM) is a significant etiological factor that contributes to the early onset of cataract formation. Studies indicate that hyperglycemia and the duration of diabetes increase the risk of development of a cataract (Brian and Taylor, 2001; Congdon et al., 2003; Ughade et al., 1998). Considering the magnitude of DM worldwide including India (King et al., 1998; Wild et al., 2004) diabetic- induced cataracts may pose a major problem in the management of blindness. Hence, medical treatment for cataract prevention or for slowing down the progression of cataracts is a highly desired alternative and discovering an effective medical treatment for cataracts is likely to have a global impact on eye health. Crystallins are the major structural proteins in the eye lens accounting for up to 90% of the total soluble protein (Harding, 1991; Horwitz, 2003; Bloemendal et al., 2004). There are three distinct families of crystallins: α-, β- and γ-crystallins, whose structure, stability and short-range interactions are thought to contribute to lens transparency (Harding, 1991; Horwitz, 2003; Bloemendal et al., 2004). However, during aging and in clinical conditions, such as

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diabetes, crystallins undergo extensive modifications due to increased oxidative stress and altered intraocular milieu.

Oxidatively challenged, unfolded crystallins are vulnerable to disulphide cross links, and prone to the formation of high molecular weight (HMW), water-insoluble aggregates. α-crystallin exists as an heterooligomer of approximately 800 kDa with two subunits, αA and αB occurring in a stoichiometry of 3:1 (Horwitz, 2003; Kumar and Reddy, 2009; Srinivas et al., 2008). α-crystallin has been shown to function like a chaperone in suppressing protein aggregation under various conditions (Horwitz, 2003; Kumar and Reddy, 2009), so as to protect other lens proteins from the adverse effects of heat, chemicals and UV irradiation. Hence, α-crystallin is instrumental in maintaining the transparency of the lens with its chaperone-like activity (CLA) (Horwitz, 2003; Kumar and Reddy, 2009). Nevertheless, α-crystallin CLA gets compromised under certain conditions. Decreased α-crystallin CLA and the appearance of α-crystallin subunits in HMW fractions are associated with the formation of cataracts. It is believed that preserving α-crystallin's CLA could be a potential means either to prevent or delay the incidence of cataracts (Kumar and Reddy, 2009). Earlier, we showed that compounds that prevent the loss of α-crystallin CLA could delay diabetic cataract formation in experimental rats (Kumar et al., 2005, 2009). We have also reported that the tannoid-enriched fraction of *Emblica officinalis* inhibits aldose reductase *in vitro* and prevents hyperglycemia-induced lens opacification in organ culture and streptozotocin-induced diabetic cataracts in rats (Suryanarayana et al., 2004, 2007).

In the present study, we have investigated the effect of tannoid principles of *E. officinalis* to prevent hyperglycemia-induced lens opacification and their efficacy to prevent the loss of CLA of α-crystallin in lens organ culture.

MATERIALS AND METHODS

ANS (8-anilinonaphthalene-1-sulphonic acid), TC-199 medium (#M3769), glucose, fructose, penicillin, streptomycin, sodium azide, sodium chloride, sodium bicarbonate, EDTA and Tris were purchased from Sigma Chemical Co (St. Louis, MO). Molecular weight markers were from Bio-Rad (Hercules, CA); Sephacryl S-300HR was from Amersham Biosciences (Uppsala, Sweden). TSK 3000 HPLC column from Tosoh (Tokyo, Japan). Tannoid-enriched extract of *E. officinalis* was obtained in the form of a standardized mixture from Indian Herbs Research and Supply Company (Saharanpur, India; U. S. patent # 6,124,268). The relative proportions of the different tannoids in the standardized extract are as follows: emblicanin A and B, 35 to 55%; punigluconin, 4 to 15%; pedunculagin, 10 to 20%; rutin, 3%; and gallic acid, 1% (Suryanarayana et al., 2004, 2007).

Lens organ culture

Eye balls from six to eight months old goats were obtained from a local abattoir and were dissected within 2 h post mortem. Dissections were performed under a flow hood by an anterior approach. Lenses devoid of any damage were cultured with their anterior surface up. The integrity of the lenses was assessed by measuring the protein content of 20 µl aliquot of the conditioned medium at 2 h after cultivation (Tumminia et al., 1994; Moghaddam et al., 2005). Quantification of the proteins in conditioned medium was performed by the Lowry method. Lens culture medium is composed of TC-199, L-glutamine, sodium bicarbonate, 1% antibiotics (penicillin/streptomycin 100 units/ml). The medium was prepared under sterile conditions adjusting the pH (7.4) and osmolarity (298 ± 2 mOSm/kg). Individual lenses were incubated in 5 ml TC-199 medium at 37°C and 5% CO_2 with 30 mM of glucose. The high glucose concentration was chosen according to published reports (Zigler and Hess, 1985; Suryanarayana et al., 2004). A stock solution of tannoids was added to the medium to produce final concentrations of 25 µg or 50 µg/ml. Lenses incubated in the medium containing 30 mM fructose were treated as osmotic controls (Tumminia et al., 1994; Kamiya and Zigler, 1996). The medium was changed every 48 h and lenses were maintained in culture for 12 days and observed for development of opacification. Visual inspection was conducted by placing lenses on a transparent glass slide with grids.

Photographing of the grid through the lenses was performed with a digital camera (Sony, DSC-P7). After 12 days of culture, lenses were homogenized in a buffer containing 25 mM Tris, 100 mM NaCl, 0.5 mM EDTA, and 0.01% NaN3, pH 8.0 (TNEN buffer). The homogenate was centrifuged at 10,000 xg for 30 min at 4°C and the supernatant was collected as lens soluble protein (LSP) and used for further analysis.

Crystallin distribution profile by HPLC

The α-crystallin distribution in LSP was analyzed by applying 20 µl (1 mg/ml concentration) of LSP on a TSK-3000 SW column (Tosoh, Tokyo, Japan) using HPLC system (Shimadzu, Japan). The column was equilibrated and the proteins were eluted with 0.05 M sodium phosphate buffer (pH 7.2) containing 0.15 M sodium chloride and 0.05% sodium azide (SPSS buffer) with a flow rate of 1 ml/min. The column was calibrated with a set of known proteins whose molecular weights ranged from 669 kDa (thyroglobulin) to 67 kDa (BSA) prior to analyzing the LSP of cultured lenses.

Isolation of α-crystallin

LSP was applied onto a Sephacryl S-300 HR preparative gel-filtration column (100 cm × 1.5 cm) connected to a FPLC system (AKTA-prime, GE biosciences). The column was equilibrated and crystallins were eluted with TNEN buffer. Fractions corresponding to αHMW (high molecular weight) and αLMW (low molecular weight) crystallins were collected separately and their purity was assessed by SDS-PAGE. Equal quantities (15 µg) of α-crystallin preparations were analyzed on self-made 12% polyacrylamide gels using a discontinuous system using a mini slab-gel apparatus. After electrophoresis, the stacking gels were removed and the separating gels were stained with Coomassie Blue solution. αHMW- and αLMW-crystallins will be referred to as αH- and αL-crystallins throughout the manuscript.

Chaperone-like activity of α-crystallin

The CLA of αL-crystallin was assessed with the βL-crystallin aggregation assay (Reddy et al., 2000, 2002). This assay employs the ability of αL-crystallin to suppress the heat-induced aggregation of βL-crystallin (purified from control rat lenses) at 60°C as

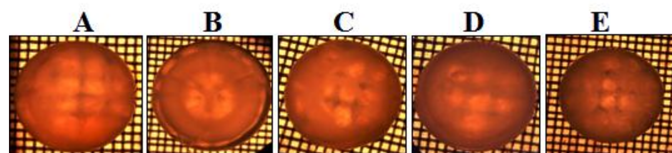

Figure 1. Effect of *E. officinalis* tannoids on lens transparency in organ culture. Representative images from 4 individual experiments are shown. Goat lenses were cultured in modified TC-199 medium in the presence of 30 mM fructose (A), 30 mM glucose (B), 30 mM glucose and 25 μg/ml (C) or 50 μg/ml tannoids (D), and 30 mM fructose +50 μg/ml tannoids (E). The ‘Y’ shaped suture in the centre and a concentric opaque ring in the cortical region of lens is prominent only in panel B.

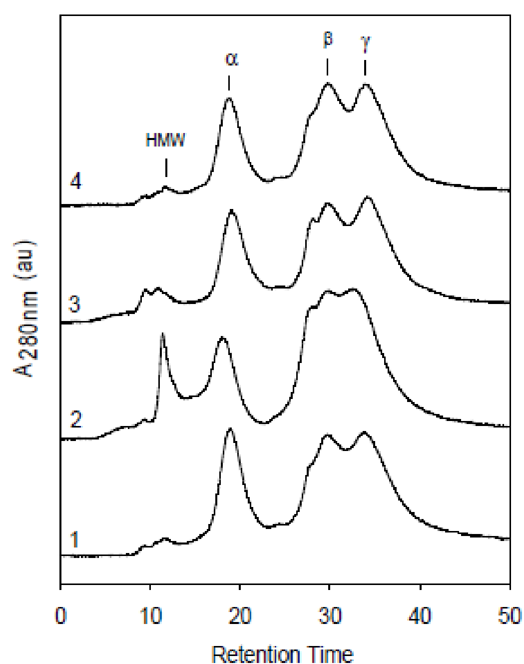

Figure 2. HPLC chromatogram of lens soluble protein. Trace 1 - control lens; Trace 2 - lens incubated with 30 mM glucose; Trace 3 - lens incubated with 30 mM glucose and tannoids 25 μg/ml; Trace 4 - lens incubated with 30 mM glucose and tannoids 50 μg/ml.

monitored by measuring the apparent absorption at 360 nm as a function of time. The relative CLA of αL-crystallin was calculated as a percentage of protection against aggregation using the formula:

$$\% \text{ protection} = ((Ao - A)/Ao) \times 100,$$

Where Ao and A represent the apparent saturation absorption (after 60 min) in the absence and presence of αL-crystallin.

Circular dichroism (CD) studies

CD spectra were recorded at room temperature using a Jasco J-810 spectropolarimeter. All spectra are the average of five accumulations. Far- and near-UV CD spectra were recorded at room temperature using cells of 0.01 and 0.02 cm path length, respectively. All spectra were corrected for their respective blanks. The protein concentration used for far- and near-UV measurements was 0.15 and 1.5 mg/ml, respectively.

Fluorescence measurements

Fluorescence measurements were performed using a Jasco spectrofluorometer (FP-6500; Tokyo, Japan). For all measurements, 0.15 mg/ml protein in 20 mM sodium phosphate buffer, pH 7.2 was used. Intrinsic tryptophan fluorescence was recorded by exciting at 280 nm and following the emission between 310 to 390 nm with slit width of 5 nm for both the excitation and the emission filters. The fluorescence of 8-anilino-1-naphthalene-sulfonic acid (ANS) bound to α-crystallin was measured by exciting samples at 390 nm and following the emission between 450 and 600 nm. For this, α-crystallin was incubated with 50 μM ANS for 30 min at room temperature and the fluorescence of the protein-bound dye was measured. The spectra were corrected with appropriate protein and buffer blanks.

Statistical analysis

The Mann-Whitney and Kruskal-Wallis nonparametric tests were performed to analyze the statistical significance of the difference between the distributions of two or multiple independent samples, respectively, using SPSS software (version 11.5).

RESULTS

Goat lenses could be kept viable in culture conditions up to 2 weeks. In this study lenses were incubated in culture for 12 days. Cultured lenses showed no acute protein leakage after 72 h. This suggests that there is no significant damage to the cultured lenses during the dissection and initial incubation. Lenses incubated in 30 mM glucose became opaque and displayed cortical cataracts, with a ‘Y’ shaped suture in the nucleus of the lens, surrounded by a concentric opaque layer (Figure 1B). Cortical cataracts are one of the predominant lens opacities among diabetic subjects (Leske et al., 1991; Miglior et al., 1994; Rowe et al., 2000; Obrosova et al., 2010). Lenses incubated with high glucose and 25 μg or 50 μg/ml tannoids (Figures 1C and D) showed some slight haziness but no evidence of cataracts compared with untreated lenses (Figure 1B). The morphology of the tannoid-treated lenses was comparable to that of control lenses (Figures 1C, D and A). Incubation of lenses with 30 mM fructose and tannoids 50 μg/ml alone did not cause any change in the morphology (Figure 1E). HPLC analysis of lens soluble protein (LSP) showed that there was an increase in the HMW fraction and a decrease in the α-crystallin fraction in lenses that are maintained in hyperglycemic conditions (Figure 2: Trace 2).

Furthermore, the β and γ fractions of LSP from hyperglycemic lenses were not resolved as distinctly as they were in the control lens (Figure 2: trace 2 vs. 1). Interestingly, the HMW fraction was decreased in lenses incubated with 30 mM glucose and tannoids (Figure 2: Traces 3 and 4) and the effect of tannoids in decreasing

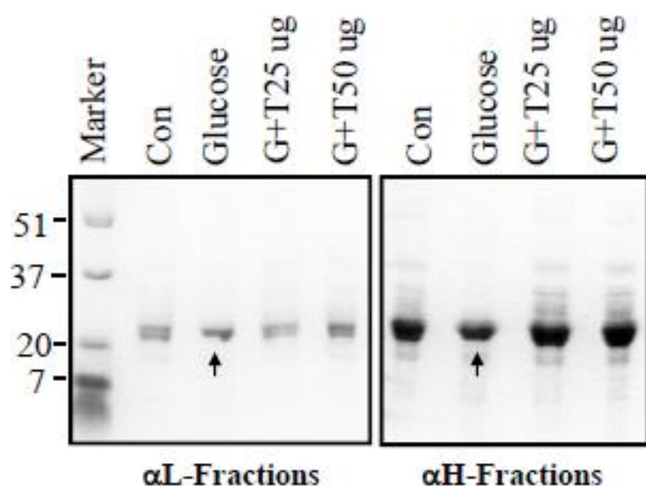

Figure 3. SDS-PAGE profile of αL- and αH-crystallins. LSP from experimental lenses was resolved on a Sephacryl S-300 HR preparative gel-filtration column to obtain αL and αH fractions. The purity of these fractions was analyzed by subjecting them to electrophoresis on 12% SDS-PAGE gels and staining with Coomassie blue (arrow indicates loss of αA subunit).

the HMW fraction was found to be dose dependent. Furthermore, the HPLC profile of the LSP from tannoid-treated samples (50 µg/ml) suggest that the β- and γ-crystallin fractions were resolved separately and the profile was comparable to control lenses (Trace 4 vs. 1).

Concurrently, the increased HMW fraction in lenses grown under hyperglycemic conditions correlated with incidence of cataracts. Furthermore, analysis of the αH- and αL-crystallin fractions on 12% SDS-PAGE revealed that the αA subunit is decreased in the LSP from lenses incubated in hyperglycemic conditions (Figure 3). αL-crystallin isolated from lenses incubated with 30 mM glucose showed a significant loss (approximately 75%) of CLA in suppressing the heat-induced aggregation of βL-crystallin over αL-crystallin isolated from control lens (Figures 4A and B). Interestingly, αL-crystallin isolated from lenses that were exposed to glucose in the presence of tannoids showed improved CLA in a dose dependent manner compared with αL-crystallin from lenses exposed to 30 mM glucose alone (Figure 4). The far-UV CD spectrum for αL-crystallin from control lenses shows negative ellipticity at 217 nm indicating a typical β-sheet structure (Figure 5) (Kumar et al., 2004). The far-UV CD signal for αL-crystallin isolated from lenses incubated with 30 mM glucose showed a loss of negative ellipticity suggesting an altered secondary structure for αL-crystallin isolated from hyperglycemic lenses (Figure 5: Trace 2 vs. 1). However, treatment with tannoids had no modulatory effect on hyperglycemia-mediated secondary structural changes to αL-crystallin. Tryptophan fluorescence of αL-crystallin isolated from lenses incubated with 30 mM glucose was decreased compared with αL-crystallin from control lenses (Figure 6: Trace 2 vs. 1). Loss of tryptophan fluorescence, an indicator of protein tertiary structure changes, suggests a loss of the native conformation of αL-crystallin isolated from hyperglycemic lenses. αL-crystallin isolated from lens incubated with high glucose and tannoids 25 or 50 µg/ml displayed increased tryptophan fluorescence compared with αL-crystallin from lenses treated with glucose alone (Figure 6: Traces 3 and 4 vs. 2). It is generally considered that surface-exposed hydrophobic sites of α-crystallin are involved in the CLA of αL-crystallin (Reddy et al., 2006). Therefore, we measured the surface hydrophobicity in terms of ANS-bound fluorescence.

αL-crystallin from lenses incubated with 30 mM glucose showed less ANS fluorescence than those from control lenses (Figure 7) indicating that there is less accessible surface hydrophobicity in αL-crystallin isolated from lenses incubated in hyperglycemic conditions. However, ANS fluorescence of αL-crystallin isolated from lenses treated with 30 mM glucose and tannoids was higher than that from lenses treated with glucose alone (Figure 7). Decreased accessible surface hydrophobicity of αL-crystallin isolated from lens incubated with hyperglycemic conditions, as observed here, correlates well with its compromised CLA (Figure 4, Trace 3 and Figure 7, Trace 2).

DISCUSSION

Cataracts, which account for nearly half of the blind population, is the most common cause of preventable blindness (Taylor, 1999). Exploring methods to discover anti-cataractogenic agents and pharmacological interventions could help overcome this avoidable blindness. In the present study we adopted a lens organ culture model to study the effect of hyperglycemia on the lens transparency and CLA of α-crystallin. Lens organ culture was used in several studies aimed at understanding the physiology and biochemistry of cataracts (Kamiya and Zigler, 1996; Zigler et al., 1985, 2003). A long-term lens organ culture was employed to monitor lens optical quality (Dovrat and Sivak, 2005) and to determine age-related effects of UV irradiation on the eye lens (Azzam and Dovrat, 2004). Cataract development in this lens culture model shows similarities to experimental cataracts in rodents, during which formation of HMW aggregates was observed (Kumar et al., 2005). Furthermore, incubation of lenses for 12 days with 30 mM glucose resulted in cataract formation that is usually seen in case of diabetic subjects. The LSP fraction of lenses incubated with high glucose has varied significantly from that of control lenses. An increased HMW crystallin fraction, decreased α-crystallin fraction, and altered β- and γ- crystallin profiles were noticed in lenses incubated under high glucose conditions. A similar profile was seen in the LSP from streptozotocin-induced diabetic cataract rat lens (Kumar et al., 2005). It should

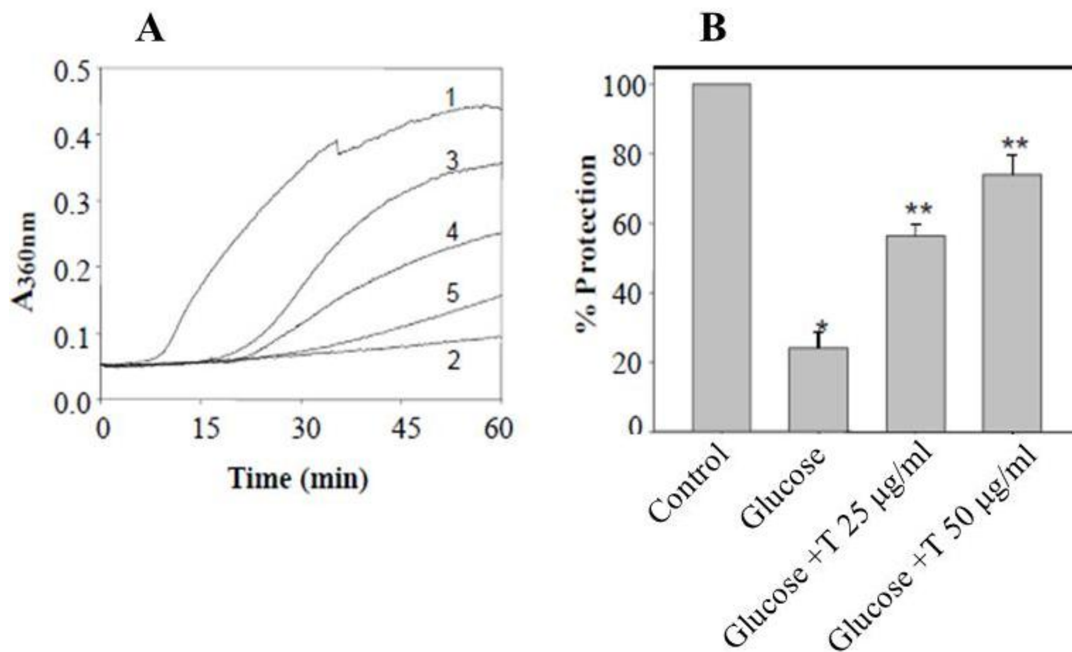

Figure 4. Chaperone-like activity (CLA) of αL-crystallin. A) Representative data of 4 to 5 individual chaperone assays. CLA of αL-crystallin in heat induced aggregation of βL-crystallin (0.2 mg/ml in 50 mM phosphate buffer, pH 7.4) at 60°C. βL-crystallin was incubated in the absence (Trace 1) or presence of 25 µg/ml of αL-crystallin isolated from control lenses (Trace 2), from lenses incubated with 30 mM glucose (Trace 3), from lenses incubated with 30 mM glucose and tannoids 25 µg/ml (Trace 4) or tannoids 50 µg/ml (Trace 5); B) percent protection of heat-induced aggregation of βL-crystallin by αL-crystallin preparations from different treatments. The percent protection by αL-crystallin from control lenses was considered as 100%. Data are expressed as mean ± SE; n = 4. *, p< 0.01, compared with control αL-crystallin, **, p<0.01, compared with α-crystallin from lens incubated with 30 mM glucose.

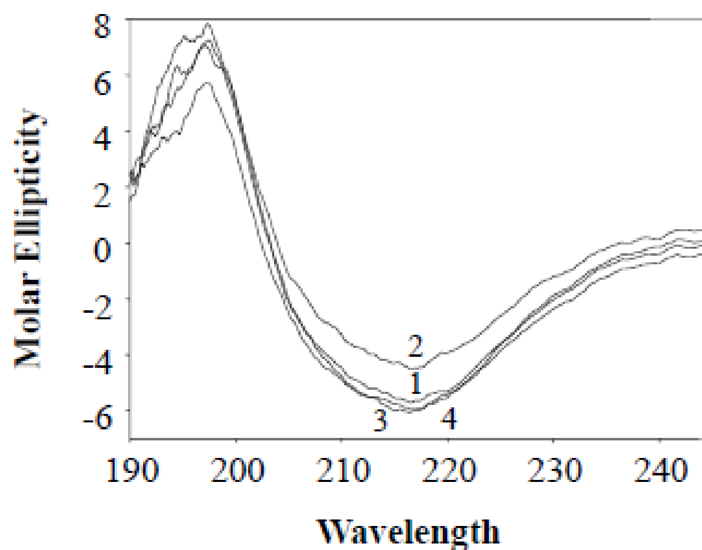

Figure 5. Secondary structure of αL-crystallin. Far-UV CD spectrum of αL-crystallin preparation from a control lens (Trace 1), from a lens incubated with 30 mM glucose (Trace 2), from lenses incubated with 30 mM glucose and tannoids 25 µg/ml (trace 3) or tannoids 50 µg/ml (Trace 4). Data were the average of four assays.

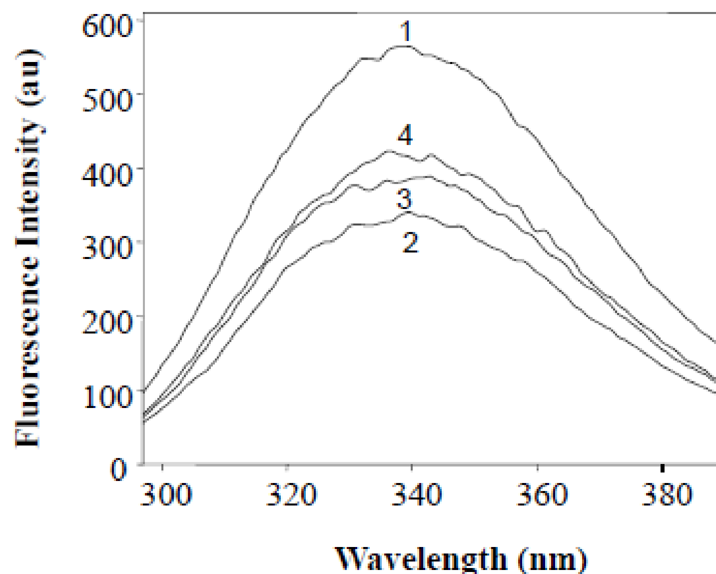

Figure 6. Tertiary structure of αL-crystallin. Tryptophan fluorescence of αL-crystallin from a control lens (Trace 1), from a lens incubated with 30 mM glucose (Trace 2), from-lenses incubated with 30 mM glucose and tannoids 25 µg/ml (Trace 3) or tannoids 50 µg/ml (Trace 4). Data were the average of four assays.

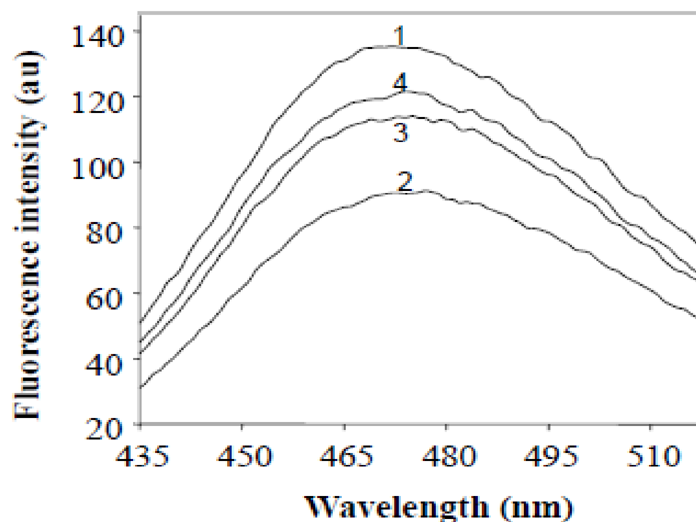

Figure 7. Hydrophobocity of αL-crystallin: ANS bound fluorescence of αL-crystallin from a control lens (Trace 1), from a lens incubated with 30 mM glucose (Trace 2), from-lenses incubated with 30 mM glucose and tannoids 25 µg/ml (Trace 3) or tannoids 50 µg/ml (Trace 4). Data were the average of four assays.

be noted that there is a loss of the αA-subunit in both αH and αL-crystallin preparations from lenses cultured with 30 mM glucose as evident from SDS-polyacrylamide gels. Further, α-crystallin isolated from lenses incubated in hyperglycemic conditions showed decreased CLA. An altered crystallin profile and compromised CLA of α-crystallin from lens incubated with hyperglycemic conditions were comparable to those changes observed with diabetic cataracts in, both, rats and humans (Cherian and Abraham, 1995b; Thampi et al., 2002; Kumar et al., 2005).

A previous study that reported lens opacification due to

lead exposure in an organ culture system also displayed a decrease in α-crystallin CLA (Neal et al., 2010). These data suggest that lens organ culture can be used for screening compounds that modulate α-crystallin CLA in relation to cataractogenesis. Under diabetic conditions, increased activity of aldose reductase leads to the accumulation of sorbitol, an osmolyte, and the resultant oxidative stress is considered as an etiological event that cause diabetic cataracts (Zigler and Hess, 1985; Kador PF 1988; Obrosova et al., 2010). The chaperoning ability of α-crystallin is known to be compromised by oxidative stress (Cherian and Abraham, 1995a; Peluso et al., 2001; Rajan et al., 2006). In addition, non-enzymatic glycation and activation of protein kinase C also contribute to cataractogenesis. Therefore, modulation of α-crystallin's CLA through the inhibition of aldose reductase or prevention of non-enzymatic glycation would provide potential targets to prevent or delay cataract formation (Kumar and Reddy, 2009). Earlier, we reported that the tannoids of *E. officinalis* inhibited aldose reductase in rat eye lenses (Suryanarayana et al., 2004). In addition, feeding tannoids to diabetic rats delayed the incidence of cataracts (Suryanarayana et al., 2007). However, it is unknown whether tannoids modulate the CLA of α-crystallin under hyperglycemic conditions. Interestingly, we demonstrate here that tannoids prevented the formation of HMW aggregates, averted the alteration in αL-crystallin's secondary and tertiary structures, and improved its CLA under hyperglycemic conditions. Therefore, we speculate that the aldose reductase inhibitory potential of tannoids might have minimized the oxidative stress and improved the high glucose-mediated loss of α-crystallin CLA. In summary, the present study suggests that the lens organ culture system can be used to study compounds that modulate α-crystallin CLA during experimental conditions.

From an investigational and potentially therapeutic standpoint, the ability of tannoids to modulate α-crystallin chaperone like activity and prevent cataract formation in a lens organ culture model has immense value. It is our anticipation that modulation of CLA of α-crystallin will provide new insight for tannoids as a novel therapy directed for the treatment of diabetic cataracts.

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