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Glycation by Ascorbic Acid Oxidation Products Leads to the Aggregation of Lens Proteins

**Running title:** Ascorbic acid-mediated lens protein aggregation and precipitation

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**Abstract**

Previous studies from this laboratory have shown that there are striking similarities between the yellow chromophores, fluorophores and modified amino acids released by proteolytic digestion from calf lens proteins ascorbylated in vitro and their counterparts isolated from aged and cataractous lens proteins. The studies reported in this communication were conducted to further investigate whether ascorbic acid-mediated modification of lens proteins could lead to the formation of lens protein aggregates capable of scattering visible light, similar to the high molecular aggregates found in aged human lenses. Ascorbic acid, but not glucose, fructose, ribose or erythrose, caused the aggregation of calf lens proteins to proteins ranging from  $2.2 \times 10^6$  up to  $3.0 \times 10^8$  Da. This compared to proteins ranging from  $1.8 \times 10^6$  up to  $3.6 \times 10^8$  Da for the water-soluble (WS) proteins isolated from aged human lenses. This aggregation was likely due to the glycation of lens crystallins because [ $^{14}\text{C}$ ] ascorbate was incorporated into the aggregate fraction and because  $\text{CNBH}_3$ , which reduces the initial Schiff base, prevented any protein aggregation.

Reactions of ascorbate with purified crystallin fractions showed little or no aggregation of  $\alpha$ -crystallin, significant aggregation of  $\beta_{\text{H}}$ -crystallin, but rapid precipitation of purified  $\beta_{\text{L}}$ - and  $\gamma$ -crystallin. The aggregation of lens proteins can be prevented by the binding of damaged crystallins to alpha-crystallin due to its chaperone activity. Depending upon the ratios between the components of the incubation mixtures,  $\alpha$ -crystallin prevented the precipitation of the purified  $\beta_{\text{L}}$ - and  $\gamma$ -crystallin fractions during ascorbylation. The addition of at least 20% of alpha-crystallin by weight into glycation mixtures with  $\beta_{\text{L}}$ -, or  $\gamma$ -crystallins completely inhibited protein precipitation,

and increased the amount of the high molecular weight aggregates in solution. Static and dynamic light scattering measurements of the supernatants from the ascorbic acid-modified mixtures of  $\alpha$ - and  $\beta_L$ -, or  $\gamma$ -crystallins showed similar molar masses (up to  $10^8$  Da) and hydrodynamic diameter (up to 80 nm). These data support the hypothesis, that if the lens reducing environment is compromised, the ascorbylation of lens crystallins can significantly change the short range interactions between different classes of crystallins leading to protein aggregation, light scattering and eventually to senile cataract formation.

## Introduction

During aging and cataractogenesis, the human lens exhibits increased aggregation of the structural proteins [1, 2], an increasing amount of water-insoluble proteins that form upon homogenization and increased lens protein-associated modifications, protein crosslinking and non-tryptophan fluorescence [3-5]. The aggregation of lens proteins is thought to initially be prevented by the binding of partially denatured crystallins to  $\alpha$ -crystallin due to its intrinsic chaperone ability, however the partially denatured crystallins over time form high molecular weight aggregates (HMW) capable of scattering light. The pioneering work by Benedek [6] and Libondi [7] showed the presence of such aggregates in human lenses during early cataract development. While aggregation of lens proteins may be caused by numerous factors (e.g. deamidation [8, 9], mutagenesis [10, 11], UV-light [12], disulfide formation [11], crosslinking [13, 14], temperature [15]), in most cases the common denominator is a chemical change in the

structure of particular amino acid residue(s) within the protein structure, which in turn causes protein structure instability and unfolding.

On the other hand, HMW protein aggregates from human aged and cataractous lens are profoundly modified according to the reports from this laboratory [16-18] and the other labs [19-22]. This fraction contains the bulk of the modifications, chromophores and fluorophores [16-18, 23-25] covalently attached to amino acid residues. The role of these modifications in the formation of HMW aggregates is not well understood.

The glycation of lenticular proteins has been suggested to be responsible for the formation of a significant fraction of lens protein modifications present in the aged human lenses and in diabetic and brunescant cataractous lenses [16-25]. These types of reactions occur primarily at Lys and Arg residues within the lens proteins by a variety of sugar carbonyls and aldehydes [16-25]. The presence of such adducts can cause lens protein destabilization, unfolding and aggregation *in vivo* as has already been shown in the rat diabetic lenses by Swamy and Abraham [26] and for the lens proteins modified by sugars *in vitro* [27].

Ascorbic acid, one of the major lenticular antioxidants, can be readily oxidized [28-30] if the lens reducing environment is compromised [31, 32]. Under chronic oxidative stress (e.g. diabetes, UV-light, age, etc.) increased formation of dehydroascorbic acid has been reported [33] and the inability of glutathione to completely reduce dehydroascorbic acid back to ascorbic acid brings about the rapid formation of highly reactive carbonyls from dehydroascorbate [34]. These events usually result in rapid glycation of lens proteins and both metal-dependent and metal-independent generation of superoxide anion as has been shown *in vitro* [16, 24, 25, 35-37] resembling

the modifications of lenticular proteins in vivo [38]. Specifically, Fan et al. showed that increased uptake of ascorbic acid into mouse lenses [38] was able to cause increased formation of modified lenticular proteins, exhibiting characteristic browning and fluorescence. These changes resemble closely those seen in crude lens proteins ascorbylated in vitro and those present in the human lens during normal aging and cataract formation. We have recently reported that the majority of the yellow chromophores, fluorophores and modified amino acids released by proteolytic digestion from calf lens proteins ascorbylated in vitro have the same or similar properties as their counterparts isolated from aging and brunescant cataractous lens proteins as has been determined by TLC, HPLC and a recently developed 2-D LC-MS method [16, 23-25, 35]. All these amino acid modifications increased with age in the normal human lens water-insoluble proteins and in the proteins from brunescant cataractous lenses [23-25].

While it has already been established that advanced glycation end products are present in human aging or cataractous lenses and some of them can result from glycation by ascorbic acid degradation products (pentosidine, LM-1, K2P, etc. [17, 20-22, 38]), very little if anything is known about their influence on lens crystallin stability and conformation. Thus, we conducted this project to evaluate the ability of ascorbic acid-derived glycation products covalently bound to the lens proteins to initiate protein aggregation and the formation of HMW aggregates capable of scattering visible light.

## **Materials and Methods**

De-ionized water (18 M $\Omega$  or greater) was used throughout this project. All phosphate buffers employed in this project were treated with Chelex resin (10.0 g/l, 200-

400 mesh (Bio-Rad Laboratories, Richmond, CA) to remove transient metal ion contaminants according the method of Dikalov, Vitek, Maples et al. [39]. D-(-) glucose (anhydrous; > 99.5%; Glc), L-(+) ascorbic acid sodium salt (>99.7%; Asc), D-(-) fructose (> 99.5%; Fru), D-(-) ribose (>99%; Rib), L- erythrulose (97%; Ery), methylglyoxal (40%; MGO), diethylenetriaminepentaacetic acid (>95%) (DTPA), Trizma (base), trichloroacetic acid (TCA) and NaCNBH<sub>3</sub> were purchased either from Sigma (St. Louis, MO), Fluka or Aldrich (Milwaukee, WI). L-[U-<sup>14</sup>C] ascorbic acid was synthesized from D-[U-<sup>14</sup>C] glucose by the method of Crawford and Crawford [40] as described previously [41]. Tricarboxyethyl phosphine (TCEP) was obtained from Molecular Probes (Eugene, OR). Superose 6 support and protein high molecular weight gel filtration calibration kit (Aldolase 158 kDa, Catalase 232 kDa, Ferritin 440 kDa, Thyroglobulin 669 kDa, and Blue Dextran 2000) were all from Amersham Biosciences (Uppsala, Sweden).

### ***Preparation of Lens Proteins***

Fetal calf lenses were shipped in ice from PelFreeze (Rogers, AR) and stored at -70°C until use. The water-soluble (WS) fraction was prepared from the outer cortex of thawed calf lenses. The cortex from 10 lenses were pooled and homogenized in a 50-ml Dounce tissue homogenizer with ice-cold de-ionized water. The homogenate was centrifuged at 27,000 x g for 30 min at +4°C. The lens supernatant (CLP) was carefully collected and dialyzed against 5 mM phosphate buffer (pH 7.0) and used for glycation experiments or for preparation of the purified  $\alpha$ -,  $\beta$ - and  $\gamma$ - crystallin fractions.

To isolate purified crystallins, calf lens water-soluble proteins (CLP) were further dialyzed against 10 mM Tris buffer (pH 7.8) containing 1.0 mM TCEP to reduce protein



disulfides and preserve the protein SH- groups from being oxidized. The reduced proteins were passed through a Sephadex G-200 (5 x 80 cm) column to separate  $\alpha$ -,  $\beta_H$ -,  $\beta_L$ - and  $\gamma$ -crystallins using gravity flow. The  $\alpha$ -,  $\beta_H$ -,  $\beta_L$ - and  $\gamma$ -crystallins were concentrated using a 10 kDa cut off filter and each pooled fraction was re-chromatographed on the Sephadex G-200 (5 x 80 cm) column under the same conditions. Each crystallin peak fraction was pooled, concentrated again and dialyzed against de-ionized water containing 0.1 mM DTPA, pH 7.0 at 4°C. The homogeneity and composition of each preparation were confirmed by SDS-PAGE analysis.

#### ***Preparation of human lens water-soluble and water-insoluble proteins***

Intact human lenses (20-75 years old) and cataractous lens nuclei of Indian origin (65-75 years old; type I-II cataract) were used. Each decapsulated lens was homogenized in a 2-ml Dounce tissue homogenizer with ice-cold de-ionized water. The homogenate was placed in 15-ml Corning centrifuge tubes and centrifuged at 27,000 x g for 30 min at +4°C. The lens supernatant was carefully collected with a pipette and dialyzed against 5 mM phosphate buffer (pH 7.0). The lens protein precipitate was washed three times with ice-cold de-ionized water and sonicated with 2.5 ml of 5 mM phosphate buffer (pH 7.0) for 10 min with cooling. A power setting of 4 (approximately 40 W) and a 30% duty cycle were used with Model 375 Cell Disruptor (Heat Systems Ultrasonics Inc., Plainview, NY). Protein concentrations in both water-soluble (WS) and water-insoluble sonicated supernatant protein preparations (WISS) were measured by the BCA method described by Pierce Chemical Co. (Rockford, IL) or by the BioRad Bradford method (BioRad, Hercules, CA).

***Glycation reactions of CLP with different sugars***

All reaction mixtures contained 1.0 mM DTPA in Chelex-treated 0.1 M phosphate buffer, pH 7.0, 10 mg/ml of CLP and either 100 mM glucose, 100 mM fructose, 100 mM ribose, 20 mM ascorbic acid, 20 mM erythrulose or 10 mM methylglyoxal in a total volume of 4.0 ml. Prior to incubation each glycation mixture was sterile-filtered through a 0.22  $\mu$  syringe filter (Whatman) into a 15-ml sterile polyethylene vial, sealed and incubated at 37°C for a period of time ranging from 0 to 4 weeks. At weekly intervals aliquots of the reaction mixtures were withdrawn, dialyzed against 5 mM sodium phosphate buffer (pH 7.0) and stored at 4°C until further use. Control reactions incubated without sugar were subjected to the same procedure.

Reaction mixtures (4.5 ml) containing CLP (10.0 mg/ml) and L-[U-<sup>14</sup>C] ascorbic acid (20 mM, 5  $\mu$ Ci per reaction mixture) in 0.1 M phosphate Chelex-treated buffer, pH 7.0 were incubated at 37°C and at weekly interval aliquots were removed and chromatographed using a Superose 6 column (1x25 cm) eluted at 1.0 ml/min flow rate with 50 mM phosphate buffer (pH 7.0). Fractions (0.5 ml) were collected and their absorbance at 280 nm and 330 nm were recorded. The radioactivity of each fraction was determined using liquid scintillation counter Tri-Carb 2100 TR (Packard Instruments Company, Downers Grove, IL).

***Glycation reactions of CLP in the presence of ascorbic acid and cyanoborohydride***

All glycation mixtures (2.0 ml) contained dialyzed CLP (10 mg/ml), either 20 mM or 1 mM ascorbic acid in 0.1 M Chelex-treated phosphate buffer (pH 7.0) containing 1 mM DTPA, both with and without 40 mM or 5 mM sodium cyanoborohydride respectively. The pH of each reaction mixture was adjusted to 7.0 if necessary prior to incubation. The controls were identical except that they contained no ascorbic acid. Each mixture was sterile-filtered through a Whatman 0.22  $\mu\text{m}$  syringe filter into 4 ml sterile polyethylene vial, sealed, and incubated at 37°C. After 4 weeks of incubation, the reaction mixtures were dialyzed against 5 mM phosphate buffer (pH 7.0) and stored at 4°C until superose chromatography.

***$\alpha$ -Crystallin inhibition of ascorbic acid-mediated  $\beta$ - and  $\gamma$ -crystallin precipitation during glycation reactions***

Mixtures (2.0 ml) containing purified  $\alpha$ -,  $\beta_{\text{H}}$ -,  $\beta_{\text{L}}$ - or  $\gamma$ -crystallin preparations (10 mg/ml) and 20 mM ascorbic acid in 0.1 M Chelex-treated phosphate buffer (pH 7.0) with 1 mM DTPA were sterile-filtered through a Whatman 0.22  $\mu\text{m}$  syringe filter into 4 ml sterile polyethylene vials, capped and incubated at 37°C for 1 week. The controls contained no ascorbic acid. Reaction mixtures containing varying levels of  $\alpha$ -crystallin with purified  $\beta_{\text{H}}$ -,  $\beta_{\text{L}}$ - or  $\gamma$ -crystallins (10 mg/ml total protein concentration) and 20 mM ascorbic acid were prepared as described above and incubated at 37°C for 1 week. The ratios of  $\alpha$ -crystallin to  $\beta$ - or  $\gamma$ -crystallins were varied from 0:1; 1:50; 1:20; 1:10; 1:5, 1:2; and 1:1 on a weight basis. The controls contained no ascorbic acid. At the end of incubation the amount of precipitate in the mixtures was estimated by light scattering of the suspended solution. Each vial was vortexed extensively and the contents were then

poured into a fluorescence cuvette. The cuvette was vortexed again and the fluorescence at excitation and emission wavelengths set at 620nm was immediately measured. When all mixtures were centrifuged, the supernatants were analyzed by Bradford and BCA assays for protein concentration and HPLC gel permeation chromatography was carried out for molecular weight determination.

***Relative 90° light scattering measurements*** of crystallins incubated both in the presence and in the absence of ascorbic acid were performed using a Hitachi model F-2500 spectrofluorometer (Hitachi, Tokyo, Japan) by a modification of the method of Tappan [42]. In brief, the excitation and emission wavelengths were set at 620 nm in order to reduce the sensitivity of the method and to prevent possible light interaction with the chromophores/fluorophores usually present in ascorbylated proteins. The light scattering values of the thoroughly vortexed reaction mixtures were taken using 1 cm path quartz cuvettes (fluorescence cells). All the measurements were performed in triplicate.

***Protein concentrations in the supernatants*** from the glycation mixtures and the corresponding controls were determined after aliquots (100 µl) of the supernatants were TCA-precipitated using ProteoPrep precipitation kit (Sigma, St. Louis, MO). The precipitates were re-dissolved in 0.1 N NaOH and protein concentrations were measured using BCA colorimetric method (Pierce Chemical Co., Rockford, IL).

***The quaternary structural parameters*** of native and ascorbylated crystallin mixtures were determined by dynamic light scattering measurements. The reaction mixtures were

centrifuged at 10,000 x g for 30 min at 4°C. The supernatants were further separated (125 µg/injection) using a TSK G5000PW<sub>XL</sub> size-exclusion column (7.8x300 mm, Tosoh Bioscience LLC, Montgomeryville, PA) equilibrated with 50 mM phosphate buffer containing 150 mM NaCl (pH 7.0) and operating at 0.75 ml/min flow rate using an HPLC system (Shimadzu Scientific Instruments Inc.). The HPLC system was equipped with refractometer and UV detectors (Shimadzu Scientific Instruments Inc.) as well as multi-angle light scattering (DAWN) and quasi-elastic scattering detectors (Wyatt Technologies Corp., Santa Barbara, CA). The molar mass moments, hydrodynamic radius and polydispersity values of each crystallin peak were determined using ASTRA software (ver. 5.1.5) developed and supplied by Wyatt Technologies Corp., Santa Barbara, CA.

*SDS-PAGE* was carried out according to Laemmli [43] using 10-14% gradient gels. The BioRad Precision Plus Protein prestained proteins standards kit was used during electrophoresis.

## Results

The incubation of calf lens proteins with 20 mM ascorbic acid in the presence of air leads to the progressive formation of protein HMW aggregates. Fig.1A shows the separation of a reaction mixture of CLP with 20 mM ascorbate on a Superose 6 size exclusion column (separation range  $5 \times 10^3$ - $5 \times 10^6$  Da for globular proteins). Samples were analyzed every week for the duration of four weeks. As can be seen in Panel A, there was a decrease in the native proteins within the first week of incubation and the

formation of a peak, with a molecular weight somewhat larger than native  $\alpha$ -crystallin. The MW of this peak could be due to the chaperone activity of  $\alpha$ -crystallin binding modified crystallins. Within the next two weeks this “larger than  $\alpha$ -crystallin peak” diminished and coincided with a significant increase in HMW protein aggregates that eluted in the void volume of the column. Panel B shows a significant time-dependent increase in the absorbance at wavelength of 330 nm of the HMW peaks from two to four weeks, which may reflect AGE chromophore formation in the HMW aggregates.

Four-week incubations of CLP with ascorbate and four other sugars (ribose, glucose, fructose and erythrose, see Fig. 2), revealed that only ascorbic acid caused the formation of a HMW peak (Fig. 2B). Based on the calibration data of the Superose 6 column with the mixture of standard proteins (see Materials and Methods) and  $\alpha$ -crystallin, all the HMW proteins eluted in the fractions prior to 12.5 ml. Glucose (Fig. 2D) and fructose (Fig. 2E) produced no HMW protein aggregates. Ribose (Fig. 2C) and erythrose (Fig. 2 F) produced proteins on average 10% larger than  $\alpha$ -crystallin, but no HMW peak was observed in these mixtures even though many sugars were present at higher concentrations than ascorbic acid.

The protein concentration profile from the Superose 6 column of the 4-week incubation mixture of CLP and ascorbate presented in Fig 2B showed that approximately 10-15% of the CLP used for this reaction was present in the HMW peak. Given that only ascorbic acid caused HMW aggregate formation in CLP, these data suggest that ascorbate and its degradation products form a unique set of modifications within the lens proteins responsible for a change in aggregation properties of these proteins. Similar results were obtained in a repeat experiment with a separate CLP preparation incubated under the

same conditions and separated on G5000PW<sub>XL</sub> size-exclusion HPLC column (separation range  $5 \times 10^4 - 1 \times 10^7$  Da) (Fig.3). The protein profiles generated during the separation of glycosylated CLP were used to determine the relative molecular mass moments and hydrodynamic radii of the protein aggregates formed.

Ascorbate was the only sugar that caused the formation of HMW aggregates with CLP with an average molecular mass moment of  $7.3 \times 10^6 \pm 0.03\%$  Da (see Fig. 3), though the left shoulder of the first peak of the CLP–ascorbate mixture extends to a molecular weight of  $1.5 \times 10^7$  Da. Ribose and erythrose caused the formation of protein aggregates with the average molecular mass moments of  $7.2-9.6 \times 10^5$  Da, which is only slightly higher in molecular mass moments than  $\alpha$ -crystallin ( $7.6 \times 10^5 \pm 0.03\%$  Da). Glucose-modified CLP exhibited no significant protein aggregation and was very similar to the elution profile of CLP alone (see Fig. 3A). Incubation of CLP with methylglyoxal showed an effect opposite to the effect of ascorbic acid on CLP. It led to a significant decrease in the  $\alpha$ -crystallin peak exhibiting a slightly lower molecular mass moment, possibly due to methylglyoxal-mediated disaggregation of the  $\alpha$ -crystallin molecule.

To verify that ascorbylation of CLP causes the formation of HMW aggregates due to the glycation by ascorbic acid, two additional experiments were conducted. We repeated the experiment described in Fig. 1 and incubated CLP with [U-<sup>14</sup>C] ascorbic acid under the conditions described in Fig. 1. The elution profile of exhaustively dialyzed ascorbylated CLP on a Superose 6 column is essentially the same as the one seen in Fig.1. Ascorbic acid was covalently incorporated into the HMW peak (approximately 10% of total radioactivity incorporated), presumably because of glycation (Fig. 4). The HMW peak shows a significant absorbance at 330 nm, which also argues that the HMW

aggregates contains chromophores formed by CLP ascorbylation. Clearly, initial glycation products represented significant incorporation of [U-<sup>14</sup>C] ascorbate, but had little or no effect on aggregation. The elution profiles of CLP mixtures incubated in the presence of ascorbate compared to ascorbate together with sodium cyanoborohydride (see Fig. 5) show that the presence of the Schiff's base reducing agent in the reaction mixture significantly reduced the amount of HMW aggregates formed in the glycation mixtures (compare Panel A and Panel C in Fig. 5) arguing that the reduction of early glycation products covalently attached to CLP prevented the formation of advanced glycation end products and inhibited HMW formation. Furthermore, the presence of the reducing agent in the ascorbic acid- CLP mixture drastically abolished the formation of UV-absorbing chromophores, which coincided in time with the decrease in the formation of HMW aggregates (see Fig. 5).

Ascorbate-induced aggregate formation was also shown when CLP proteins were incubated at 37°C under aerobic conditions in the presence of 1.0 mM ascorbic acid (replenished weekly) (see Fig 6). Our data show that the incubation of CLP with physiological concentrations of ascorbic acid [33] under air produced HMW protein aggregates chromatographically identical to those produced during the incubation of CLP with 20 mM ascorbate (see Fig.2B and Fig.3). Similar to the mixtures containing 20 mM ascorbic acid (Fig. 5C), the presence of NaCNBH<sub>3</sub> in the 4-week incubation mixtures containing 1 mM ascorbate completely inhibited the formation of the HMW aggregates in ascorbylated CLP (Fig. 6).

Fig. 7A compares the profiles for ascorbate-modified CLP and the water soluble (WS) fraction from aged human lenses eluting from the TSK G5000PW<sub>XL</sub> column..



Again, the ascorbate-modified CLP exhibited a broad peak of absorbance at 280 nm, whereas the human lens WS fraction showed a plateau of protein with little discernable peak. Analysis of the molecular weight showed increasing aggregate size up to  $10^8$  Da in the earliest portions of both elution profiles. The WS proteins had considerable amounts of native-sized crystallins, whereas few of these remained in the ascorbate-modified CLP sample. The large amount of aggregates in fraction II and III in the ascorbate-modified CLP was not present in the WS fraction from aged human lens. It was possible that these proteins became selectively precipitated upon homogenization and entered the water insoluble (WI) fraction. We, therefore, solubilized a WI fraction from aged human lenses by sonication and separated these solubilized proteins (WISS) on the TSK G5000PW<sub>XL</sub> column. Panel 7B shows a comparison of this profile with the ascorbate-modified CLP preparation. As can be seen, the WISS fraction seemed to contain a distribution of HMW aggregates similar to the ascorbate-modified CLP proteins. Apparently, ascorbate glycation could account for the protein aggregation seen in aged human lens, but the data for the WI fraction must be viewed with caution, because the sonication, used for solubilization may have led to disruption of larger aggregates present in the lens WI fraction. The average molecular mass of the various fractions are shown in Table I.

SDS-PAGE gel analysis under reducing conditions of 4-week incubations of CLP in the presence of 20 or 1 mM ascorbic acid shows the presence of similar crosslinked proteins on the top of the gel (see lanes 2 and 3 in Fig. 8). This protein crosslinking is concentration-dependent, because CLP incubated in the presence of 20 mM ascorbic acid showed a higher intensity HMW bands near the top of the gel compared to the HMW bands from 1 mM ascorbic acid-incubated CLP. The appearance of highly polymerized

HMW aggregates from 4-week ascorbylated proteins on the SDS-PAGE gel resembles very closely the electrophoretic behavior of WI proteins from human aged (lanes 5 and 6) and cataractous lenses (lanes 7 and 8), which may reflect the similarities in their formation mechanisms. The presence of traces of highly crosslinked HMW proteins can be seen even in the electrophoresed WI lens proteins from young human lenses (27 years old; see lane 4 in Fig. 8).

To further examine how ascorbylation influences the aggregation properties of lenticular crystallins, purified  $\alpha$ -,  $\beta_H$ -,  $\beta_L$ - and  $\gamma$ -crystallin fractions were incubated for up to 3 weeks in the presence of 20 mM ascorbic acid under aerobic conditions. We observed that the incubation of  $\beta_L$ - and  $\gamma$ -crystallin fractions in the presence of ascorbate under air quickly led to the precipitation of these proteins in the first 3-4 days of the experiment (data not shown). The precipitation was ascorbate-dependent, since no precipitate was formed in the  $\alpha$ -,  $\beta_H$ -,  $\beta_L$ - and  $\gamma$ -crystallin fractions even after 3 weeks of incubation in the absence of ascorbic acid (data not shown). The precipitation within the ascorbylated  $\beta_L$ - and  $\gamma$ -crystallin fractions coincided with an increase in the total light scattering of the re-suspended precipitates according to 90° light scattering measurements of ascorbylated crystallin solutions (see insets in Panel C and Panel D in Fig. 9). No protein precipitates were formed with the  $\alpha$  and  $\beta_H$ -crystallin fractions incubated with ascorbic acid for up to three weeks (data not shown).

Static and dynamic light scattering measurements were performed for fractions separated on an HPLC G5000PW<sub>XL</sub> size-exclusion column. Precipitates from ascorbylated  $\beta_L$ - and  $\gamma$ -crystallins were removed by centrifugation at 10,000xg and the supernatants were analyzed. Measurements (Fig. 9A, Table II) show that ascorbylation of

$\alpha$ -crystallin for three weeks caused only a slight increase in the molecular mass of the aggregates (from  $8 \times 10^5$  Da in the native  $\alpha$ -crystallin to  $1.00 \times 10^6$  Da in the ascorbylated  $\alpha$ -crystallin) and only to a marginal increase in hydrodynamic radius of ascorbylated  $\alpha$ -crystallin (9.3 nm for the native  $\alpha$ -crystallin vs. 10.3 nm for the ascorbylated  $\alpha$ -crystallin). At the same time, ascorbylation for 0 to 3 weeks of the  $\beta_H$ -crystallin fraction showed a progressive increase in hydrodynamic radii of the HMW peaks with  $R_t$  8.5-10.0 min to 17.4 nm in the 3-week ascorbylated  $\beta_H$ -crystallin fraction. This corresponds to almost a two-fold increase in the average molecular mass moment of the HMW aggregates in  $\beta_H$ -crystallins (molecular mass moment for 3 weeks  $\beta_H$  control of  $1.6 \times 10^6 \pm 0.02\%$  Da vs. molecular mass moment 3-week ascorbylated  $\beta_H$  of  $2.1 \times 10^6 \pm 0.01\%$  Da; see Fig. 9B and Table II). The 2-3 fold increase in the HMW species correlated with an almost 10-fold increase in  $90^\circ$  light scattering of 3-week ascorbylated  $\beta_H$ -crystallin solutions (see inset in Fig. 9B).

Separation of the proteins remaining in the supernatant of the ascorbylated  $\beta_L$ -crystallin fractions on the G5000PW<sub>XL</sub> size-exclusion column shows that almost 69 -78% of the protein was lost due to ascorbate-mediated precipitation of the proteins within the first week of the experiment (see inset in Fig. 9C). The relative average molecular mass moment determination for the prevalent species in these fractions ( $R_t$  12-13 min) shows that while the remaining  $\beta_L$ -crystallins didn't increase in mass, the average hydrodynamic radius of the 3-week ascorbylated species in the major peak is increased by 3-fold, reaching a value of 13.7 nm as compared to 3-week native  $\beta_L$ -crystallin fraction incubated without ascorbate under the same conditions (Fig. 9C). This increase in ascorbylated  $\beta_L$ -crystallin aggregation with time is in good agreement with 5-fold

increase in the total 90° light scattering of whole 3-week ascorbylated  $\beta_{\text{H}}$ -crystallin solution (see inset in Fig. 9C).

Similar to  $\beta_{\text{L}}$ -crystallins, ascorbylation of  $\gamma$ -crystallins causes precipitation of almost 60% of the total protein within one week (see inset in Fig. 9D). The chromatographic separation of the 0-3 week supernatants supports this finding (see Fig. 9D). The major species that remained soluble ( $R_{\text{t}}$  12-13 min) in the reaction mixtures exhibited an average molecular mass moment of  $2.7 \times 10^4 \pm 0.02\%$  Da for 0 week incubation compared to  $3.2 \times 10^4 \pm 0.03\%$  Da at 3 weeks. Hydrodynamic radii ranged from 1.1 to 7.8 nm after 3 weeks ascorbylation. It should be noted, that  $\gamma$ -crystallin mixtures ascorbylated for 2-3 weeks contained trace amounts of aggregates with hydrodynamic radii ranging up to 35 nm (see species with  $R_{\text{t}}$  9.5-10.5 min), which are of sufficient size to scatter visible light. The presence of these species was probably responsible for excessive total 90° light scattering of 1-3 week ascorbylated gamma-crystallin solutions (see inset in Fig. 9D). Given that the major ascorbylated  $\gamma$ -crystallins with hydrodynamic radii of 8 nm or less were found in the 1-3 week ascorbylated  $\gamma$ -crystallin supernatants, it seems that aggregates with larger radii were prone to precipitation.

Both the presence of protein precipitates in the 1-3 week ascorbylated  $\beta_{\text{L}}$ - and  $\gamma$ -crystallin fractions and the absence of precipitates in ascorbylated CLP (see insets in Fig. 9C and 9D) suggested that during glycation of CLP by ascorbate,  $\alpha$ -crystallin in the CLP interacted with the modified  $\beta_{\text{L}}$  and  $\gamma$ -crystallins because of its chaperone activity thereby preventing their precipitation. We tested this hypothesis by ascorbylating  $\beta_{\text{L}}$  and  $\gamma$ -crystallin fractions in the presence of increasing amounts of added  $\alpha$ -crystallin for one

week under air and monitoring the effect on aggregation (see Fig. 10 and Table II). At ratios of  $\alpha$ -crystallin:  $\beta_L$ - or  $\gamma$ -crystallin less than 1:10 we have observed only a partial protection of ascorbylated  $\beta_L$ - and  $\gamma$ -crystallins by  $\alpha$ -crystallin. Depending upon the concentration of  $\alpha$ -crystallin added to these mixtures approximately 30% to 50% of the protein precipitated in these solutions within one week and they were characterized by a 500-800 fold increase in the total  $90^\circ$  light scattering compared with the control solutions incubated without ascorbic acid under the same conditions (see Fig. 10C). Separation of the proteins remaining in the supernatants of the ascorbylated  $\beta_L$ -crystallin and  $\gamma$ -crystallin samples in the presence of  $\alpha$ -crystallin showed the presence of aggregates with molecular mass moments ranging from  $5 \times 10^7$  Da to  $1.5 \times 10^8$  D and hydrodynamic radii ranging up to 40 nm ( $R_t$  6.5-8.2 min; see Fig. 10 Panel A and Panel B and Table II). It should be emphasized, that similar to the HMW aggregates from  $\alpha+\beta_L$ - and  $\alpha+\gamma$ -crystallins solutions (with ratios higher than 1:5), human water-soluble crystallin fraction from old (70-75 years old) lenses exhibited similar chromatographic characteristics under the same separation conditions. Depending upon the preparation, the peak at  $R_t$  6.8-8.0 min from the WS fraction from human lens showed visible light scattering aggregates with molecular mass moments ranging from  $3.0 \times 10^7 \pm 0.03\%$  Da to  $6.6 \times 10^7 \pm 0.03\%$  Da with hydrodynamic radii up to 40 nm.

With an increase in  $\alpha$ -crystallin concentrations in both the ascorbylated  $\alpha$ -: $\beta_L$  and  $\alpha$ -: $\gamma$ -crystallins mixtures at ratios higher than 1:5 we observed the complete disappearance of protein precipitates, which coincided with a significant decrease in the total  $90^\circ$  light scattering of the solutions (see Fig.10C). At the same time, at the ratios of  $\alpha$ -:  $\beta_L$ - and  $\alpha$ -:  $\gamma$ -crystallins of 1:2 the major HMW species had hydrodynamic radii

within the range of 22-30 nm the average molecular mass moments ranging from  $7 \times 10^6 \pm 0.02\%$  Da to  $1.5 \times 10^7 \pm 0.03\%$  Da (Fig. 10A and 10B). These species are very similar to the ones detected in the protein water-insoluble sonicated (WISS) fractions from aged lenses. The disappearance of the protein precipitates and significant decrease in the size of the protein aggregates in the ascorbylated  $\alpha + \beta_L$ - and  $\alpha + \gamma$ -crystallin mixtures clearly shows that at high  $\alpha$ -crystallin to  $\beta_L$ - and  $\gamma$ -crystallin ratios  $\alpha$ -crystallin prevented the formation of glycation-mediated  $\beta_L$ - and  $\gamma$ -crystallin precipitation, probably by binding (chaperoning) ascorbylated  $\beta_L$ - and  $\gamma$ -crystallins and preventing their aggregation. An opposite effect occurred when the  $\alpha$ -crystallin to  $\beta_L$  and  $\gamma$ -crystallin ratios drop lower than 1:5. Under these conditions  $\alpha$ -crystallin not only is ineffective as a chaperone for ascorbylated  $\beta_L$  and  $\gamma$ -crystallin, but also facilitates the formation of HMW lens protein aggregates similar in mass and size to the HMW aggregates from aged human lens water soluble protein. (Fig. 10, Table II).

## Discussion

Short-range protein-protein interactions of lens crystallins within the lens fiber cell are considered to be responsible for lens transparency [44]. These forces are thought to be compromised in the lens due to post-translational crystallin modifications that occur during normal aging of the lens and during cataractogenesis [2, 6, and 7]. Usually, such modifications (e.g. methionine oxidation, formation of protein-protein disulfides and GSH-protein mixed disulfides, non-enzymatic glycation, phosphorylation, deamidation, truncation, etc.) lead to increased protein-protein interactions and cause fluctuations in the protein density within the lens [9, 12, 16, 23, 26, 38, 45]. Eventually, as the

magnitude of these modifications increases, it results in the formation of HMW aggregates capable of scattering visible light impinging on the lens [2, 6, 7, 45- 47].

Interestingly, even a small number of such aggregates present in apparently undamaged lens fiber cells can cause light scattering in the guinea pig lenses treated with hyperbaric oxygen [48]. Similar HMW aggregates (slow diffusing species) were observed in the human lenses with early cataracts and according to Benedek [6] they correspond to aggregates with approximate molecular mass of  $5.0 \times 10^7$  Da (approximately 50 nm in diameter) and higher [46]. According to Bettelheim et al. [49] and Liem et al. [50] these aggregates could reach 300-500 nm in diameter (apparent molecular mass  $5 \times 10^8$  Da) and higher in nuclear cataract and up to 1000 nm in diameter in the X-ray induced cataract in rabbit lenses [50], respectively. The data presented in this communication show that even WS proteins from aged human lenses (70-75 years old) and early (Type I) cataracts (data not shown) contain aggregates with average mass moments ranging from 3.0 to  $6.5 \times 10^7$  Da with hydrodynamic radii ranging up to 40 nm (Fig. 9).

In their pioneering work Stevens et al. were the first to recognize that sugar-mediated modifications of the Lys  $\epsilon$ -amino groups in lens crystallins can influence their functional properties, and they hypothesized that non-enzymatic glycation can cause structural changes in lens proteins leading to their insolubilization [51]. According to Swamy and Abraham, there is a progressive and significant increase in the glycated HMW proteins within rat lenses with time and with the severity of sugar cataract in diabetic rats [26, 52]. Similarly, human cataractous lenses also show an increase in early glycation products with age and an increase in fluorescent substances in certain types of cataract [53]. There

is a positive correlation between the decrease in the content of Lys, Arg and His residues in lens crystallins [53, 54] and type I-II cataract formation [23-25, 54, 55]. A similar correlation between lens protein glycation, browning, fluorescence and age has been observed in human lenses [16, 23-25, 56]. Based on data published in the literature, the total levels of AGE modifications (e.g. CML, CEL etc.) and AGE crosslinks (e.g. K2P, MOLD, glucosepane, etc.) can reach levels up to 7.5 nmol/mg of lens protein in cataractous lenses and 4.1 nmol/mg of lens protein in aged human lenses (ages 60 years old and older) [16-25, 57-66]. It should be noted that most of these AGEs have been detected in WI lens proteins and may represent a contributory factor in lens protein unfolding and aggregation.

There is a growing body of evidence that ascorbic acid can contribute to an overall lens predisposition to cataract formation. On one hand, aged and cataractous human lenses contain increased levels of dehydroascorbic acid [33]. On the other hand, the old and cataractous human lenses usually provide a suitable environment for ascorbic acid oxidation. The conditions that facilitate the oxidation of ascorbic acid in the lens include diabetes and the formation of a dense barrier around the lens nucleus [67], which causes a significant decrease in GSH concentration in the lens nucleus. The other factors include the low GSH levels within the lens and the lack of hexose monophosphate shunt needed to synthesize NADPH, which is needed for the normal function of glutathione reductase [68]. It is quite possible that the low GSH levels in the nucleus lead to a build up of ascorbic acid degradation products, including dehydroascorbic acid (DHA), 2,3-diketogulonic acid and erythrulose [34]. These intermediates are far more potent as glycation agents, and under air ascorbic acid modifies lens proteins at rates 9-fold faster



and crosslinks proteins 90-fold more rapidly than glucose [35]. The incubation of rabbit lens explants in the presence of dehydroascorbic acid, along with suppressed glutathione disulfide reduction by glutathione reductase, therefore culminated in the lens opacification within hours from the beginning of the experiment [31]. Furthermore, an increased influx of ascorbic acid to the mouse lens by an overexpressed vitamin C SVCT2 transporter led to marked lens browning [38].

While the modifications of CLP by ascorbic acid produces modified amino acids similar to those in aged human lenses and cataract [16-18], it has not been shown that these glycation modifications play a causal role in cataract formation. In the communication presented here we have shown that ascorbic acid-mediated modifications of individual calf lens crystallins can induce their aggregation (Fig. 1) and precipitation (Fig. 9). These results are in good agreement with the data from our previous publication, which showed that ascorbic acid can induce lens protein crosslinking and precipitation of lens extracts at physiological vitamin C concentrations at pH 7 and under limiting oxygen conditions [69]. This effect is sugar-specific, because no other carbohydrate tested in this study was able to produce HMW aggregates of CLP upon incubation under aerobic conditions. Further, the aggregates produced by ascorbate were similar in size to the combined aggregates present in the sum of the WS and WI lens proteins from aged human lenses (Fig. 7). Also, our results show that the presence of  $\text{NaCNBH}_3$ , an effective and specific Schiff's base reducing agent, in the incubation mixtures almost completely prevented the formation of the HMW aggregates in ascorbylated CLP. This reagent reduces the early glycation products on proteins thereby preventing the formation of AGEs. Consistent with this effect was the diminished formation of UVA-absorbing

AGEs (see Fig. 5), which is indirect evidence for the presence of nitrogen-containing heterocycles within ascorbylated CLP. The appearance and the electrophoretic mobility of ascorbylated calf lens proteins on the SDS-PAGE gel resemble closely those from the WI proteins from aged and cataractous lenses (Fig. 8). These coincides with the fact that there are close structural similarities between the adducts covalently bound to proteins in ascorbylated CLP and the lens protein modifications found in aged human lenses and in brunescant cataracts of Indian origin [16-18, 23-25].

The precipitation of  $\beta_L$ - and  $\gamma$ -crystallins by ascorbic acid occurs rather rapidly (within 2-3 days of incubation) and it seems ascorbate-induced, since no precipitates were formed in the  $\beta_L$ - and  $\gamma$ -crystallin solutions kept under air and in the absence of ascorbate even after 4 weeks of incubation at 37°C (Fig. 9 and Fig. 10). This effect resembles that observed by Swamy and Abraham in the lenses of diabetic rats [26]. The cataractous lenses of older hyperglycemic animals were shown to lose  $\gamma$ -crystallins in the lens protein WS fraction with subsequent accumulation of  $\gamma$ -crystallins in the urea-soluble fraction (water-insoluble proteins). The effect was probably due to lens protein glycation as well as oxidation, because the acetylation of rat lens proteins  $\epsilon$ -amino groups by aspirin significantly diminished the formation of HMW aggregates and cataract formation in the diabetic rats [52].

The interactions between  $\alpha$ -crystallin and  $\beta$ - and  $\gamma$ -crystallins are weak and usually do not produce any stable complexes under physiological conditions [70, 71]. This situation changes when  $\alpha$ -crystallin is present in  $\beta_L$ - or  $\gamma$ -crystallins solution under air in the presence of ascorbic acid.  $\alpha$ -Crystallin diminished the formation of ascorbylated  $\beta$ - and  $\gamma$ -crystallins precipitates (Fig. 10). At lower  $\alpha$ -crystallin to  $\beta_L$  and  $\gamma$ -crystallins

ratios, there was a strong tendency to form aggregates with molecular mass  $3.0 \times 10^7$  Da to  $7.0 \times 10^7$  Da and hydrodynamic radii up to 40 nm similar to HMW aggregates in the aged human lenses (70-75 years old) and early (Type I) cataracts. Thus, it seems that  $\alpha$ -crystallin exhibits its chaperone function toward ascorbylated  $\beta_L$  or  $\gamma$ -crystallins and forms complexes maintaining them in solution. Once the chaperoning capacity of  $\alpha$ -crystallin is apparently overwhelmed, soluble HMW aggregates are formed. The proposed mechanism may also take place in rat diabetic lenses. Lenses from rats with persistent moderate hyperglycemia have a significant amount of light scattering HMW aggregates similar in composition to HMW aggregates from human lens [72-74]. Crystallins isolated from those lenses contain AGEs within their framework and show a tendency to form HMW aggregates that scatter visible light [72-74], however, unlike these aggregates, the ascorbylated aggregates were completely resistant to reduction by the TCEP reducing reagent.

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### Figure Legends

**Figure 1.** Aggregation of CLP during incubation with 20 mM ascorbic acid in air at 37°C. Samples were taken weekly and chromatographed over a Superose 6 column. The dashed curve – 0 day incubation, black rectangles – 1 week incubation, white rectangles –

2 week incubation, solid bold trace – 4 week of incubation. Panel A shows profile of absorbance at 280 nm whereas panel B shows the profile at 330 nm.

**Figure 2.** Superose 6 column profiles of CLP incubated both in the absence of any glycating agent (panel A) and in the presence of 20 mM ascorbic acid (panel B), 100 mM ribose (panel C), 100 mM glucose (panel D), 100 mM fructose (panel E), 20 mM erythrose (panel F) under aerobic conditions at 37°C. The dashed curves show protein concentration profiles (mg/ml) determined by BCA assay; the bold solid curves show absorbance at 280 nm; the thin solid curves show absorbance at 330 nm.

**Figure 3.** TSK G5000PW<sub>XL</sub> size-exclusion column elution profiles of CLP mixtures incubated for four weeks under aerobic conditions at 37°C in the absence and in the presence of 20 mM ascorbic acid, 100 mM ribose, 100 mM glucose, 20 mM erythrose, 20 mM methylglyoxal are shown in panel A. Panel B shows the average relative molecular weights of the protein peaks calculated using ASTRA software. For convenience, the elution profiles are divided into four zones: 8-9.5 min (HMW aggregates), 9.5-11.5 min ( $\alpha$ -crystallin-like fraction), 12.5-13.5 min ( $\beta$ -crystallin fraction) and 13.5-14.5 min ( $\gamma$ -crystallin fraction) and are shown as shaded bars in panel A.

**Figure 4.** Superose 6 profile of CLP incubated in the presence of 20 mM [U-<sup>14</sup>C] ascorbic acid for 4 weeks under aerobic conditions at 37°C. Bold solid line – elution

profiles measured at 280 nm, thin solid line – elution profiles measured at 330 nm and bold dash line shows radioactivity [ $U\text{-}^{14}\text{C}$ ] ascorbic acid incorporation into the CLP.

**Figure 5.** Superose 6 elution profiles of CLP mixtures incubated for four weeks under aerobic conditions at 37°C in the presence of 20 mM ascorbic acid (panel A), without ascorbate (panel B) and in the presence of 20 mM ascorbate together with 40 mM sodium cyanoborohydride (panel C). Black line – elution profiles measured at 330 nm, grey line – elution profiles measured at 280 nm.

**Figure 6.** Superose 6 column profiles of CLP incubated both in the absence (dashed bold line) and in the presence of 1.0 mM ascorbic acid for one week (black triangles), four weeks (thin solid line) and four weeks with 1.0 mM ascorbic acid in the presence of 5 mM  $\text{NaCNBH}_3$  (white rectangles) under aerobic conditions at 37°C. Bold solid line in this figure shows a profile of CLP incubated with 20 mM ascorbate under identical incubation conditions.

**Figure 7.** Comparisons of the TSK G5000PW<sub>XL</sub> size-exclusion elution profiles for ascorbate-modified CLP and aged human lens WS proteins (Panel A) and ascorbate-modified CLP and aged human lens WISS proteins (Panel B). Average molecular weights of the proteins eluting in the shaded areas were determined by light scattering and analyzed by ASTRA software.



**Figure 8.** Gradient (10-14.5%) SDS-PAGE (reducing) of CLP incubated in the absence (lane 1) and in the presence of 20 mM (lane 2) or 1 mM (lane 3) ascorbic acid for one week at 37° C; human aged WI sonicated lens proteins from 27 year old (lane 4), 60 year old (lane 5), 70 year old (lane 6) lenses; human cataractous (type I-II cataract) WI sonicated lens proteins from 60 year old (lane 7) and 69 year old (lane 8).

**Figure 9.** HPLC size-exclusion chromatography of ascorbylated  $\alpha$ - (panel A),  $\beta_H$  (panel B),  $\beta_L$  (panel C) and  $\gamma$ -crystallin (panel D) fractions separated on the TSK G5000PW<sub>XL</sub> size-exclusion column. The inserts in the panels show light scattering (measured as a fluorescent intensity at excitation 620 nm and emission 620 nm) of ascorbylated crystallin reaction mixtures, vortexed immediately before measurements to suspend precipitated WI proteins. Numbers above the bars in the insets show the protein concentrations (mg/ml) determined by a BCA assay in supernatants of the reaction mixtures. Dark bars show CLP incubated with ascorbate, whereas white bars show CLP incubated without ascorbate.

**Figure 10.** HPLC profiles of undialyzed non- and ascorbylated  $\alpha + \gamma$  - (panel A), and  $\alpha + \beta_L$  - crystallin mixtures (panel B) separated on the TSK G5000PW<sub>XL</sub> size-exclusion column (peaks numbers are shown above the bars). Panel C shows the light scattering (measured as a fluorescent intensity at excitation 620 nm and emission 620 nm) of crystallin mixtures (I –  $\alpha + \gamma$ ; II –  $\alpha + \beta_L$ ; III –  $\alpha + \beta_L + \beta_H + \gamma$ ) incubated for 1 week at 37°C both in the presence and in the absence of ascorbic acid. Crystallin ratios were calculated by dividing the total concentration (mg/ml) of  $\beta_L$  or  $\gamma$  or  $\beta_L + \beta_H + \gamma$  crystallins

by the concentration (mg/ml) of  $\alpha$ -crystallin in the corresponding reaction. The first number in crystallin ratios stands for  $\alpha$  and the second for  $\beta_L$  or  $\gamma$  or  $\beta_L + \beta_H + \gamma$ .

ACCEPTED MANUSCRIPT

**Table I. Protein distribution and molecular weights of different lens preparations.**

<b>Crystallins</b>	<b>Peak number *</b>	<b>Peak average MW** (Da)</b>	<b>Protein distribution** (% by weight)</b>
CLP	III	8.0E+05	51
	IV	3.0E+05	17
	V	1.0E+05	18
	VI	6.1E+04	13
CLP+Asc	I	2.4E+07	<3
	II	1.1E+07	21
	III	2.8E+06	42
	IV	1.6E+06	17
	V	8.7E+05	10
	VI	5.4E+05	9
WS(human)	I	8.0E+07	3
	II	1.4E+07	6
	III	2.3E+06	17
	IV	9.7E+05	26
	V	6.1E+05	22
	VI	7.3E+05	27
WISS(human)	I	3.4E+07	8
	II	6.2E+06	75
	III	4.9E+06	17

\* Peak I (6.8-8.5 min, HMW1); peak II (8.5-10 min, HMW2); peak III (10-11.5 min,  $\alpha$  – like); peak IV (11.5-12.5 min,  $\beta_H$  – like); peak V (12.5-13.5 min,  $\beta_L$  – like); peak VI (13.5-14.5 min,  $\gamma$  – like).

\*\* Results shown are the average of four experiments with different protein preparations.

**Table II.** Quaternary properties of native and ascorbic acid-modified calf lens crystallins

Crystallins	Peak number *	MW (Da)	Protein distribution (% by weight) **
$\alpha$ (calf)	III	7.79E+05	
$\alpha$ +Asc	III	8.9E+05	
$\beta_H$ (calf)	IV	1.59E+05	
$\beta_H$ +Asc	II-III	2.16E+06	
$\beta_L$ (calf)	V	5.8E+04	100**
$\beta_L$ +Asc	V	5.8E+04	22**
$\gamma$ (calf)	VI	2.2E+04	100**
$\gamma$ +Asc	VI	2.76E+04	35**
$\alpha+\beta_L(1:10)$ +Asc	I	1.8E+07	15
	II	1.32E+07	24
$\alpha+\beta_L(1:5)$ +Asc	I	1.5E+07	8
	II	5.54E+06	53
$\alpha+\gamma(1:20)$ +Asc	I	4.77E+07	6
	II	2.1E+07	16
$\alpha+\gamma(1:10)$ +Asc	I	4.98E+07	4
	II	1.04E+07	20
$\alpha+\gamma(1:5)$ +Asc	I	5.7E+07	2
	II	8.69E+06	22

\* Peak I (6.8-8.5 min, HMW1); peak II (8.5-10 min, HMW2); peak III (10-11.5 min,  $\alpha$  – like); peak IV (11.5-12.5 min,  $\beta_H$  – like); peak V (12.5-13.5 min,  $\beta_L$  – like); peak VI (13.5-14.5 min,  $\gamma$  – like)

\*\* Most of the crystallins was precipitated during glycation reactions; only a fraction of the starting material (22% of starting 100%  $\beta_L$  and 35% of starting 100% $\gamma$ ) was found in the solution at the end of the incubation period.

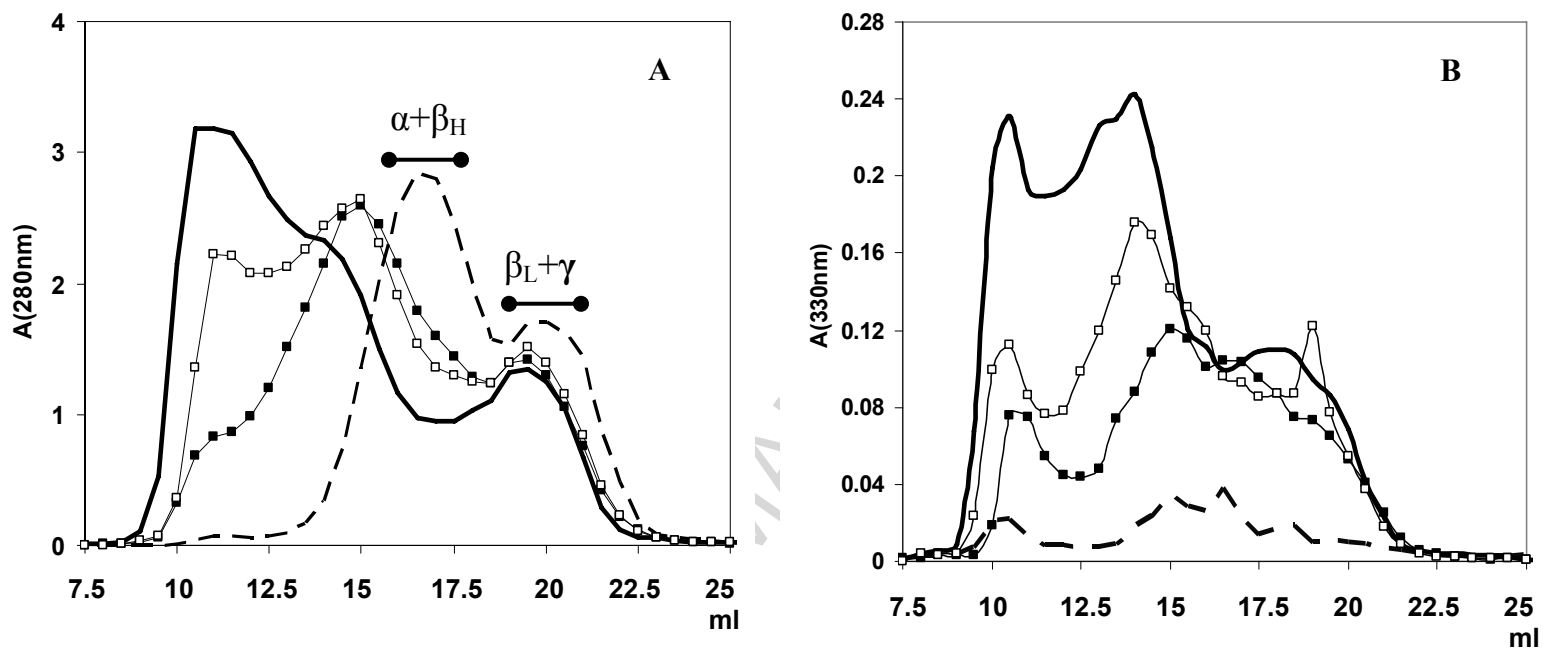


Figure 1

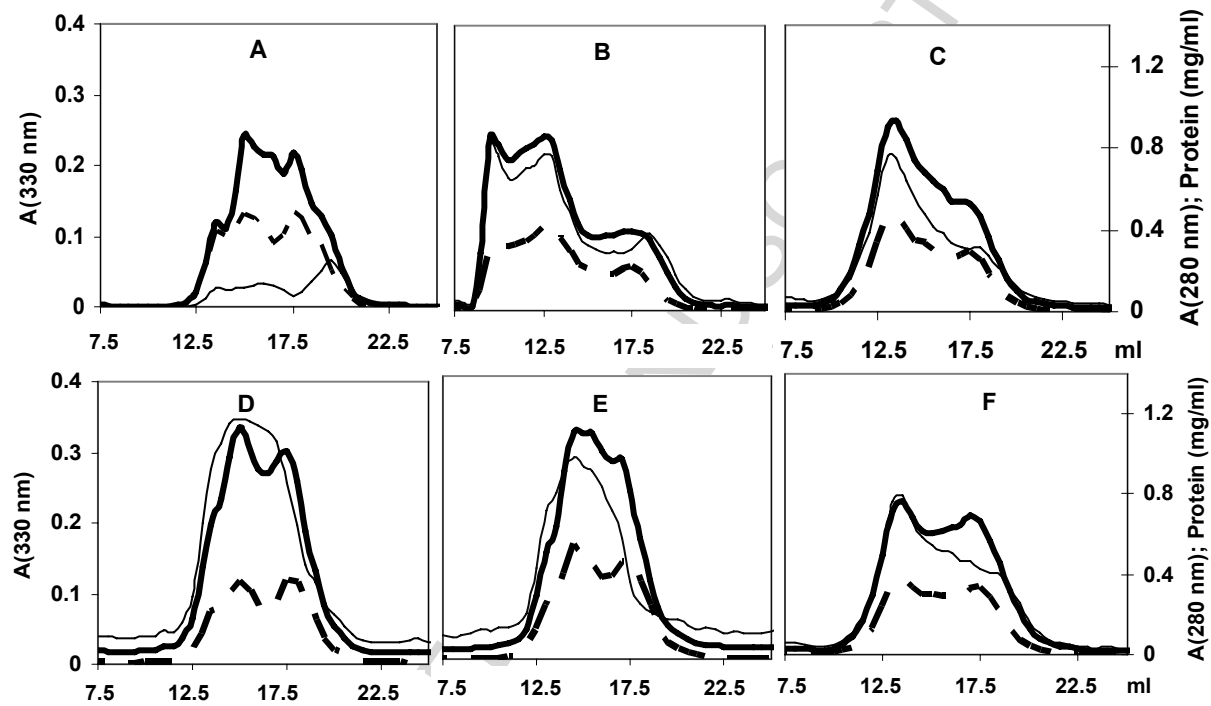


Figure 2

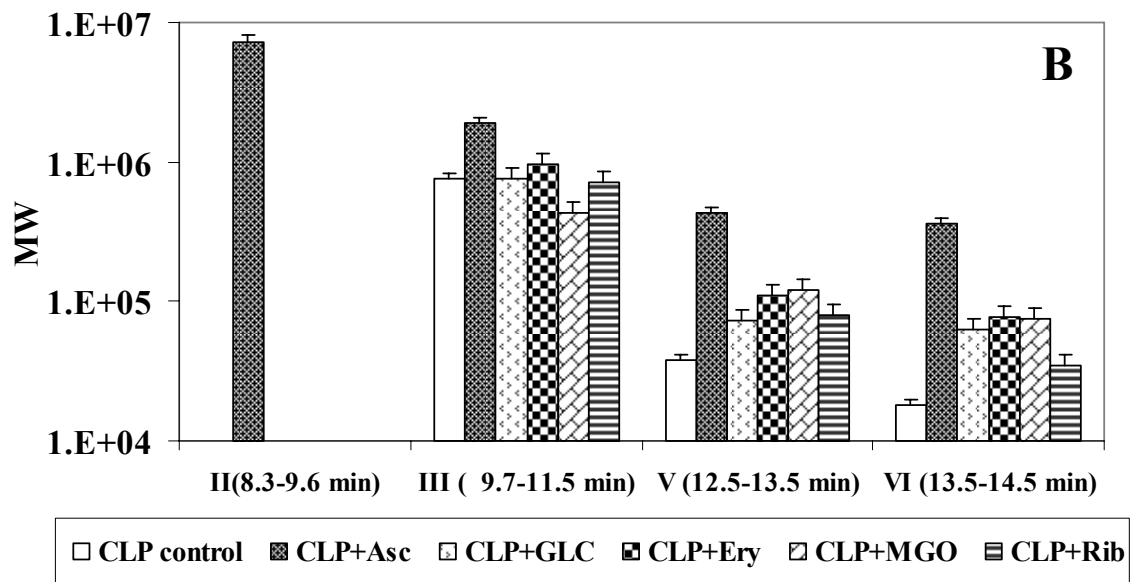
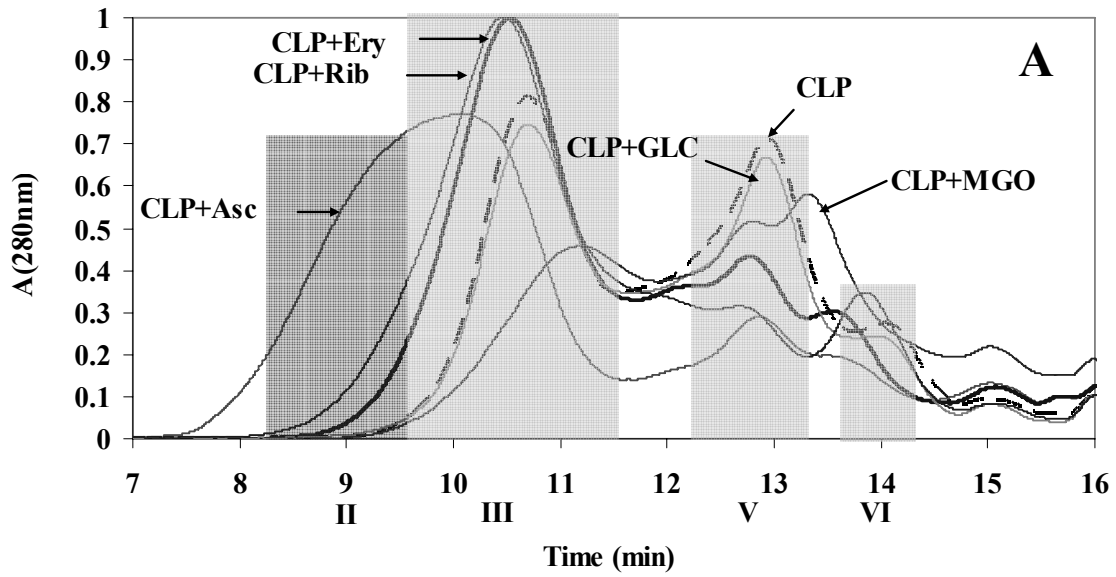


Figure 3



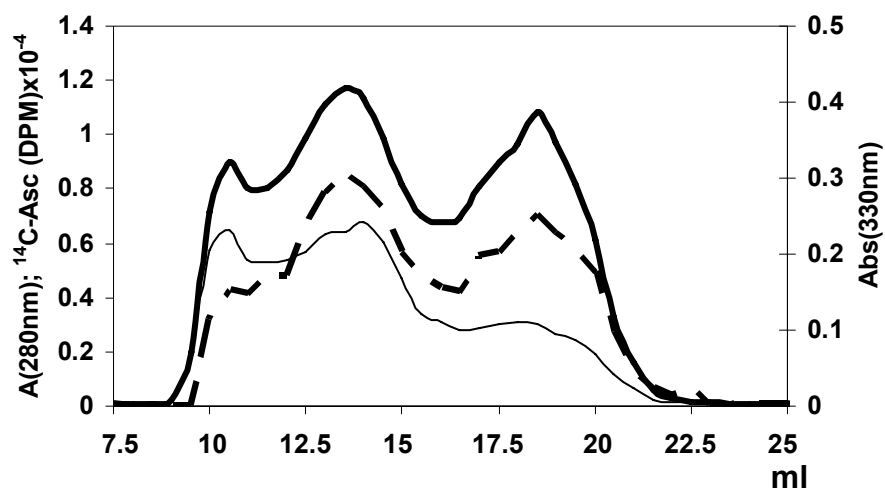


Figure 4

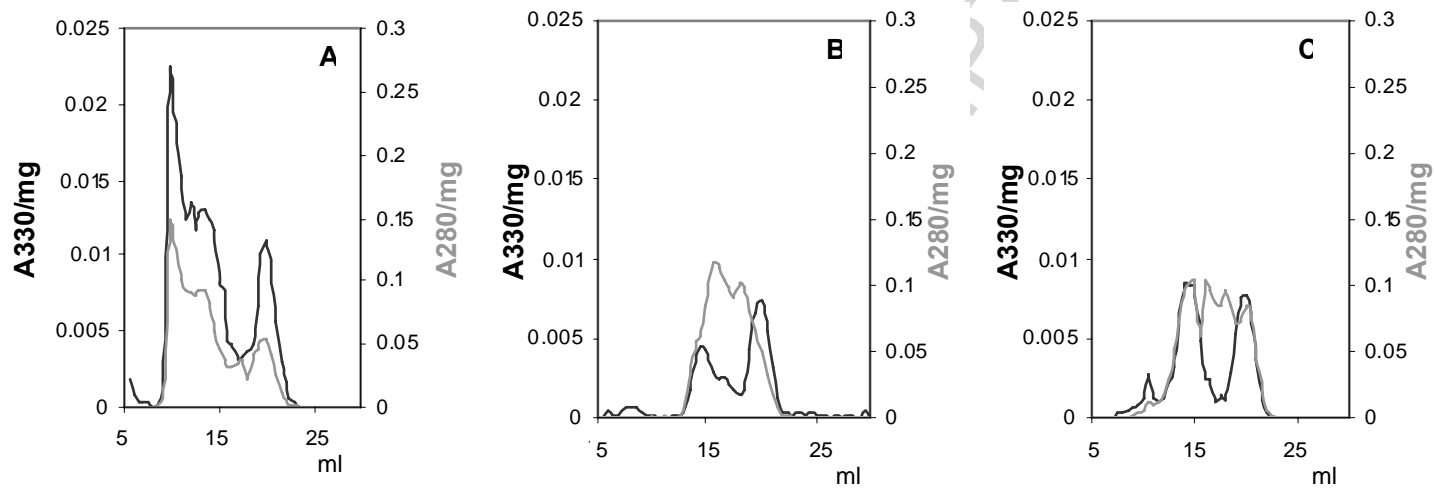


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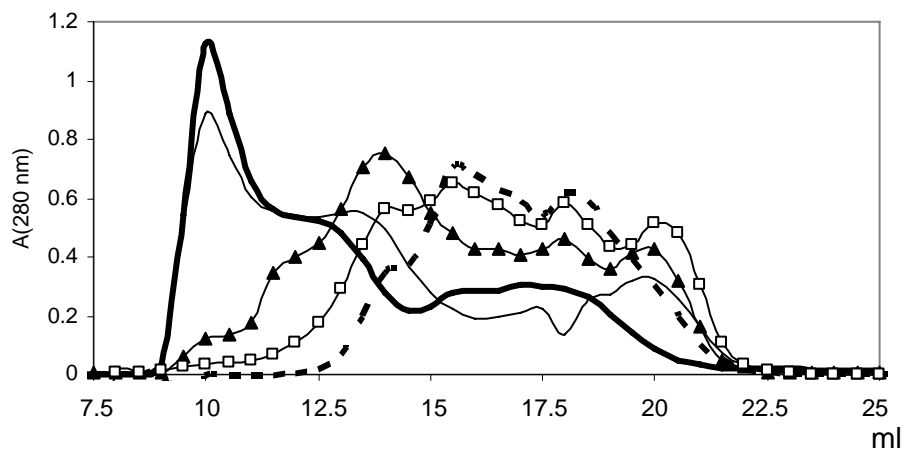


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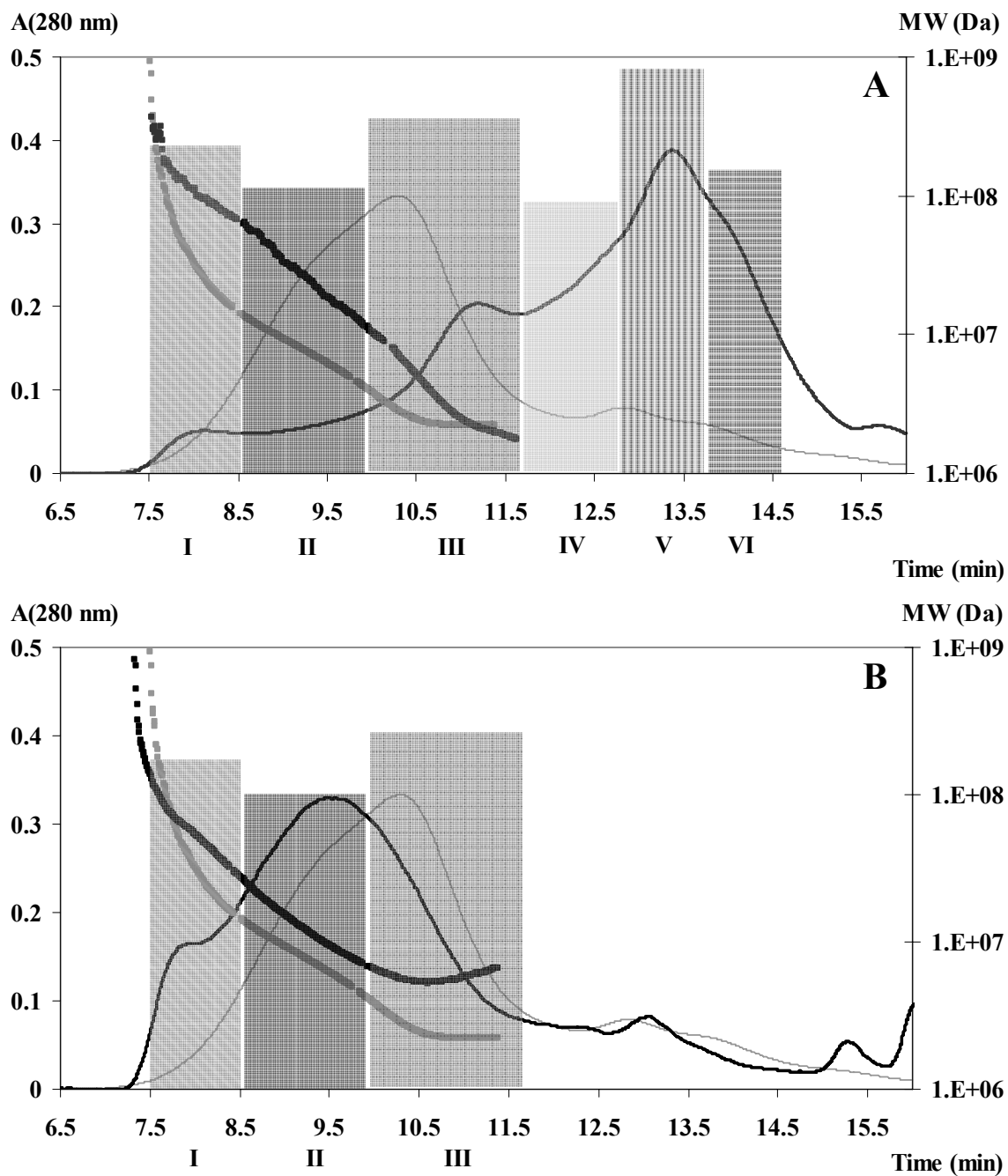


Figure 7

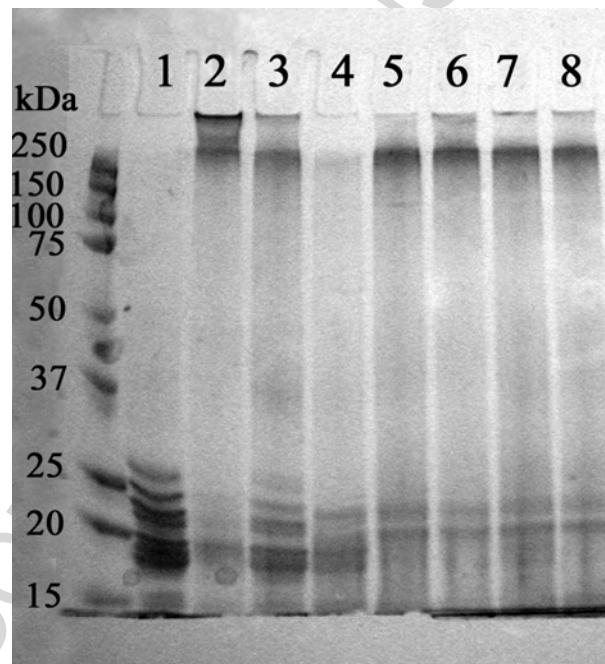


Figure 8

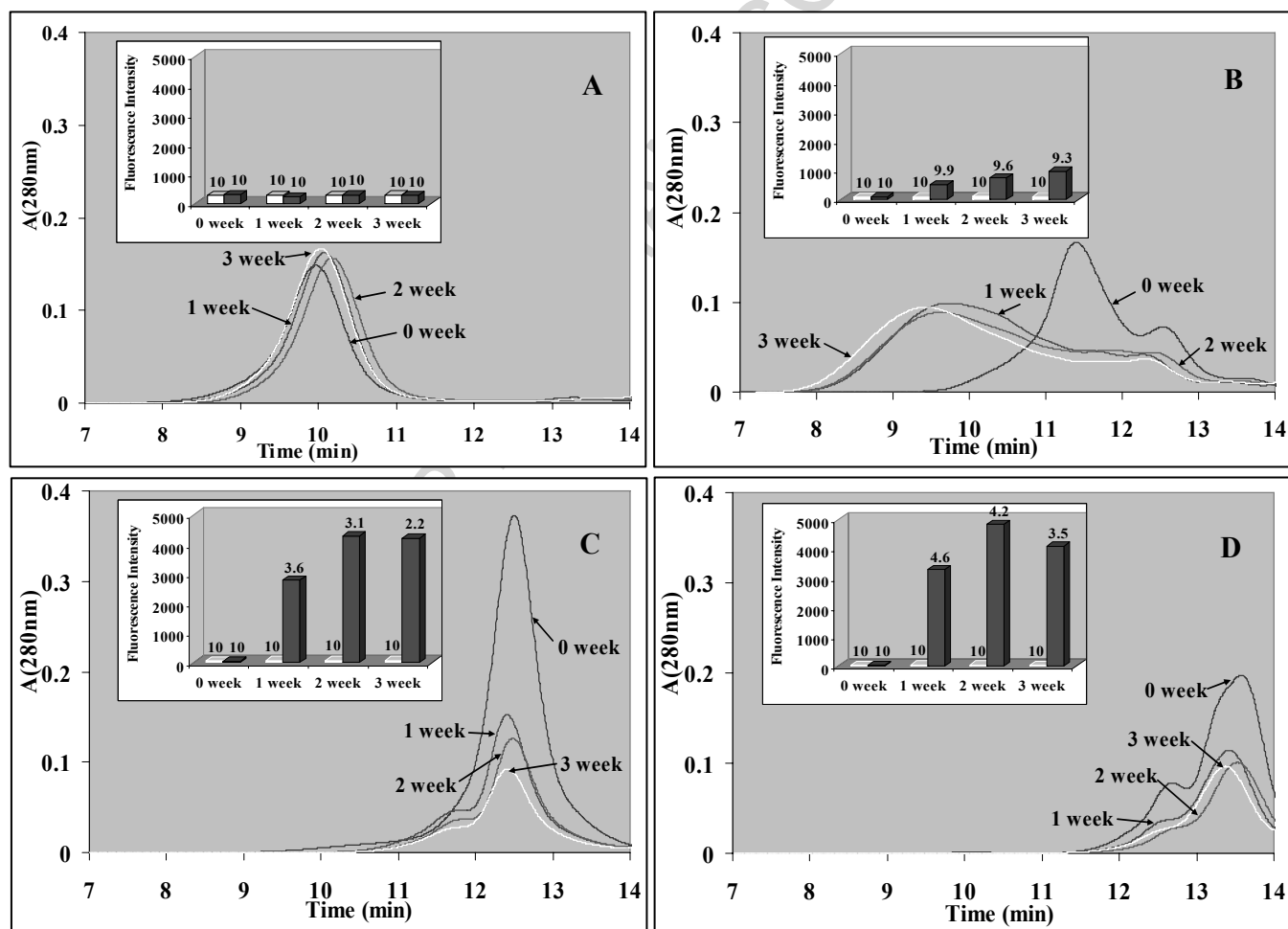


Figure 9

