Molecular Vision 2003; 9:644-56 <http://www.molvis.org/molvis/v9/a78>
Received 20 August 2003 | Accepted 5 December 2003 | Published 8 December 2003

Crosslinking of human lens 9 kDa γD-crystallin fragment in vitro and in vivo

Om P. Srivastava, Kiran Srivastava

Department of Physiological Optics, School of Optometry, University of Alabama at Birmingham, Birmingham, AL

Purpose: The aims of this study were to determine in vitro crosslinking of a 9 kDa γD-crystallin fragment alone and with α-, β-, or γ-crystallins, the existence of covalent multimers of the polypeptide in vivo, and posttranslational modifications in the three isoforms of the polypeptide.

Methods: A mixture of crystallin fragments (3-14 kDa), a 9 kDa γD-crystallin polypeptide or the polypeptide and individual α-, β-, or γ-crystallins, were incubated at 37 °C for a desired length of time and the crosslinked species were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), size exclusion Agarose A 1.5 gel chromatography, and western blot analysis. In addition, the existence of covalent multimers of the 9 kDa polypeptide in human lens water soluble (WS) and water insoluble (WI) protein fractions of normal and cataractous human lenses was determined by western blot analyses. The posttranslationally modified amino acids of three isofroms of the polypeptide were identified by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) and ES-MS/MS mass spectrometric analyses.

Results: Following incubation of a mixture of the crystallin fragments or the 9 kDa polypeptide, covalently crosslinked species held via non-disulfide bonding were seen on SDS-PAGE analysis. The polypeptide also exhibited crosslinking with individual α-, β-, and γ-crystallins. After western blot analysis with site specific anti-9 kDa antibodies, both WS and WI protein fractions from normal and cataractous lenses showed immunoreactive 27 and 45 kDa multimers. The mass spectrometric analysis of the three isoforms of the polypeptide (with identical molecular weight but different charges) showed oxidized methionine and tryptophan residues, with the latter residue containing two oxygens.

Conclusions: The data suggest that a 9 kDa γD-crystallin fragment demonstrated crosslinking properties, which might be due to oxidation of its methionine and tryptophan residues.

Mammalian lenses contain three major structural proteins known as α, β, and γ-crystallins. Among these, α- and β-crystallins exist as oligomers whereas γ-crystallin is found as a monomer. The crystallins, because of their special structural interactions and high concentrations, contribute to the transparency of the lens and provide the needed refractive power to focus light on to the retina. With aging, crystallins undergo several posttranslational modifications that cause aggregation, crosslinking, water insolubilization, and lead to cataract development. A recent study of water insoluble (WI) proteins from human lenses [1,2] showed that the major in vivo modifications of human WI crystallins occurred in αA-crystallin, which included disulfide bonding, deamidation, methionine oxidation, and backbone cleavage.

One common end product of crystallin modifications in aging and cataractous lenses is the crosslinked supramolecular weight species that plays a central role in the cataractogenic process [3]. The WI protein fraction contains aggregated and covalently crosslinked supramolecular protein species that could cause light scattering. Benedeck [4] has shown that the protein aggregates of M_r >5x10^6 would cause significant light scattering. Indeed, the high molecular weight aggregates have been demonstrated in vivo by NMR spectroscopy [5] and by quasielastic light scattering [6], and the sizes of these particles were determined to be between 0.12-0.9 mm in both normal and cataractous lenses with an estimated M_r of 2x10^9 Dalton. An intermediate state between WS and WI proteins exists in the form of the water soluble-high molecular weight (WS-HMW) proteins [7-9], and its existence in vivo has also been demonstrated by light scattering methods [7].

Several reports have described the composition of both WS-HMW and WI proteins, and potential mechanisms for aggregation and crosslinking of crystallins. The WS-HMW protein of human lenses contain the three crystallins (α-, β-, and γ-crystallins), and also crystallin fragments [7-9]. The WI protein fraction is much more complex because it contains modified crystallins and their fragments in aggregated and crosslinked forms [1]. Based on posttranslational modifications of crystallins, a variety of mechanisms for the formation of the crosslinked species are proposed [3,10], but the relative roles of modifications remain unclear. The modifications of crystallins include disulfide bonding [11], glycation [12], oxidation of Trp and His residues [13,14], deamidation [15], transglutaminase mediated crosslinking [16], racemization [17], streoinversion [18], and phosphorylation [19].

Past studies have suggested a potential role of crystallin fragments in protein aggregation and the crosslinking processes. This evidence includes reports from our laboratory

Correspondence to: Om P. Srivastava, Department of Physiological Optics, Worrell Building, 924 S-18th Street, University of Alabama at Birmingham, Birmingham, AL, 35294; Phone: (205) 975-7630; FAX: (205) 934-5725; email: Srivasta@uab.edu

demonstrating an age-related increase in the levels of crystallin fragments in both WS-HMW proteins (5-6% of total protein in 16 to 19 year old compared to 27% in the 60 to 80 year old) [9], and also in the WI proteins (up to 20% of total protein) [20]. Further, the fragments of α-, β-, and γ-crystallins were found to accumulate in both WS-HMW and WI protein fractions with aging. The fragments showed crosslinking in vitro via non-disulfide bonding and their crosslinked species existed at increasing levels in the WI proteins with aging [20]. Several additional reports suggesting such a potential role of crystallin fragments included: (i) the crystallin fragments were present in the opaque but not in the clear portion of a human brunescent cataractous lens [21], (ii) the C-terminally truncated bovine αA-crystallin species formed oligomeric complexes of a much higher molecular weight compared to those formed by the native species [22], (iii) the in vitro proteolysis of rat lens soluble proteins by calpain caused a rapid increase in turbidity that was inhibited by E-64, an inhibitor of calpain-type cysteine proteases [23-25], (iv) human cataract specific HMW-aggregates contained a heterogeneous 10 kDa breakdown product in addition to 20 and 43 kDa components [26], (v) a human lens 10 kDa polypeptides was found to be glycated and was believed to play a major role in protein aggregation and insolubilization [27], and (vi) recently, we identified a covalent multimer (M_r>90 kDa) in aging human lenses, which contained only crystallin fragments (unpublished).

To ascertain a potential role of crystallin fragments in aggregation and crosslinking of crystallins, it is important to identify and characterize properties of those fragments that may initiate such process in vivo. We have isolated and purified an in vivo generated 9 kDa γD-crystallin fragment containing residues 87-173 [28]. The polypeptide showed covalent modification at the C-terminal region [29], and existed as three isoforms due to posttranslational modifications [30]. The results suggested that the polypeptide might serve as model species to determine the potential role of an in vivo generated crystallin fragment in the crosslinking process. In this report, we describe the crosslinking properties of the 9 kDa γD-crystallin fragment alone and with crystallins, identify the polypeptide-derived crosslinked multimers in vivo, and investigate posttranslational modifications in the polypeptide.

METHODS

Materials: Normal human lenses with no apparent opacity were obtained from either the Lions Eye Tissue Bank of Missouri or from Dr. Robert Church, Emory University. The lenses were retrieved within 48 h post mortem and stored in Medium-199 without phenol red at -20 °C until utilized.

Prestained and unstained molecular weight protein markers were from GIBCO/BRL (Life Technologies, Rockville, MD) and Amersham Biosciences (Piscataway, NJ), respectively. Unless indicated otherwise, all remaining chemicals used in this study were purchased from Sigma (St. Louis, MO) or Fisher (Atlanta, GA).

Isolation of water soluble (WS) and water insoluble (WI) proteins: The WS and WI proteins were isolated essentially by a method previously described [28]. Briefly, the WS proteins were recovered following disruption of lens cells and solubilization in buffer A (50 mM Tris-HCl, pH 7.9, containing 1 mM phenylmethylsufonyl fluoride, 1 mM iodoacetamide and 10 mM dithiothreitol) [28] and centrifugation at 27,000x g for 15 min at 5 °C. The pellet was solubilized in buffer A and centrifuged as above two additional times. The supernatants recovered after each centrifugation were pooled and designated as the water soluble (WS) protein fraction, and the pellet recovered after the third centrifugation was designated as the water insoluble (WI) protein fraction [20].

The WS protein preparation from a 10 year old donor was fractionated by size-exclusion HPLC using a TSK G-3000 PW_{XL} column to recover α-, β_H-, and γ-crystallins. The column equilibration and sample elution were carried out by 50 mM Tris-HCl, pH 7.9. The crystallins were identified in the column fractions by SDS-PAGE [31], concentrated by lyophilization, dialyzed at 5 °C against 0.05 M phosphate buffer, pH 7.0, and used for crosslinking experiments as described below.

Isolation of a mixture of crystallin fragments and purification of a 9 kDa polypeptide: A mixture of crystallin fragments was isolated from the WS protein fraction of human lenses (60-80 year old donors) by a preparative SDS-PAGE method as described previously [28]. The preparative SDS-PAGE [31] was carried out using a 3-mm thick gel and, by using the prestained molecular weight protein markers, the gel portion containing 3-14 kDa polypeptides was excised and suspended in buffer A. The gel was broken into tiny pieces by passage through a 10 mm syringe (without needle). The preparation was incubated at 37 °C for 3 h to solubilize polypeptides, and then was filtered through a 0.45 μm filter. The solubilized polypeptides were dialyzed against double distilled water at 5 °C, concentrated by lyophilization, and dissolved in 0.05 M phosphate buffer, pH 7.0.

To isolate the 9 kDa γD fragment, a previously published method was used [28]. The WS protein fraction from lenses of 60-80 year old donors was lyophilized to dryness, dissolved in buffer B (50 mM Tris-HCI, pH 7.9 containing 7 M urea and 5 mM β-mercaptoethanol), and subjected to Sephadex G-50 column chromatography under denaturing conditions at room temperature. Column equilibration and elution were carried out using buffer B. The column fractions containing the 9 kDa polypeptide (ascertained by SDS-PAGE) were pooled, dialyzed to remove urea at 5 °C against three changes of 2,000 volume excess of 0.05 M Tris-HCI, pH 7.9 for 48 h, and concentrated by lyophilization. The preparation was further separated by a preparative SDS-PAGE method [28], and the 9 kDa containing fractions were pooled, concentrated as above, dialyzed against 1,000 volume excess of 0.05 M phosphate buffer, pH 7.0, and the purified polypeptide was used for crosslinking experiment as described below. The three isoforms of the 9 kDa polypeptide were also isolated as described previously [29,30] by a non denaturing gel electrophoretic method using a Bio-Rad Prep Cell (Model 509, Bio-Rad, Hercules, CA). The eluted fractions, containing the three isoforms, were subjected to SDS-PAGE using a 3 mm thick, 15% polyacrylamide gel. The three isoforms were eluted from the gel using a

Bio-Rad whole gel eluter. The eluted proteins were examined by SDS-PAGE for their purity.

Incubation of a mixture of crystallin fragments, the 9 kDa γD-crystallin polypeptide, or 9 kDa polypeptide plus individual crystallins during crosslinking experiments: Preparations of a mixture of crystallin fragments, the purified 9 kDa γD-polypeptide, or the 9 kDa polypeptide plus individual crystallins isolated from lenses of a 10 year old donor were incubated at 37 °C using an identical protocol. Each preparation was filter sterilized by passage through a 0.22 μm filter (Whatman, Clifton, NJ), and incubated under sterile conditions at 37 °C in a water bath or an incubator. The preparations (in 0.05 M phosphate buffer) were incubated under fluorescent day light lamp (15 W) with a radiation intensity of about 2-5 Wm^{-1} in light or in the dark (tubes wrapped with aluminum foil). The samples were withdrawn at the desired time intervals under sterile conditions, and kept frozen at -20 °C until utilized. The preparations were examined for crosslinked products by the SDS-PAGE method of Laemmli [31] following treatments with sample buffer containing 200 mM β-mercaptoethanol to disrupt disulfide linked species. The gels were either stained with Coomassie blue or with silver stain using a kit from BioRad.

Western blot analysis: From the published sequence of human lens γ-crystallins [32], it was identified that the G_{86}-S_{87} bond in γD-crystallin was cleaved to produce a 9 kDa polypeptide with residues 87 to 173 [28]. Based on the N- and C-terminal sequences of the 9 kDa polypeptide (residues 87-173 of human γD-crystallin), individual nonapeptides representing the N- and C-terminal regions were synthesized by Research Genetics (Huntsville, Alabama). The tenth amino acid in each peptide was cysteine, and it was utilized for peptide linkage to Keyhole Limpet Hemocyanin (KLH). The N-terminal peptide (S-H-R-I-R-L-Y-E-R-C; residues 87-95 of human γD-crystallin) and the C-terminal peptide (C-S-L-R-R-V-I-D-F-S; residues 166-174 of human γD-crystallin) were linked to maleimide activated KLH (Pierce Chemical Company, Rockford, IL) by the procedure provided by manufacturer (Bulletin 77125). These KLH-conjugated peptides were injected in rabbits by standard procedures to develop polyclonal antibodies. Using exactly the above described procedure, two additional site specific polyclonal antibodies, anti-γD (residues 1-9) antibody and anti-γD (residues 78-86 [minus 9 kDa polypeptide] antibody) were also raised. Western blot analysis was performed by the procedure of Towbin et al. [33]. Each antibody was utilized at a dilution of 1:200. Preimmune serum was always utilized as a control. The immunoblots were stained for peroxidase by a standard procedure following the secondary antibody (anti-rabbit IgG-peroxidase conjugate) reaction. The immunoreactive species were visualized either following reactivity with [^{125}I]-protein A as described by Burnett [34] or by immunoreactivity with peroxidase-conjugated anti-rabbit IgG prepared in goat (diluted 1:1000). Protein A was radioiodinated with Iodo-beads using the procedure described by Pierce. To quantify the immunoreactive spots, the autoradiogram was overlapped onto the nitrocellulose sheet and the desired lighted (immunoreactive) spots were excised with a scalpel and counted in an LSC radioactive counter.

Two dimensional gel electrophoresis and mass spectrometric analyses: The 9 kDa isoforms were analyzed by two-dimensional (2D) gel electrophoresis prior to mass spectrometric analysis. Each isoform was dissolved in resolubilization buffer [35] (5 M urea, 2 M thiourea, 2% 3-[C3-cholamidoproyl] dimethyl-ammonio-1-propansulfonate [CHAPS], 2% caprylyl sulfobetaine 3-10, 2 mM tri-butyl phosphine, 40 mM Tris, pH 8.0), and subjected to 2D gel electrophoresis (IEF in the first dimension and SDS-PAGE in the second dimension). IEF separation was carried out using Immobiline Dry Strips (pH range of 3-10) following the manufacturer's method (Amersham Biosciences), and SDS-PAGE was performed by the Laemmli [31] method. Prior to the first dimension IEF, the dry strips were incubated overnight with desired WI protein fraction at room temperature. Following the IEF separation, the strips were consecutively treated for 15 min each, first with 100 mM dithioerythreitol (in equilibration buffer, 0.1 M Tris, pH 6.8, containing 6 M urea, 30% glycerol, and 1% SDS) and next with 300 mM iodoacetamide (also dissolved in equilibration buffer). During SDS-PAGE, a 15% polyacrylamide gel of 16x14 cm (WxH) was used. The protein spots on a gel were stained with Coomassie blue.

For mass spectrometric analyses, the individual protein spots were excised from a polyacrylamide gel, washed with doubly deionized water, and destained after treating with ammonium bicarbonate and acetonitrile. Trypsin solution (12 ng/μl) was added, and the preparation was resuspended in 25 mM ammonium bicarbonate, pH 7.8. The samples were digested by trypsin at 37 °C overnight, and the next day they were analyzed by matrix assisted laser desorption ionization-time of flight (MALDI-TOF, Model Voyager-DE2 PRO, Pespective Biosciences, Foster City, CA). The MALDI-analysis and ES-MS/MS sequencing (Micromass QTOF-2) were performed at the Comprehensive Cancer Center Mass Spectrometric Shared Facility of the University of Alabama at Birmingham. The MALDI-TOF identity of proteins was established by using mass fingerprinting as implimented in the Mascot program at (Matrix Science). During the ES-MS/MS analysis of the tryptic fragments, the "Proteinlynx Global Server" data base (Micromass software, Micromass UK, Ltd., Manchester, UK) was used along with manual interpretations as needed in certain cases.

Miscellaneous methods: Protein concentrations were determined by a modified Lowry method using bovine serum albumin as a standard [36]. The amino acid composition analysis of a mixture of crystallin fragments was performed by the method of Gerhke et al. [37] at the Agriculture Experimental Station of the University of Missouri-Columbia. Proteins on gels were stained with silver stain using a kit purchased from BioRad or with Coomassie blue.

RESULTS

Crosslinking of WS crystallin fragments on incubation: In a previous report [20], we demonstrated extensive covalent crosslinking of a mixture of crystallin fragments (M_r 3-14 kDa,

isolated from either the WS or WI protein fractions of human lenses [60-80 year old donors]) following storage at 4 °C for 10 days. The crosslinked fraction, on size exclusion Agarose A 1.5 column chromatopgraphy, eluted as a void volume peak (M_r>1.5x10^6 Dalton) along with intermediate species between 10 kDa and 1,500 kDa (not shown). On SDS-PAGE analysis of the void volume peak fractions (after treatment with 200 mM β-mercaptoethanol), protein bands between 10 kDa to 100 kDa were seen (not shown). Furthermore, an incubation of a mixture of crystallin fragments for an extended period of time (30 days), either at 4 °C or at 37 °C, the mixture became water insoluble. Together, the results suggested that the crystalin fragments crosslinked via non-disulfide bonding during in vitro incubation, and also became water insoluble.

In a separate experiment, a mixture of crystallin fragments (M_r 3-14 kDa) was incubated at 37 °C for 21 days under sterile conditions and a time dependent crosslinking into multimeric species was observed during SDS-PAGE analysis. These multimers, because of their large sizes, failed to enter a 15% polacrylamide gel (Figure 1A, lanes 8 and 9). These multimers were held via non-disulfide covalent bonding because the preparations were treated with 200 mM β-mercaptoethanol prior to SDS-PAGE analysis.

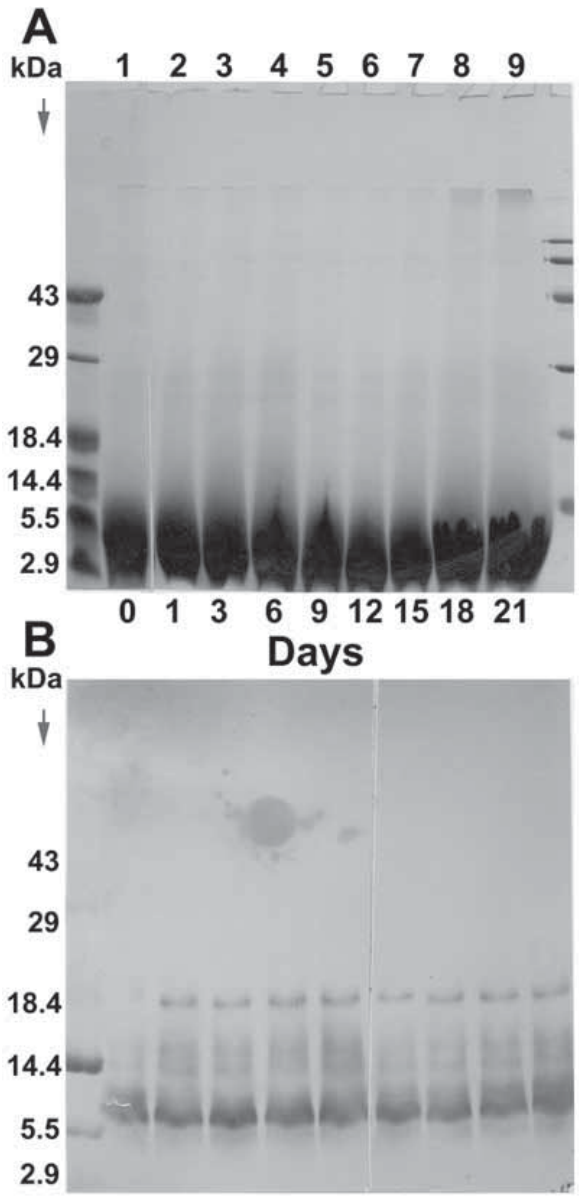


Figure 1. Analysis of crosslinked products of a mixture of crystallin fragments. A mixture of crystallin fragments was incubated at 37 °C under sterile conditions and aliquots were withdrawn at time intervals indicated at the bottom of gel **A**. **A**: Silver staining of a mixture of crystallin fragments (15 μg) following SDS-PAGE. **B**: A western blot of crosslinked fractions of **A** following immunoreactivity with anti-γ-crystallin antibody.

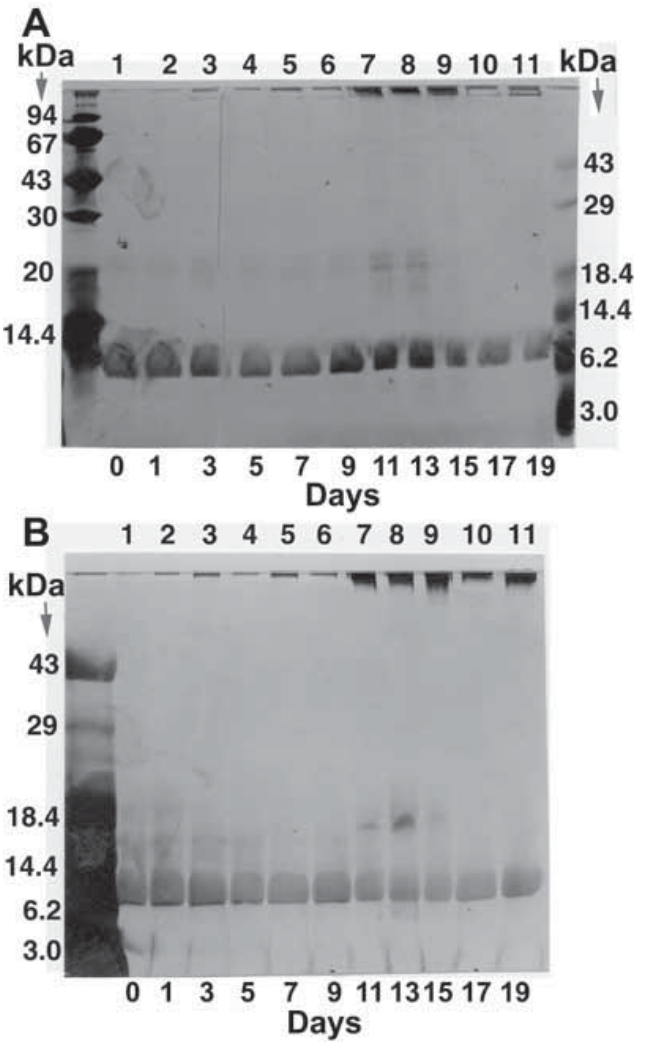


Figure 2. Analysis of products generated during incubation of purified 9 kDa γD-crystallin polypeptide. The purified 9 kDa polypeptide (10 μg) was incubated at 37 °C for up to 19 days under light and dark conditions. Aliquots were withdrawn at the time intervals as shown at the bottom of the gels. **A**: Silver staining of the crosslinked species of the 9 kDa polypeptide produced during incubation in light, and **B**; Silver staining of crosslinked species produced during incubation in dark.

The temporally derived crosslinked multimers of crystallin fragments were analyzed by western blot to determine whether a 9 kDa γD-crystallin polypeptide (previously studied in our laboratory [28-30]), was involved in the crosslinking process. The immunoblot with anti-γ-crystallin antibody showed that an 18 kDa species (possibly a dimer of the 9 kDa polypeptide) appeared within 24 h of incubation (Figure 1B, compare lanes 1 and 2). Additional multimer species of higher M_r also appeared during further incubation, but they showed a lack of immunoreactivity with the antibody (Figure 1A,B, lanes 8 and 9).

Crosslinking of a 9 kDa γD-crystallin fragment: To further ascertain whether the 9 kDa γD-crystallin fragment had a crosslinking property, crosslinking of the 9 kDa γD-polypeptide in vitro was determined. The 9 kDa polypeptide, purified as described previously [28], showed increased crosslinking in a time dependent manner with incubation at 37 °C under sterile conditions in either light or dark conditions (Figure 2A,B). These crosslinked products were held via non-disulfide bonding as the multimers did not dissociate following treatment with 200 mM β-mercaptoethanol. In addition, as levels of the crosslinked species increased with time, a concomitant decrease in the levels of the 9 kDa polypeptide occurred (Figure 2A,B). A major crosslinked species of 18 kDa (a possible dimer of the 9 kDa polypeptide) appeared during day 3 to 13 (Figure 2A,B, lanes 3 to 8) of incubation but the

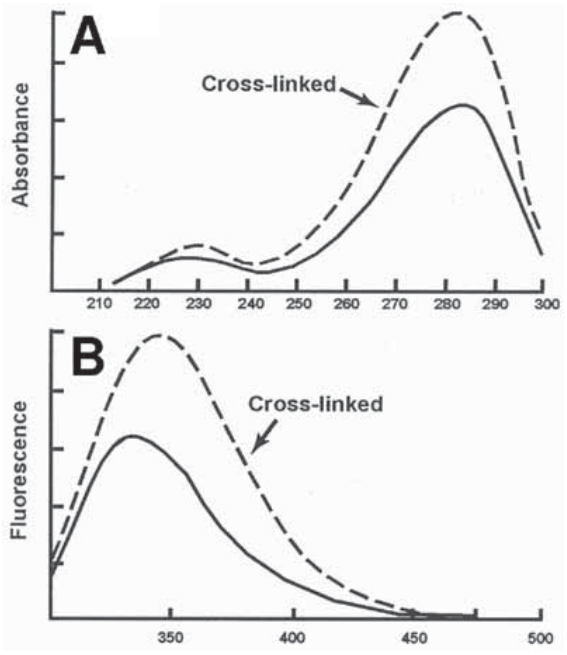
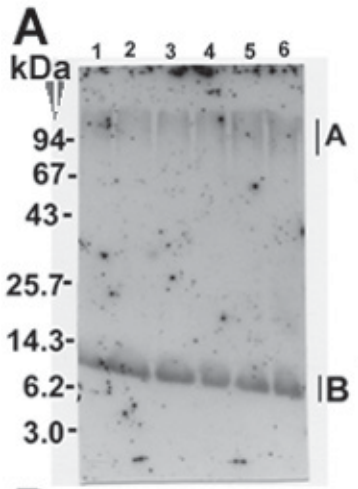
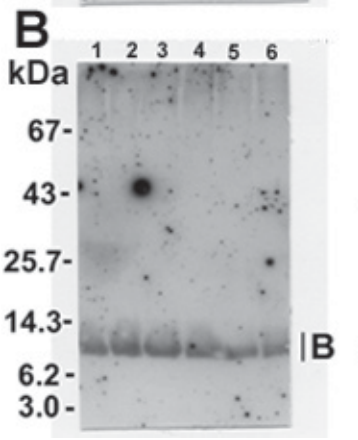


Figure 4. Absorption and fluorescence spectra of native and crosslinked species of the 9 kDa polypeptide. **A**: Absorption spectra, and **B**: Fluorescence spectra following excitation at 280 nm. The dotted and solid lines represent the crosslinked (19 days of incubation in the dark) and native (zero time) species of the 9 kDa polypeptide, respectively.



Lanes	1	2	3	4	5	6
Slice A (cpm x 10-3)	14.2	14.5	18	20.5	22.5	22
Slice B (cpm x 10-3)	20.6	18.2	18	16	12.8	14.3
Days	0	3	5	7	11	15



Lanes	1	2	3	4	5	6
Slice B (cpm x 10^{-3})	22	18	14	13	12	11
Days	0	3	5	7	11	15

Figure 3. Analysis of crosslinked 9 kDa polypeptide species. Immunoblots following reactivity with: **A** anti-γ-crystallin antibody and **B** anti-9 kDa polypeptide antibody. Both blots were reacted with radioiodinated protein A as a secondary antibody to visualize and quantify the immunoreactive proteins. The A and B portions of immunoblot **A**, and the B portion of immunoblot **B** were excised and counted for radioactivity. These counts are shown in the adjacent tables. Preparations used for western blot analysis in lanes 1 to 6 included the following samples incubated for the length of time as shown in Figure 2B: lane 1 is day 3, lane 2 is day 7, lane 3 is day 9, lane 4 is day 13, lane 5 is day 15, and lane 6 is day 19.

level dramatically increased at day 11 to 13 under both light and dark conditions (lanes 7 and 8 in Figure 2A,B). A further incubation after 13 days generated major crosslinked species having a M_r>100 kDa, which failed to enter the gel (Lanes 7-11 in Figure 2A,B). Additional minor multimeric species having intermediate molecular weights between 18 kDa and 100 kDa were also observed during the time course of incubation. Under both light and dark conditions the time course of appearance of multimers and their M_r were almost identical, suggesting that crosslinking was not a light dependent process.

Because the 9 kDa polypeptide was a fragment of γD-crystallin (cleavage at G_{86}-S_{87}, residues 87-173) [28], the crosslinked species of the polypeptide was quantified by western blot analysis using anti-γ-crystallin and anti-9 kDa polypeptide antibodies and radioiodinated protein A as a secondary antibody. Although the 9 kDa species showed the strongest immunoreactivity with both antibodies, the immunoreactivity of the crosslinked species with M_r>100 kDa (at the top of the gel) was greater with only the anti-γ-crystallin antibody (Figure 3A,B). In order to quantify, the two immunoreactive regions representing bound [^{125}I]-protein A in the autoradiogram (region A [representing crosslinked species of >90 kDa] and region B [representing the monomeric 9 kDa species]), were excised from the nitrocellulose paper and counted. A time dependent decrease in cpm associated with the monomeric 9 kDa species with a concomitant increase in the radioactivity of crosslinked multimers of >90 kDa was observed (Figure 3A,B and the adjacent tables).

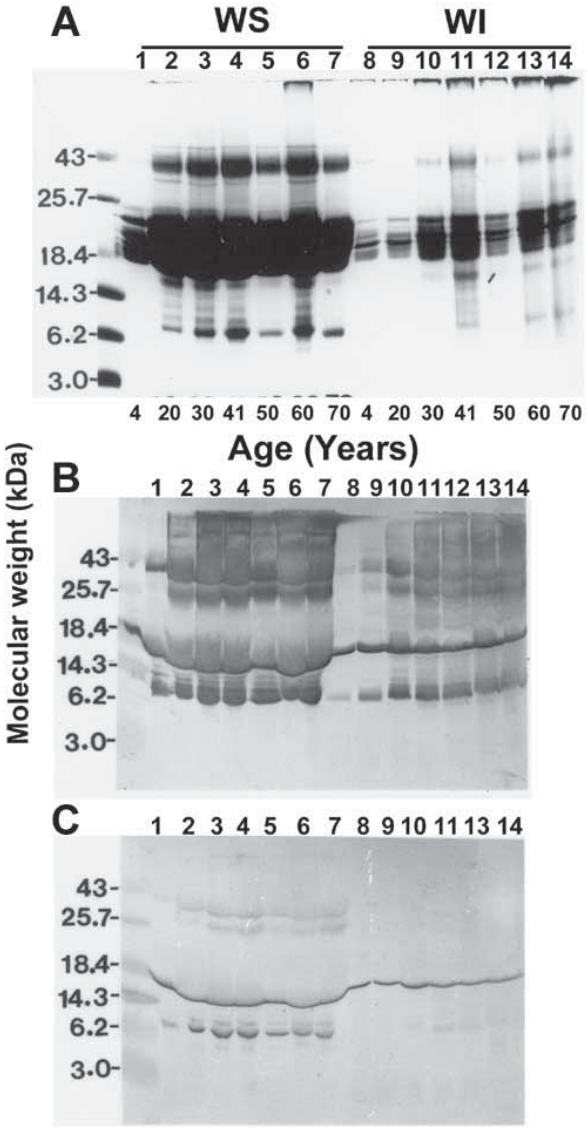


Figure 6. Analyses of WS and WI proteins isolated from lenses of donors of different ages. **A**: Coomassie blue stained protein species (20-50 μg proteins used). **B** and **C** are immunoblots of the protein fractions as shown in **A** following immunoreactivity with anti-9 kDa N-terminal and anti-9 kDa C-terminal antibodies, respectively. Proteins in **A** were stained with Coomassie blue.

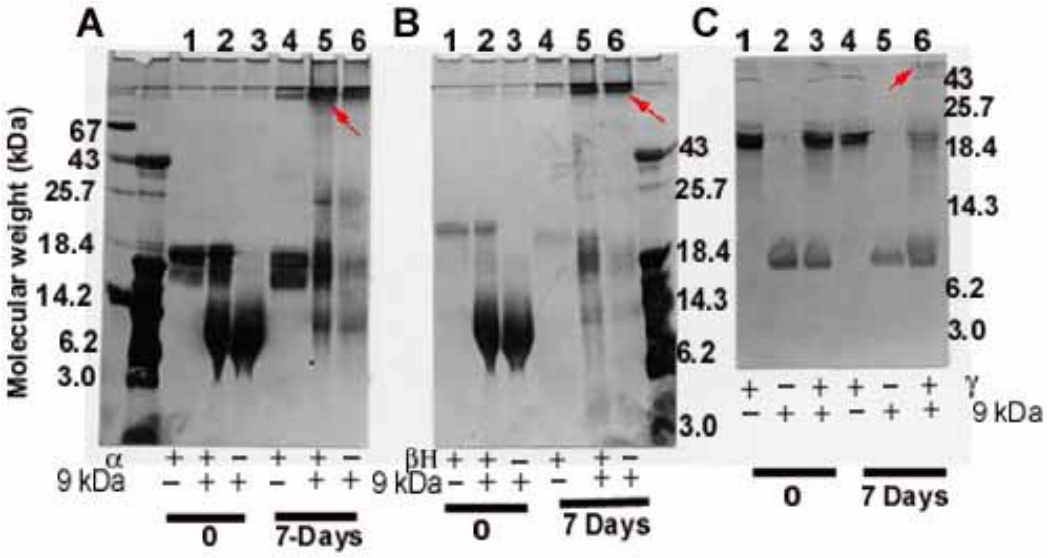


Figure 5. SDS-PAGE analysis of crosslinked species produced following incubation of the 9 kDa polypeptide with crystallins. **A**: α-Crystallin (15 μg) and 9 kDa polypeptide (15 μg). **B**: β_H-crystallin (6 μg) and 9 kDa polypeptide (15 μg) and **C**: γ-Crystallin (10 μg) and 9 kDa polypeptide (10 μg). The constituents of each of the incubated mixture are shown at the bottom of the gel. In these experiments, as apparent from the above quantities, the individual crystallins and the polypeptide were incubated at the ratio of 1:1 or 1:2. The protein species on gels were stained with silver stain.

A change in the absorption spectra of the crosslinked 9 kDa polypeptide compared to non-crosslinked species was also observed (Figure 4). The absorption spectra showed an increased absorption at 280 nm following crosslinking (Figure 4A), and following excitation of both the preparations at 280 nm, a shift in the emission peak from 334 nm to 342 nm following crosslinking was also observed (Figure 4B). The data suggested an exposed tryptophan residue after crosslinking.

Crosslinking of the 9 kDa polypeptide with human lens crystallins: The purified 9 kDa polypeptide was incubated with either α-, β_H-, or γ-crystallins (isolated from lenses of a 10 year old donor) for 7 days at 37 °C under the above described sterile and dark conditions and the crosslinked species were examined by SDS-PAGE. The 9 kDa polypeptide and α-crystallin produced crosslinked multimers with M_r of about 27 kDa and those >90 kDa (Figure 5A, identified by an arrow at the top of the gel), and levels of these crosslinked species were lower in the individually incubated preparations of 9 kDa polypeptide or α-crystallin (Figure 5A, compare lane 5 with lanes 4 and 6). Similarly, the 9 kDa polypeptide incubated with β_H-crystallin produced multimers with M_r >90 kDa, but the multimer level was almost the same as seen with the 9 kDa polypeptide alone (Figure 5B, compare lanes 5 and 6). Following incubation of γ-crystallin with 9 kDa polypeptide the multimers of M_r >90 kDa (Figure 5C, lane 6, identified with an arrow), were at relatively higher levels than those from individual γ-crystallin or the 9 kDa preparations (Figure 5C, compare lane 6 with 4 and 5). Further, following incubation of the 9 kDa polypeptide alone, generally three major species of 18 kDa (a dimer), 29 kDa (a trimer) and >90 kDa (a multimer) were observed (lane 6 in Figure 5A,B). However, as shown in Figure 5C, on a similar incubation of the 9 kDa polypeptide alone, the 18 kDa and >90 kDa species were observed but the 27 kDa species was not seen. This could be due to a lower quantity of the 27 kDa species, which could not be stained. This is evident from the comparison of the relative quantities of 9 kDa polypeptide used during incubation with α- and β_H-crystallins and with γ-crystallin (Compare lane 5 in Figure 5A,B with lane 6 in Figure 5C).

Determination if crosslinked species of the 9 kDa γD-crystallin fragment exist in the WS and WI proteins: The existence of covalent multimers of the 9 kDa polypeptide in the WS and WI proteins, isolated from lenses of donors of different ages, was determined by western blot using anti-9 kDa N-

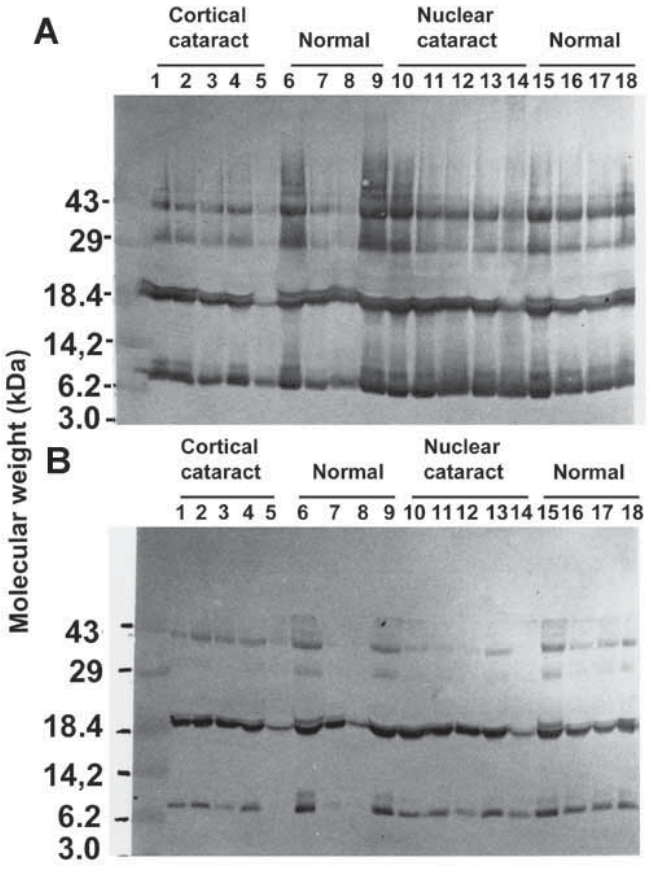


Figure 7. Analysis of WS proteins from lenses with cortical and nuclear cataracts. Age matched with normal lenses using **A** anti-9 kDa N-terminal- antibody and **B** anti-9 kDa C-terminal antibody. Identical quantities of WS proteins (40 μg) were used from these lenses.

Table 1. Total amino acid composition of crystallin fragments, lens MIP26K, αA-, and αB-crystallin

Amino acids	Residues per 1000 residues: Crystallin fragments	MIP26 (bovine)	αA2 (bovine)	αB2 (bovine)
Non-polar				
Ala	79.6	121.7	38.2	55.9
Val	19.0	95.1	50.9	68.3
Leu	61.1	144.5	70.1	74.5
He	10.9	34.2	57.3	62.1
Pro	151	49.4	70.1	80.7
Met	10.9	19.0	6.3	6.2
Phe	118	68.4	82.8	74.5
Trp	27.6	19.0	6.4	12.4
Polar (uncharged)				
Gly	121	98.9	63.7	49.6
Ser	75	72.2	140.1	105.6
Thr	12.1	49.4	31.9	37.3
Cys	15	11.4	6.4	-
Tyr	27.6	30.4	31.8	12.4
Gln	-	30.4	12.7	12.4
Asn	-	30.0	19.11	18.6
Negatively charged				
Asp	151	7.6	82.8	62.11
Glu	27.1	34.2	70.1	86.9
Positively charged				
Lys	14.6	11.4	31.8	43.5
Arg	35	49.8	82.8	80.8
His	8.1	22.8	44.6	55.9
Percent hydrophobic amino acids	61.8	69	38.2	44.7

The crystallin sequence was calculated from published amino acid sequences. Hydrophobic amino acids were calculated based on Eisenberg method [37].

terminal and anti-9 kDa C-terminal antibodies as previously described [29,30]. The Coomassie blue stained protein profiles of WS and WI proteins from lenses of different ages is shown in Figure 6A, and western blot profiles with anti-9 kDa N-terminal and anti-9 kDa C-terminal antibodies are shown in Figure 6B,C. Following immunoreactivity with anti-9 kDa N-antibody, both WS and WI proteins from lenses of different ages typically showed species of 9, 20, 27, and 45 kDa, but the 27 kDa species was absent in the WS and WI proteins of lenses from a 4 year old donor (Figure 6B lanes 1 and 8). This suggested a posttranslational appearance of the 27 kDa species, and it could represent a trimer of the 9 kDa polypeptide (previously seen during in vitro incubation as in Figure 4) or the 9 kDa plus a yet unknown crystallin fragment.

Western blot analysis of both WS and WI protein fractions with anti-9 kDa C-terminal antibody showed a different immunoreactive protein profile (Figure 6C). The WS protein fractions contained four major immunoreactive species of 9, 20, 27, and 45 kDa (Figure 6C, lanes 1-7). In the WS proteins from the 4 year old donor, the 27 and 45 kDa species were absent, again suggesting their posttranslational appearance (Figure 6C, lane 1). Further, the WI proteins of these lenses showed only an immunoreactive 20 kDa γ-crystallin The 27 and 45 kDa species were absent, and the 9 kDa species was seen only in the WI protein fraction of lenses of >20 year old (Figure 6C, lanes 8-14). A previous report from our laboratory showed that the lack of immunoreactive anti-9 kDa C-terminal antibody was due to modifications of amino acids at the C-terminal region of the 9 kDa polypeptide [29,30].

To ascertain the species that may not be generated from the 9 kDa polypeptide, western blot analysis of WS and WI protein fractions from lenses of different ages (21, 30, 42, 50, and 61 year old donors) was carried out using two additional site specific polyclonal antibodies (anti-γD [residues 1-9] antibody and anti-γD [residues 78-86, minus the 9 kDa polypeptide] antibody). The rationale was that these antibodies would immunoreact with species that were not produced by crosslinking of the 9 kDa γD polypeptide containing residues 87-173 of the γD-crystallin. Western blot results with the WS proteins showed immunoreactive species of 11 and 14 kDa along with species of 20 kDa and 43 kDa, but the 27 kDa species was not observed. This is consistent with the previous western blot results from Figure 6B with anti-N-terminal (residues 87-95) 9 kDa γD-polypeptide antibody and anti-C-terminal (residues 166-174) 9 kDa γD-polypeptide antibody, suggesting that a major immunoreactive species of 27 kDa might be derived from the 9 kDa polypeptide as a result of crosslinking.

The crosslinked species of the 9 kDa polypeptide in the WS proteins from lenses with cortical or nuclear cataracts, or age matched normal lenses was also examined using the above anti-9 kDa N-terminal and anti-C-terminal antibodies (Figure 7). After immunoreactivity with anti-9 kDa N-terminal antibody, both WS proteins from cataractous and normal lenses showed multimers of 27 and 45 kDa species as well as immunoreactivity with 9 kDa and 20 kDa species (Figure 7A). Following immunoreactivity with anti-9 kDa C-terminal antibody, the 45 kDa species was the major immunoreactive species with a reactive 27 kDa species in both cortical and nuclear cataractous lenses (Figure 7B lanes 1-5 and 10-14), but normal lenses

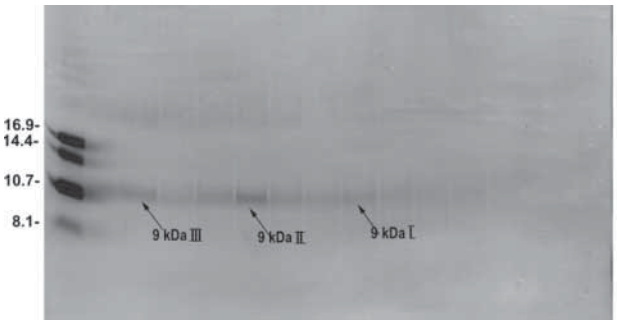


Figure 8. Analysis of three isoforms of the 9 kDa polypeptide (shown as isoform I, II and III). The isoforms were isolated by a preparative non-denaturing gel electrophoretic method as described in Methods. The isoforms were collected following electrophoresis in different fractions and the fractions were examined by SDS-PAGE to locate the fraction containing the three isoforms. The 9 kDa isoform I eluted first from a non-denaturing gel during electrophoresis followed by isoforms II and III.

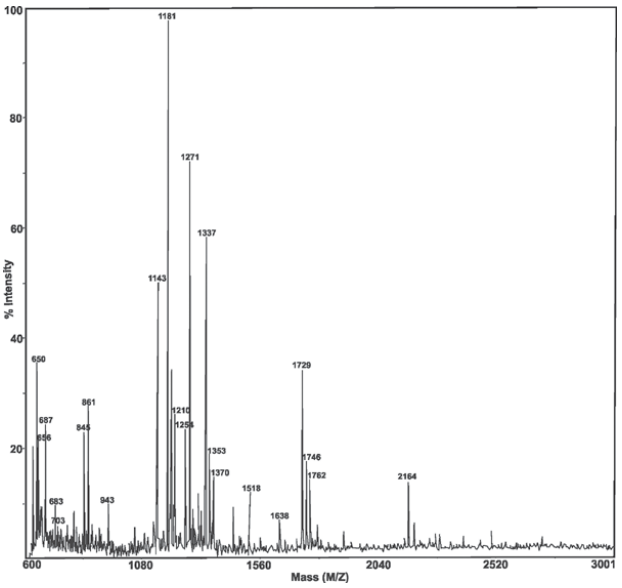


Figure 9. MALDI-TOF profile of 9 kDa isoform I. The MALDI-TOF profiles of the 9 kDa isoform II and III were similar to the profile 9 kDa isoform I. Note that among the four expected tryptic fragments of 9 kDa polypeptide at the C-terminal regions (fragments RYQDWGATNAR [residues 153-163, mass 1,337], YQDWGATNAR [residues 154-163, mass 1,181], YQDWGATNARVGSLR [residues 154-168, mass 1,693], and VGSLRRVIDFS [residues 164-174, mass 1,248]), the tryptic fragments with mass of 1181 and 1337 were present but tryptic fragments with mass of 1248 and 1693 were absent. The results suggested that the tryptic fragment after residue number 163 was absent in the 9 kDa isoforms.

showed low immunoreactive 27 and 45 kDa species (Figure 7B, lanes 6-9 and 15-18), and also very low reactivity with the 9 kDa polypeptide.

Determination of amino acid modifications in the 9 kDa polypeptide: The assumption in this study was that some of the crystallin fragments must have certain unique characteristics that might lead to their crosslinking upon incubation. To ascertain this, the amino acid composition of a mixture of crystallins, isolated from WS and WI urea soluble protein fractions of lenses from 50-70 year old donors was determined (Table 1). The hydrophobic amino acid contents were determined by the Eisenberg et al., method [38]. The hydrophobic amino acid content of WS crystallin fragments was 40.2%, similar to hydrophobic amino acid contents of the most hydrophobic crystallin, αA- (38.2%) and αB-crystallin (44.7%) [39]. More interestingly, the crystallin fragments from the WI protein fraction showed an even higher hydrophobic amino acid contents, (50%) but it was lower than MIP-26 (69%). These data suggest the possibility that the high hydrophobicity of the polypeptides cause their aggregation and subsequent crosslinking.

To ascertain whether posttranslational modifications of amino acids in the 9 kDa polypeptide were responsible for its crosslinking property, amino acid modifications were examined by MALDI-TOF and ES-MS/MS methods. Because the polypeptide existed as three isoforms [31], these isoforms were purified and used in the analysis. The 9 kDa I, II, and III frations were isolated by an alternative method than reported previously [30]. The three isoforms were eluted after non-denaturing preparative gel electrophoresis as shown in Figure 8, and described in Methods. Examination of the 9 kDa isoforms I, II, and III by 2D gel electrophoresis showed a single major 9 kDa spot on a gel (not shown). The individual isoform spots (Figure 8) were excised from 2D gels, digested with trypsin, and the tryptic fragments were examined by MALDI-TOF. The results showed that isoforms I, II, and III were fragments of human γD-crystallin. A typical MALDI-TOF profile represented by 9 kDa isoform I is shown in Figure 9. In a previous report [31], we showed that the three isoform contained an identical N- terminal sequence of SHRIR, (with cleavage at G_{86}-S_{87}) in γD-crystallin with residues 87-173. The tryptic peptides recovered were analyzed for their C-terminal regions. A database search for tryptic fragments from the 9 kDa polypeptide showed the presence of the following four tryptic peptides from the C-terminal regions; RYQDWGATNAR (residues 153-163, mass 1,337), YQDWGATNAR (residues 154-163, mass 1,181), YQDWGATNARVGSLR (residues 154-168, mass 1,693), and VGSLRRVIDFS (residues 164-174, mass 1,248). An examination of the MALDI-TOF tryptic profiles of the three isoforms showed an absence of tryptic fragments with a mass of 1,248 and 1,693, but fragments with a mass of 1,181 and 1,337 were present. The data suggested that a tryptic fragment after residue number 163 was absent, and therefore, each isoform was C-terminally truncated and contained only residues 87 to 163, instead of residues 87 to 173 as originally believed [30]. Upon determination of amino acid modifications by ES-MS/MS using QTOF-2, the Methionine residue was found to be oxidized in the tryptic fragment with a sequence of QYllMPGDYR, having a mass of 1,412 (Figure 10). A similar analysis showed oxidized tryptophan

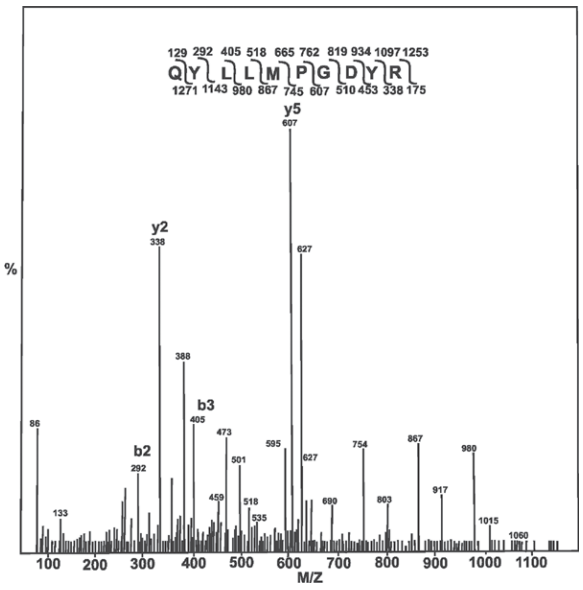


Figure 10. ES-MS/MS analysis of a tryptic fragment with mass of 628 (in QTOF) and 1,272 (in MALDI-TOF) from the 9 kDa I isoform. Note the oxidation of M-146 residue in γD-crystallin isofrom I. A similar oxidation of methionine residue was also seen in the 9 kDa isoforms II and isoform III (not shown).

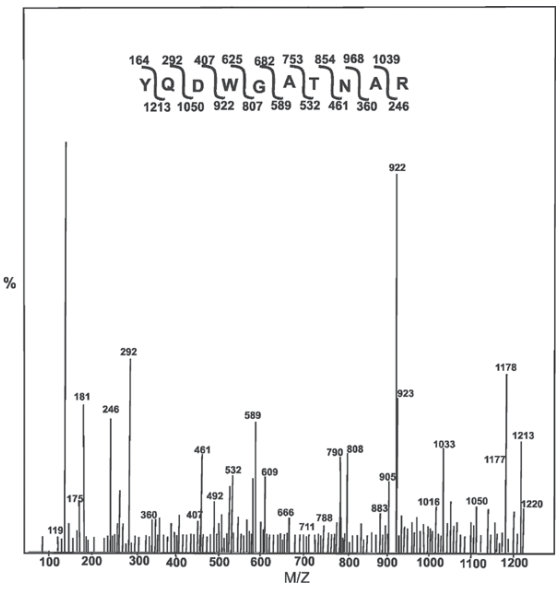


Figure 11. ES-MS/MS analysis of a tryptic fragment with mass of 591.97 (in QTOF) and 1,180.53 (in MALDI-TOF) of the 9 kDa isoform I. Note the oxidation of the tryptophan-156 residue in the γD-9 kDa isoform I. A similar oxidation of tryptophan was also seen in the 9 kDa isoforms II and isoform III (not shown).

residues containing two oxygens in a tryptic fragment with a sequence of YQDWGATNAR (Figure 11). It is speculated that the oxidative modifications of methionine and tryptophan residues in the three isoforms of the 9 kDa polypeptide might partly cause differences in their isoelectric points.

DISCUSSION

One major difference between aging and cataractous human lenses is the presence of higher levels of crosslinked species in the latter [1,3], and these species are believed to play a major role in development of lens opacity. Presently, it is believed that posttranslational modifications in crystallins cause changes in their conformations that in turn might lead to aggregation and crosslinking. Although several such modifications have been identified (see Introduction), their relative mechanistic roles in aggregation and crosslinking of crystallins remain unclear.

As described in the Introduction, several reports suggest that crystallin fragments, produced as a result of in vivo truncation, might play a role in their aggregation and crosslinking. Indeed, a recent study of water insoluble (WI) proteins from human lenses [1,2] showed that the major in vivo modifications of human WI crystallins occurred mainly in αA- and αB-crystallins, which included disulfide bonding, deamidation, methionine oxidation, and backbone cleavage. These modifications might represent the major causative factors for aggregation and crosslinking of crystallins. Our previous results have suggested that the crystallin fragments per se might be involved in the crosslinking process [20]. To ascertain this, the present study was undertaken using an in vivo produced 9 kDa γD-crystallin fragment (residues 87-173) as a model polypeptide. The choice of the polypeptide was also based on our studies which showed its existence as a major polypeptide with posttranslational modifications and crosslinking properties in aging human lenses [28-30].

The major findings of this report are: (1) A mixture of crystallin fragments (M_r 3-14 kDa) incubated at 37 °C crosslinked forming non-disulfide bonded multimers, and with further incubation caused water insolubilization. The sizes of multimers produced ranged in M_r from about 10,000 to >$1x10^6$ daltons. (2) A WS 9 kDa γD crystallin fragment also exhibited crosslinking in a time dependent manner with incubation at 37 °C under both light and dark conditions. The data suggested that the crosslinked species were mostly held via non-disulfide bonds. (3) A mixture of crystallin fragments, isolated from the WS and WI protein fractions of human lenses, showed a higher content of hydrophobic amino acids compared to native crystallins, suggesting that their hydrophobicity might facilitate aggregation, and in turn the crosslinking process. (4) The 9 kDa polypeptide existed as three isoforms, and all three species exhibited oxidation of methionine and tryptophan residues, and truncation after residue number 163. (5) Western blot analyses identified a putative in vivo generated 27 kDa multimers of the 9 kDa polypeptide in both WS proteins of aging human lenses. (6) The 27 kDa species was absent in both WS and WI proteins of young human (4 year old) lenses, suggesting its posttranslational appearance.

During in vitro incubation of the crystallin fragments, a progressive time dependent crosslinking of the fragments was observed, and western blot data suggested the involvement of a 9 kDa γD-crystallin fragment (Figure 1). Because the focus of the present study was to determine the crosslinking property of the 9 kDa polypeptide, additional polypeptides that might contain similar crosslinking properties were not examined. The purified 9 kDa polypeptide showed a time dependent crosslinking via non-disulfide bonding during in vitro incubation because the crosslinked species were not disrupted even after treatment with 200 mM β-mercaptoethanol prior to SDS-PAGE analysis. However, the potential role of disulfide bonding during crosslinking cannot be completely ruled out. During a time course experiment, first an 18 kDa dimer of the 9 kDa polypeptide appeared followed by multimers of >100 kDa, which stayed at the top of a 15% polyacrylamide gel (Figure 2). Because the 9 kDa species showed a time dependent decrease in concentration with a concomitant increase in the levels of crosslinked species (Figure 2), and a similar result was obtained during semi quantitative western blot analysis using radioiodinated protein A (Figure 3), the crosslinked species were indeed derived from the 9 kDa γD-polypeptide. Further, following excitation at 280 nm, the emission of the crosslinked species shifted from 334 nm to 342 nm compared to non-crosslinked species. The emission at 330-340 nm is primarily due to tryptophan, and a shift indicated that the tryptophan residues became more exposed following crosslinking. The 9 kDa γD-crystallin fragment contained two tryptophan residues at positions 130 and 156. The mass spectrometric results showed that the tryptophan-156 residue was oxidized, and therefore, the observed emission shift from 334 to 342 nm in the crosslinked species must be due to exposure of the unoxidized tryptophan-130 residue.

Upon incubation of the 9 kDa polypeptide with α-, β-, or γ-crystallin, the α- and γ-crystallins were more reactive with the polypeptide than β-crystallin. The crosslinked multimers were >100 kDa in their M_r and therefore extensive crosslinking occurred. The polypeptide exhibited a maximum crosslinking with α-crystallin compared to β_H- or γ-crystallin which might be significant with respect to the chaperone function of α-crystallin [40]. The hydrophobic portions of α-crystallin molecules have been implicated in its chaperone function [41,42]. The high hydrophobicity of the degraded polypeptides may contribute to a greater interaction between the crystallin and the polypeptides.

Upon determination of the existence of 9 kDa polypeptide derived multimers in vivo in human lenses by western blot analysis with anti-9 kDa N-terminal and C-terminal antibodies, two multimers of 27 and 45 kDa were observed in the WS proteins from both normal and cataractous lenses (Figure 6 and Figure 7). The 27 kDa crosslinked species was also seen on crosslinking of the 9 kDa polypeptide with α-crystallin (Figure 5A, lane 5). The 9 kDa polypeptide and the 27 and 45 kDa multimers showed a lack of immunoreactivity with anti-9 kDa C-terminal antibody, suggesting modifications in this region. This was consistent with the MALDI-TOF data (Figure 9), which suggested that the 9 kDa polypeptide was trun-

cated after residue number 163. As stated in the Results, western blot analysis of WS and WI protein fractions from lenses of different ages (21, 30, 42, 50, and 61 year old donors) with site specific polyclonal antibodies (anti-γD [residues 1-9] and anti-γD [residues 78-86, minus the 9 kDa polypeptide]) showed an absence of the 27 kDa species, suggesting that the 27 kDa species indeed was a crosslinked species of the 9 kDa polypeptide. Together, the western blot results suggested that the two species of 27 trimer of the 9 kDa polypeptide existed in vivo.

Two factors, the increased hydrophobicity and posttranslational oxidative modifications of methionine and tryptophan residues might play a major role in crosslinking property of crystallin fragments. A distributional shift of crystallin fragments from the WS proteins to the WS-HMW and then to WI protein fractions [9,20] might be due to their higher hydrophobic amino acid content compared to native crystallins, as shown in Table 1. An age related increase in hydrophobic amino acids from crystallin fragments in the WS-HMW proteins was also observed. The hydrophobic amino acid content increased by 4% in lenses from 60 year old compared to lenses from 20 year old donors (unpublished). The fact the fragments could be separated following SDS-PAGE or following urea treatment further suggests a role of hydrophobic interaction among HMW protein aggregates. The high hydrophobicity of the fragments may facilitate proximity among them, and provide greater opportunity for aggregation and crosslinking.

The mass spectrometric data showed oxidation of Methionine-146 and Tryptophan-156 residues, and truncation after residue 163 in the three isoforms of the 9 kDa polypeptide. The precise reason for the difference in the pI of three isoforms of the 9 kDa is presently not well understood. Based on results, we speculate that the oxidation of methionine and tryptophan residues might contribute to the difference in the pI of the three isoforms. However, it is not known whether the oxidative modifications in the 9 kDa polypeptide precede the cleavage at G_{86}-S_{87} in γD-crystallin to generate the 9 kDa polypeptide. One of the characteristics of such modification is an increase in non-tryptophan fluorescence as previously observed for γD-crystallin in nuclear cataracts [43]. The present study also showed a similar shift in fluorescence following crosslinking in the 9 kDa polypeptide.

Oxidation is one of the most prevalent forms of chemical modifications of proteins. The oxidation products of Tryptophan residues of αA- and αB-crystallins have been identified in a previous study [44]. The tryptophan residue (molecular weight 186), upon oxidation acquires one oxygen and becomes hydroxyltryptophan (HRTP, molecular weight 202), and upon acquiring two oxygens produces N-formylkynurenine (NFK, Molecular weight 218) [44]. As shown in Figure 11, tryptophan residues in a tryptic fragment of the 9 kDa polypeptide with a sequence of YQDWGATNAR contained two oxygens, suggesting its conversion to HRTP and NFK. Because oxidation is believed to play a major role in the development of senile cataract [45], and both tryptophan oxidation products, HRTP and TFK, act as a photosensitizer, capable of producing reactive oxygen species [46]. Their presence in the in vivo produced multimers provides further evidence for a role of oxidative process in multimer formation. Because the metal catalyzed oxidation is a primary cause of biomolecular oxidation, it may be responsible for the observed tryptophan oxidation. However, the tryptophan residue has been shown to be oxidized by ionizing radiation and it is not a site for metal binding [47]. With exposure of α-crystallin to iron, no oxidation of tryptophan was reported [48].

The oxidation of sulfur containing amino acids (Methionine and Cysteine residues) in proteins could occur by a wide variety of oxidants [49] in proteins. The oxidation of methionine could lead to protein fragmentation, protein-protein crosslinking [49], increase in protein hydrophobicity [50], and loss of enzyme activity [51]. Some of the properties of the in vivo generated crystallin fragments and the 9 kDa polypeptide might be involved in these processes.

The observed aggregation and crosslinking, induced by the lens crystallin fragments or 9 kDa γD-crystallin fragment have potential significance in the formation of crosslinked products during human senile cataractogenesis. The polypeptide may act as an inducer for crosslinking, and this is consistent with our recent results that the levels of crystallin fragments increase with aging and more so during cataractogenesis.

ACKNOWLEDGEMENTS

The authors express their sincere appreciation to Ms. Martha Robbins for her expert help in preparation of the manuscript. This research is supported by grants from the Retirement Research Foundation, Inc. and PHS grants EY06400 and P30 EY03039.

REFERENCES

1. Lund AL, Smith JB, Smith DL. Modifications of the water-insoluble human lens alpha-crystallins. Exp Eye Res 1996; 63:661-72.
2. Hanson SR, Hasan A, Smith DL, Smith JB. The major in vivo modifications of the human water-insoluble lens crystallins are disulfide bonds, deamidation, methionine oxidation and backbone cleavage. Exp Eye Res 2000; 71:195-207.
3. Spector A. The search for a solution to senile cataracts. Proctor lecture. Invest Ophthalmol Vis Sci 1984; 25:130-46.
4. Benedek G. Theory of transparency of the eye. Applied Optics 1971; 10:459-73.
5. Morgan CF, Schleich T, Caines GH, Farnsworth PN. Elucidation of intermediate (mobile) and slow (solidlike) protein motions in bovine lens homogenates by carbon-13 NMR spectroscopy. Biochemistry 1989; 28:5065-74.
6. Benedek GB, Chylack LT Jr, Libondi T, Magnante P, Pennett M. Quantitative detection of the molecular changes associated with early cataractogenesis in the living human lens using quasielastic light scattering. Curr Eye Res 1987; 6:1421-32.
7. Jedziniak JA, Nicoli DF, Baram H, Benedek GB. Quantitative verification of the existence of high molecular weight protein aggregates in the intact normal human lens by light-scattering spectroscopy. Invest Ophthalmol Vis Sci 1978; 17:51-7.
8. Jedziniak JA, Kinoshita JH, Yates EM, Benedek GB. The concentration and localization of heavy molecular weight aggregates in aging normal and cataractous human lenses. Exp Eye Res 1975; 20:367-9.

9. Srivastava OP, Srivastava K, Silney C. Levels of crystallin fragments and identification of their origin in water soluble high molecular weight (HMW) proteins of human lenses. Curr Eye Res 1996; 15:511-20.
10. Harding J. Cataract: biochemistry, epidemiology, and pharmacology. New York: Chapman & Hall; 1991.
11. Takemoto L. Increase in the intramolecular disulfide bonding of alpha-A crystallin during aging of the human lens. Exp Eye Res 1996; 63:585-90.
12. Nagaraj RH, Sell DR, Prabhakaram M, Ortwerth BJ, Monnier VM. High correlation between pentosidine protein crosslinks and pigmentation implicates ascorbate oxidation in human lens senescence and cataractogenesis. Proc Natl Acad Sci U S A 1991; 88:10257-61.
13. McDermott M, Chiesa R, Roberts JE, Dillon J. Photooxidation of specific residues in alpha-crystallin polypeptides. Biochemistry 1991; 30:8653-60.
14. Ervin LA, Dillon J, Gaillard ER. Photochemically modified alpha-crystallin: a model system for aging in the primate lens. Photochem Photobiol 2001; 73:685-91.
15. Takemoto L, Boyle D. Increased deamidation of asparagine during human senile cataractogenesis. Mol Vis 2000; 6:164-8 .
16. Lorand L. Transglutaminase mediated cross-linking of proteins and cell aging: The erythrocyte and lens models. In: Zappia V, Galleti P, Porta R, Wold F, editors. Advances in post-translational modifications of proteins and aging. New York: Plenum Press; 1988. p 79-94.
17. Fujii N, Ishibashi Y, Satoh K, Fujino M, Harada K. Simultaneous racemization and isomerization at specific aspartic acid residues in alpha B-crystallin from the aged human lens. Biochim Biophys Acta 1994; 1204:157-63.
18. Fujii N, Matsumoto S, Hiroki K, Takemoto L. Inversion and isomerization of Asp-58 residue in human alphaA-crystallin from normal aged lenses and cataractous lenses. Biochim Biophys Acta 2001; 1549:179-87.
19. Takemoto LJ. Differential phosphorylation of alpha-A crystallin in human lens of different age. Exp Eye Res 1996; 62:499-504.
20. Srivastava OP. Age-related increase in concentration and aggregation of degraded polypeptides in human lenses. Exp Eye Res 1988; 47:525-43.
21. Horwitz J, Hansen JS, Cheung CC, Ding LL, Straatsma BR, Lightfoot DO, Takemoto LJ. Presence of low molecular weight polypeptides in human brunescent cataracts. Biochem Biophys Res Commun 1983; 113:65-71.
22. Yoshida H, Yumoto N, Tsukahara I, Murachi T. The degradation of alpha-crystallin at its carboxyl-terminal portion by calpain in bovine lens. Invest Ophthalmol Vis Sci 1986; 27:1269-73.
23. David LL, Azuma M, Shearer TR. Cataract and the acceleration of calpain-induced beta-crystallin insolubilization occurring during normal maturation of rat lens. Invest Ophthalmol Vis Sci 1994; 35:785-93.
24. David LL, Shearer TR, Shih M. Sequence analysis of lens beta-crystallins suggests involvement of calpain in cataract formation. J Biol Chem 1993; 268:1937-40.
25. Shearer TR, David LL, Anderson RS, Azuma M. Review of selenite cataract. Curr Eye Res 1992; 11:357-69.
26. Spector A. Aspects of biochemistry of cataract. In: Maisel H, editor. The ocular lens: structure, function, and pathology. New York: Dekker; 1985. p. 405-438.
27. Ramalho J, Marques C, Pereira P, Mota MC. Crystallin composition of human cataractous lens may be modulated by protein glycation. Graefes Arch Clin Exp Ophthalmol 1996; 234 Suppl 1:S232-8.
28. Srivastava OP, McEntire JE, Srivastava K. Identification of a 9 kDa gamma-crystallin fragment in human lenses. Exp Eye Res 1992; 54:893-901.
29. Srivastava OP, Srivastava K, Silney C. Covalent modification at the C-terminal end of a 9 kDa gamma D-crystallin fragment in human lenses. Exp Eye Res 1994; 58:595-603.
30. Srivastava OP, Srivastava K. Characterization of three isoforms of a 9 kDa gamma D-crystallin fragment isolated from human lenses. Exp Eye Res 1996; 62:593-604.
31. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680-5.
32. Meakin SO, Du RP, Tsui LC, Breitman ML. Gamma-crystallins of the human eye lens: expression analysis of five members of the gene family. Mol Cell Biol 1987; 7:2671-9.
33. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 1979; 76:4350-4.
34. Burnette WN. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate—polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem 1981; 112:195-203.
35. Herbert B. Advances in protein solubilisation for two-dimensional electrophoresis. Electrophoresis 1999 Apr-May; 20:660-3.
36. Peterson GL. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 1977; 83:346-56.
37. Gehrke CW, Rexroad PR, Schisla RM, Absheer JS, Zumwalt RW. Quantitative analysis of cystine, methionine, lysine, and nine other amino acids by a single oxidation—4 hour hydrolysis method. J Assoc Off Anal Chem 1987 Jan-Feb; 70:171-4.
38. Eisenberg D, Schwarz E, Komaromy M, Wall R. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. J Mol Biol 1984; 179:125-42.
39. Lawson EQ, Schubert CF, Lewis RV, Middaugh CR. The solubility of bovine lens crystallins. J Biol Chem 1981; 256:6523-5.
40. Horwitz J. Alpha-crystallin can function as a molecular chaperone. Proc Natl Acad Sci U S A 1992; 89:10449-53.
41. Raman B, Rao CM. Chaperone-like activity and quaternary structure of alpha-crystallin. J Biol Chem 1994; 269:27264-8.
42. Das KP, Surewicz WK. Temperature-induced exposure of hydrophobic surfaces and its effect on the chaperone activity of alpha-crystallin. FEBS Lett 1995; 369:321-5.
43. Bessems GJ, Keizer E, Wollensak J, Hoenders HJ. Non-tryptophan fluorescence of crystallins from normal and cataractous human lenses. Invest Ophthalmol Vis Sci 1987; 28:1157-63.
44. Finley EL, Dillon J, Crouch RK, Schey KL. Identification of tryptophan oxidation products in bovine alpha-crystallin. Protein Sci 1998; 7:2391-7.
45. Spector A. Oxidative stress-induced cataract: mechanism of action. FASEB J 1995; 9:1173-82.
46. Reszka KJ, Bilski P, Chignell CF, Dillon J. Free radical reactions photosensitized by the human lens component, kynurenine: an EPR and spin trapping investigation. Free Radic Biol Med 1996; 20:23-34.
47. Stadtman ER. Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. Annu Rev Biochem 1993; 62:797-821.
48. Smith JB, Jiang X, Abraham EC. Identification of hydrogen peroxide oxidation sites of alpha A- and alpha B-crystallins. Free Radic Res 1997; 26:103-11.

49. Berlett BS, Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. J Biol Chem 1997; 272:20313-6.
50. Chao CC, Ma YS, Stadtman ER. Modification of protein surface hydrophobicity and methionine oxidation by oxidative systems. Proc Natl Acad Sci U S A 1997; 94:2969-74.
51. Griffiths SW, Cooney CL. Relationship between protein structure and methionine oxidation in recombinant human alpha 1-antitrypsin. Biochemistry 2002; 41:6245-52.

The print version of this article was created on 8 Dec 2003. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.