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Proteomic analysis of water insoluble proteins from normal and cataractous human lenses

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Purpose: The purpose of the study was to compare and analyze the composition of crystallin species that exist in the water insoluble-urea soluble (WI-US) and water insoluble-urea insoluble (WI-UI) protein fractions of a human cataractous lens and an age-matched normal lens.

Methods: The water soluble (WS) and water insoluble (WI) protein fractions from a 68-year-old normal lens and a 61-year-old cataractous lens were isolated, and the WI proteins were further solubilized in urea to separate WI-US and WI-UI protein fractions. The WI-US and WI-UI protein fractions from normal and cataractous lenses were individually analyzed by two-dimensional (2D) gel electrophoresis. The protein spots were excised from 2D gels, digested with trypsin, and analyzed by the matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) method. The tryptic peptides from individual spots were further analyzed by the electrospray tandem mass spectrometry (ES-MS/MS) method to determine their amino acid sequences.

Results: The comparative 2D gel electrophoretic analyses of WI-US proteins of normal and cataractous lenses showed that the majority of species in a normal lens (68 years old) and a cataractous lens (61 years old) had M_r between 20 to 30 kDa. The ES-MS/MS analyses showed that the individual WI-US protein spots from normal and cataractous lenses contained mostly either αA- or αB-crystallin with β-crystallins, or α- and β-crystallins with filensin as well as vimentin. Similar sequence analyses of tryptic fragments of 2D gel spots of WI-UI proteins revealed that the normal lens showed either individual αA- and αB-crystallins, a mixture of βA3/A1-, βB1-, and βB2-crystallins and filensin, βA4-, βB1-, βB2-, βS-crystallins and filensin, or αA-, αB1-, filensin, and vimentin or αB-, βA3-, βA4-, βB1-, βB2-, and βS-crystallins. In contrast, the WI-UI proteins from a cataractous lens showed three intact crystallins (αB-, γS-, and βB2-crystallins), and three spots containing a mixture of β-crystallins (the first containing βB1- and βB2-crystallins, the second γS-, βB1-, and βB2-crystallins, and the third βA3-, βA4-, and βB1-crystallins).

Conclusions: The compositions of WI-US and WI-UI proteins, isolated from one normal and one cataractous lens, were different. The absence of αA- but not of αB-crystallin and preferential insolubilization mostly of β-crystallins in the WI-US protein fraction from the cataractous lens but not in the normal lens was observed. Similarly, in contrast to the normal lens, the WI-UI proteins of the cataractous lens contained αB-crystallin while αA-crystallin was absent, which suggested a major role of αB-crystallin in the insolubilization process of crystallins.

The mammalian lens contains three major structural proteins, known as α-, β-, and γ-crystallins. Among these, the α- and β-crystallins exist as oligomers, whereas the γ-crystallin is a monomer. These structural proteins, by virtue of their specific structural interactions and high concentrations, contribute to the transparency of the lens and provide the needed refractive index to focus light on the retina. The crystallins aggregation, cross-linking, and water insolubilization processes may contribute to the development of age-related lens opacity. However, the sequence of these events and their relative importance in the development of lens opacity are not well understood. It also remains unclear how the relative mechanism of water insolubilization of lens crystallins during cataract development differs from the normal aging process. Present literature suggests that a variety of posttranslational modifications cause aggregation and cross-linking of crystallins and lead to their water insolubilization. Because posttranslational modifications occur during aging as well as during cataract development, the identification of a single or combination of potential modifications as the initiating factor(s) during the development of lens opacity has not been identified. However, it is now believed that the development of lens opacity might involve mechanisms induced by more than one such modification.

Recent studies of water insoluble (WI) proteins from normal human lenses showed that crystallins undergo in vivo modifications, which included disulfide bonding, deamidation, oxidation, and backbone cleavage [1,2]. However, additional modifications in crystallins are also believed to contribute to aggregation and cross-linking, which included disulfide bonding [3], glycation [4], oxidation of Trp and His residues [5,6], deamidation [7-10], transglutaminase-mediated cross-linking [11], racemization [12,13], and phosphorylation [14]. Attempts to determine the relative importance of individual modifications in the mechanism of age-related cataractogenic process

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have resulted in limited success. Certain cataract-specific modifications (i.e., either not observed or they occur at relatively lower levels during aging) have been identified, which include, among others, increased degradation of α-crystallin in diabetic cataracts [15], presence of abnormal αB-crystallin species in human nuclear cataracts [16], and increased deamidation of γS-crystallin [17]. To distinguish between cataract- and age-specific modifications, we recently compared the crystallin species present in the water soluble-high molecular weight (WS-HMW), and water-insoluble (WI) proteins of human cataractous and age-matched normal lenses [18]. The results showed that the crystallin species of WS-HMW- and WI-protein fractions of cataractous lenses were different from those of normal lenses, and the fragments of βA3/A1- and βB1-crystallins were selectively insolubilized during cataract development compared to normal aging. Additionally, the crystallin species of cataractous lenses revealed increased truncation, deamidation of asparagine to aspartic acid residues, and oxidation of W residues. In a second recent study [19], we analyzed compositions of the covalent multimers (M_r >90 kDa), separated as individual spots by two-dimensional (2D)-gel electrophoresis from human lenses from 25-, 41-, 52-, and 72-year-old human donors. Because of the existence of non-descript and diffused WI protein spots with M_r >90 kDa in the 52- and 72-year-old lenses, the spots from 25- and 41-year-old lenses were analyzed by ES-MS/MS method. Two types of covalent multimers in these lenses were observed. The first type was composed of fragments from eight different crystallins (αA-, αB-, βA3-, βA4-, βB1-, βB2-, γS-, and γD-crystallin), and the second type was from α-, β-, and γ-crystallins (possibly fragments) and two beaded filament proteins (phakinin and filensin). The αA-crystallin fragments exhibited three posttranslational modifications (oxidation of methionine and tryptophan residues, conversion of serine residues to dehydroalanine, and formylation of histidine residues), and among these, the first two modifications are known to cause cross-linking in proteins. Together, the results suggested that covalent multimers appeared early in life in vivo (i.e., 25 years of age) and their numbers increased with aging. Some of these covalent complexes were formed between crystallin fragments and filensin and phakinin (the two beaded filament proteins).

We undertook the present study as an extension of our earlier studies [18,19]. The purpose was to analyze by comparison the species composition present in WI-US and WI-UI proteins of a 68-year-old normal lens and a 61-year-old cataractous lens to distinguish those species that were cataract-specific but not age-specific.

METHODS

Materials: A healthy lens from a 68-year-old donor was obtained from the Shared Ocular Tissue Module at the University of Alabama at Birmingham. The lens was retrieved within 48 h postmortem, visually examined for opacity, and stored in medium-199 without phenol red at -20 °C until used. A cataractous lens (removed extracapsularly) with only nuclear opacity was obtained from a 61-year-old donor within 4-5 h following surgery, and stored under the same conditions as that described for the healthy lens. A local ophthalmologist examined the cataractous lens prior to its surgical removal and determined it contained a nuclear cataract. Phacoemulsification was employed to remove cortical region and was followed by irrigation and aspiration to remove the nuclear region. The recovered lens contained 10% of the original cortex and 90% of the nucleus. The prestained and unstained molecular weight protein markers were from Invitrogen (Carlsbad, CA) and Amersham Biosciences (Piscataway, NJ), respectively. All chemicals for 2D gel electrophoresis were from either Amersham Biosciences or Bio Rad (Hercules, CA). Unless indicated otherwise, other chemicals used in this study were purchased from Sigma (St. Louis, MO) or Fisher (Atlanta, GA).

Isolation of water soluble- and water insoluble-protein fractions from normal and cataractous human lenses and their analysis by two-dimensional gel electrophoresis: All procedures were performed at 5 °C unless indicated otherwise. The WS and WI protein fractions from a 68-year-old normal lens and a 61-year-old cataractous lens were isolated by a procedure as previously described [18,19]. Each lens was thawed on ice, decapsulated, suspended (2 ml/lens) in buffer A (50 mM Tris-HCl, pH 7.9 containing 1 mM dithiothreitol, 1 mM iodoacetamide, which is a cysteine proteinase inhibitor, 1 mM phenylmethylsulfonyl fluoride, which is a serine proteinase inhibitor), and homogenized using a tissue grinder. DTT was included to prevent disulfide bonding, and iodoacetamide not only acted as a cysteine proteinase inhibitor but also alkylated sulfhydryl groups. The lens homogenate was centrifuged at 15,000x g for 15 min. The supernatant was recovered, and the pellet was homogenized and centrifuged twice as described in the previous sentence. The supernatants recovered after each centrifugation, were pooled and designated as WS protein fraction, and the pellet was designated as the WI protein fraction. The WI protein fraction was suspended (2 ml/lens) in buffer B (50 mM Tris-HCl, pH 7.9, containing 6 M urea and 5 mM dithiothreitol) and homogenized. The supernatants were designated as the water-insoluble-urea-soluble (WI-US) protein fraction. The pellet was designated as the water-insoluble-urea-insoluble (WI-UI) protein fraction. The process was repeated twice to recover the WI-US and WI-UI protein fractions and combined with above similar fractions.

Aliquots of the WI-US and WI-UI protein fractions (containing between 500 to 800 μg of protein) from the individual lenses were dissolved in resolubilization buffer (5 M urea, 2 M thiourea, 2% 3-[(3-cholamidoproyl)-dimethyl-ammonio-1-propane sulfonate] (CHAPS), 2% caprylyl sulfobetaine 3-10, 2 mM tri-butyl phosphine, 40 mM Tris, pH 8.0) [20] and incubated with Immobiline Dry Strips (pH range of 3-10, Amersham Biosciences) overnight at room temperature. Each preparation was subjected to 2D gel electrophoresis (IEF in the first dimension followed by SDS-PAGE in the second dimension) by exactly following the manufacturer's suggested method (Amersham Biosciences). Following the IEF separation, the second dimension SDS-PAGE was performed by the Laemmli [21] method using a 15% polyacrylamide gel of

16x14 cm (width x height). After the first dimensional IEF separation, the strips were consecutively treated for 15 min each, first with 100 mM dithiothreitol (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 the equilibration buffer). The protein spots on a gel were stained with Coomassie blue.

Analysis of spots on two-dimensional gels by mass spectrometric methods: The MALDI-TOF analysis and ES-MS/MS sequencing (Micromass QTOF-2) were performed at the Comprehensive Cancer Center Mass Spectrometry Shared Facility of the University of Alabama at Birmingham. For mass spectrometric analysis, the individual protein spots were excised from a SDS-polyacrylamide gel using pipette microtips. The polyacrylamide pieces containing individual spots were destained with three consecutive washes containing a mixture of 50% of 25 mM ammonium bicarbonate/50% of acetonitrile for 30 min. Next, the samples were washed for ten min with 25 mM ammonium bicarbonate prior to digestion with trypsin (12 ng/µl; sequencing grade from Roche) for 16 h at 37 °C. Peptide solutions were then extracted using 100 µl of a 50:50 solution of 5% formic acid and acetonitrile for 30 min. Supernatants were collected and dried in a Savant SpeedVac. Samples were resuspended in 10 µl of 0.1% formic acid. The C-18 ZipTips (Millipore) were used to desalt peptide mixtures before applying samples to the MALDI-TOF-96x2 well target plates. Peptides were mixed in 1:10 dilutions with a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA) matrix. Samples were allowed to dry before undergoing MALDI-TOF MS utilizing the Voyager DE-Pro in positive mode. Spectra were then analyzed using Voyager Explorer software, and peptide masses were submitted to MASCOT database for protein identifications. The MALDI-TOF-identity of proteins was established by using the NCBInr database of Matrix Science. Tandem mass spectral analyses were performed with Q-TOF 2 mass spectrometer (Micromass, Manchester, UK) using electrospray ionization. The tryptic peptides were concentrated and desalted using ZipTips as described. The samples were then analyzed by LC-MS/MS. Liquid chromatography was performed using a LC Packings Ultimate LC Switchos microcolumn switching unit and Famos autosampler (LC Packings, San Francisco, CA). The samples were concentrated on a 300 µm i.d. C-8 precolumn at a flow rate of ten µl/min with 0.1% formic acid and then flushed onto a 75 µm i.d. C-8 column at 200 µl/min with a gradient of 5-100% acetonitrile (0.1% formic acid) for 30 min. The nano-LC interface was used to transfer the LC eluent into the mass spectrometer. The Q-TOF was operated in the automatic switching mode whereby multiple-charged ions were subjected to MS/MS if their intensities rose above six counts. Protein identification was performed by either the ProteinLynx Global Server software or by manual interpretations in certain cases. Protein concentration was determined by a modified method of Lowry using a protein determination kit (Pierce Chemicals, Rockford, IL).

RESULTS

Two-dimensional gel electrophoretic profiles of water insoluble-urea soluble proteins from normal and cataractous human lenses: The 2D gel electrophoretic protein profiles of WI-US proteins from a 68-year-old normal human lens and from a 61-year-old cataractous lens are shown in Figure 1A,B, respectively. Fifteen major spots were observed in the 2D gels of the normal lens (Figure 1A), and the same number were also present in the cataractous lens (Figure 1B). Although most of the spots from the normal lens and the cataractous lens exhibited molecular weights between 20 to 30 kDa, the ES-MS/MS analysis of their tryptic peptides showed that the majority among the 15 spots from the normal lens (spots 1 to 15), and the nine spots from the cataractous lens (3, 4, 5, 6, 8, 11, 12, 13, and 15) contained multiple crystallins (Table 1). Because of the recovery of limited quantities of proteins in certain spots from the cataractous lens (1, 2, 7, 9, and 10), their tryptic peptide sequences could not be determined. Spots 13 and 15 among WI-US spots from the normal lens were of filensin, whereas the remaining spots contained multiple crystallins. Additionally, spots 7, 11, and 14, like our previous study [19], contained a mixture of filensin and α- and β-crystallins. Further, none of the spots showed any γ-crystallin, except spot 1, which contained γD-crystallin.

The majority of spots from the WI-US proteins of the cataractous lens also exhibited molecular weights between 20 to 30 kDa, and few among these were of βA3- or βB1-crystallins; other spots contained two or more crystallins. Spot 5 contained αB-, βA3-, βA4-, βB1-, and βB2-crystallins, while spot 8 had βA3-, βA4-, βB1-crystallins, and spot 12 contained βB1- and βB2-crystallins (Table 1).

Tryptic peptides sequences of water insoluble-urea soluble protein spots from two-dimensional gels of normal and cataractous lenses: Different crystallins present in individual spots of WI-US protein fractions of normal and cataractous lenses are reported in Table 1. Following ES-MS/MS analyses, the amino acid sequences of tryptic peptides of individual spots are shown in Table 2 and the posttranslationally modified amino acids in these peptide sequences are also identified. Because individual spots contained amino acid sequences of tryptic peptides belonging to multiple crystallin species, these sequences are listed under corresponding crystallins in Table 2. Additionally, these tryptic peptides with residue numbers representing their locations within individual crystallins are described below. Spot 1 (Figure 1A) had αB- and γD-crystallins because it contained the following tryptic peptide sequences: entire sequence of αB-crystallin (residue 1-175; with oxidation of methionine-1, methionine-68, tryptophan-60, and phosphorylation serine-66 residues) and residue 118-140 of γD-crystallin (Table 2). Spot number 2 contained αB- and βB1-crystallins because it showed tryptic peptide sequences of the entire αB-crystallin (residue 1-175; with oxidation of methionine-1, methionine-68, tryptophan-60, and phosphorylation serine-66 residues) and of βB1-crystallin peptide with residue 60-71. Spot 3 contained a mixture of αB-

, βA3-, βB2-, and γS-crystallins and showed the following sequences of their tryptic peptides: entire sequence of αB-crystallin (residue 1-175; with oxidation of methionine-1, methionine-68, tryptophan-60, and phosphorylation serine-66 residues), βA3- (residue 33-44 and 126-137; with modified methionine-126), βB2- (residue 108-119), and γS-crystallins (residue 131-145). Spot 4, a mixture of αB-, βA3-, βA4- and βB1-crystallins, showed the following tryptic peptide sequences: αB-crystallin (residue 1-149), and peptides representing partial sequences of βA3- (residue 33-44, 44-64; with oxidized methionine-44, 91-109, 96-109, and 163-177), βA4- (residue 107-118), and βB1- (residue 60-71,150-159, 170-181,187-201, 202-213, and 214-229; with oxidized methionine-226)-crystallins. Spot 5 contained a mixture of αB-, βA3-, βA4-, and βB1-crystallins and showed the following tryptic fragment sequences: αB- (residue 1-11, 57-69, with oxidized methionine-68, 57-69 with oxidized methionine-68, and phosphorylation of serine-69, 57-69 with phosphorylated serine-59, and 83-90), βA3- (residue 33-21, 46-64, 53-64, 91-109, 96-109, 100-109, 126-137, with oxidized methionine-126, 126-137,129-137, 138-162, with oxidized methionine-161, 163-177, and 197-211), βA4- (residue 106-117), and βB1- (residue 60-71 and 202-213) crystallins. Spot 6 was a mixture of βA3- and βA4-crystallins and showed the following tryptic peptide sequences: βA3- (residue 33-44, 34-45, 35-45, 96-109, 98-109, 126-137, with oxidized methionine-46, and 163-177), and βA4- (residue 48-61 and 106-117) crystallins. Spot 7 was a mixture of αA-, αB-, βA3, βA4-, and βB1-crystallins and filensin and showed the following tryptic peptide sequences: αA- (residue 55-65), αB- (residue 83-90), βA3- (residue 33-44, 34-45, 34-45, 35-45, 45-64, with oxidized methionine-46, 46-64, with oxidized methionine-46, 91-109, 96-109, 126-137, with oxidized methionine-126, and 197-211), βA4-

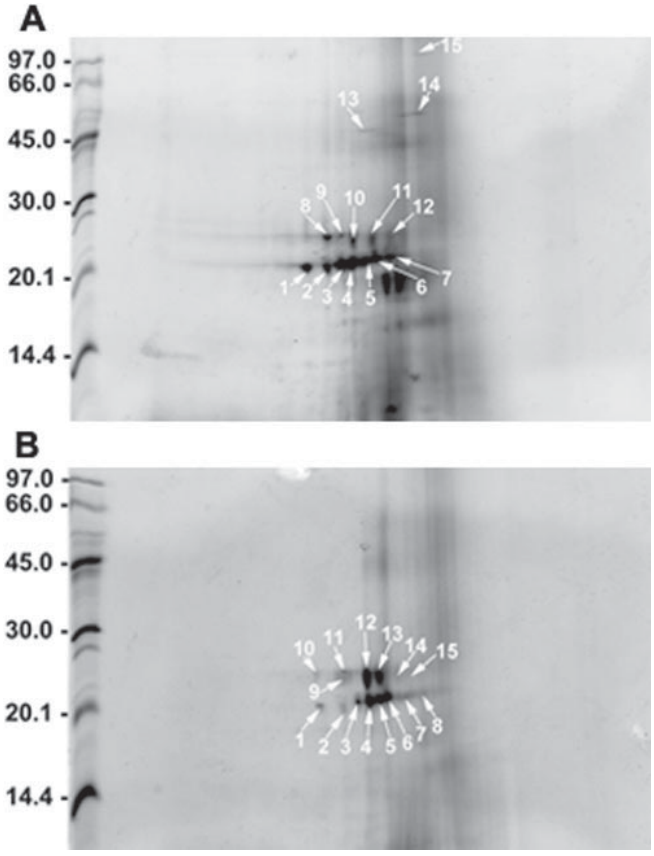

Figure 1. Two-dimensional gel electrophoretic separation of individual protein spots present in WI-US protein fractions of a normal lens and a cataractous lens. Two-dimensional gel electrophoretic profiles of water insoluble-urea soluble-protein fractions from (**A**) normal lens from a 68-year-old donor, and (**B**) cataractous lens with nuclear opacity from a 61-year-old donor. The numbers with arrows point to individual protein spots (starting from the lowest to the highest molecular weights) of a normal lens and a cataractous lens.

Table 1. Majority of spots on a 2D-gel contained a mixture of crystallins in the WI-US proteins of a normal lens and a cataractous lens

SPOT NUMBER 68-YO-Normal WI-US	COMPONENTS
1	αB and γD
2	αB and βB1
3	αB, βA3, βB2, and βS
4	αB, βA3, βA4, and βB1
5	αB, βA3, βA4, and βB1
6	βA3 and βA4
7	αA, αB, βA3, βA4, βB1, and filensin
8	βB1 and βB2
9	βA3, βB1, and βB2
10	βB1 and βB2
11	βB1, βB2, and filensin
12	αB, βA4, βB1, and filensin
13	filensin
14	αA, βB1, filensin, and vimentin
15	filensin
61-YO-Cataractous WI-US	
3	βA3
4	βA3
5	αB, βA3, βA4, βB1, and βB2
6	βA3
8	βA3, βA4, and βB1
11	βB1
12	βB1 and βB2
13	βB1
15	βB1

Summary of the components identified in the two-dimensional gel separated spots in the water insoluble-urea soluble-proteins from a 68-year-old normal and a 61-year-old cataractous lens. Majority of spots on a 2D-gel contained a mixture of crystallins in the WI-US proteins of a normal lens and a cataractous lens

(residue 13-24, 48-70, 108-118, and 109-119), βB1- (residue 60-71, 150-159, 187-201, and 202-213)-crystallins and filensin (residue 78-90). Spots 8, 9, and 10 contained only β-crystallin species. Spot 8, a mixture of βB1- and βB2-crystallins, showed the following tryptic peptide sequences: βB1- (residue 60-71, 150-159, 202-213, 214-229, with oxidized methionine-226, and 233-251), and βB2- (residue 1-17, 48-80, 81-88, 90-107, 94-107, 108-119, 110-120, 120-139, 121-139, with oxidized

Table 2. Identification of crystallins present in protein spots of a 2D-gel of the WI-US protein fraction of a normal lens by the ES-MS/MS method

Spot number	α-Crystallin	α- and α-Crystallin	Filensin	Vimentin
1	αB: Ac-MDIAIHHPWIR (#1-11, 2 Oxid. M) αB: Ac-MDIAIHHPWIR (#1-11, Oxid. M) αB: DIAIHHPWIR (#2-11) αB: APSWFDTGLSEMR (#57-69, Oxid. M) αB: APSWFDTGLSEMR (#57-69, Oxid. M, 2 OH groups on W αB: APSWFDTGLSEMRLE (#57-71, Oxid. M; PO3H on S66 αB: APSWFDTGLSEMRLEK (#57-72, Oxid. M) αB: APSWFDTGLSEMRLEKDR (#57-74, Oxid. M) αB: DRFSVNLDVK (#73-82) αB: FSVNLDVK (#75-82) αB: KYRIPADVDPLTITSSLSSDGVLTVNGPR (#121-149) αB: KYRIPADVDPLTITSSLSSDGVLTVNGPRKQVSGPER (#121-157 αB: YRIPADVDPLTITSSLSSDGVLTVNGPRKQVSGPER (#122-157) αB: IPADVDPLTITSSLSSDGVLTVNGPRK (#124-150) αB: IPADVDPLTITSSLSSDGVLTVNGPR (#124-149) αB: TIPITREEKPAVTAAPK (#158-174) αB: PITREEKPAVTAAPK (#160-174) αB: EEKPAVTAAPK (#164-174)	γD: FNEIHSLNVLEGSWVLYELSNYR (#118-140)		
2	αB: Ac-MDIAIHHPWIR (#1-11, Oxid. M) αB: DIAIHHPWIR (#2-11) αB: AIHHPWIR (#4-11) αB: RPFFPFHSPSR (#12-22) αB: PFHSPSR (#16-22) αB: APSWFDTGLSEMR (#57-69) αB: APSWFDTGLSEMR (#57-69, 2 Oxid. M) αB: APSWFDTGLSEMR (#57-69, Oxid. M; OH on W) αB: APSWFDTGLSEMR (#57-69, Oxid. M) αB: APSWFDTGLSEMRLE (#57-71, Oxid. M; PO3H on S66) αB: APSWFDTGLSEMRLEK (#57-72, Oxid. M) αB: APSWFDTGLSEMRLEKDR (#57-74, Oxid. M) αB: SEMRLEKDR (#66-74, Oxid. M) αB: LEKDRFSVNLDVK (#70-82) αB: FSVNLDVK (#75-82) αB: VLGDVIEVHGKHEERQDEHGFISR (#93-116) αB: GDVIEVHGKHEERQDEHGFISR (#95-116) αB: IPADVDPLTITSSLSSDGVLTVNGPR (#124-149) αB: TIPITREEKPAVTAAPK (#158-174) αB: PITREEKPAVTAAPK (#160-174)	βB1: LVVFELENFQGR (#60-71)		
3	αB: Ac-MDIAIHHPWIR (#1-11, Oxid. M; OH on W) αB: Ac-MDIAIHHPWIR (#1-11, Oxid. M) αB: DIAIHHPWIR (#2-11, OH on W) αB: DIAIHHPWIR (#2-11) αB: AIHHPWIR (#4-11) αB: RPFFPFHSPSR (#12-22, PO3H on S19) αB: RPFFPFHSPSR (#12-22) αB: PFHSPSR (#16-22) αB: LFDQFFGEHLLESDLFPTSTSLSPFYLRPPSFLR (#23-56) αB: APSWFDTGLSEMR (#57-69, Oxid. M; PO3H on S59) αB: APSWFDTGLSEMR (#57-69, Oxid. M) αB: APSWFDTGLSEMR (#57-69, Oxid. M) αB: APSWFDTGLSEMRLE (#57-71, Oxid. M; PO3H on S66) αB: APSWFDTGLSEMRLEK (#57-72, Oxid. M) αB: APSWFDTGLSEMRLEK (#57-72, Oxid. M; PO3H on S59) αB: APSWFDTGLSEMRLEKDR (#57-74, Oxid. M; PO3H on S59) αB: APSWFDTGLSEMRLEKDR (#57-74, Oxid. M) αB: SEMRLEKDR (#66-74, Oxid. M) αB: DRFSVNLDVK (#73-82) αB: LEKDRFSVNLDVK (#70-82) αB: LEKDRFSVNLDVK (#70-82, PO3H on S76) αB: HFSPEELK (#83-90) αB: VLGDVIEVHGKHEERQDEHGFISR (#93-116) αB: GDVIEVHGKHEERQDEHGFISR (#95-116) αB: KYRIPADVDPLTITSSLSSDGVLTVNGPR (#121-149) αB: YRIPADVDPLTITSSLSSDGVLTVNGPR (#122-149) αB: IPADVDPLTITSSLSSDGVLTVNGPR (#124-149) αB: IPADVDPLTITSSLSSDGVLTVNGPR (#124-149) αB: IPADVDPLTITSSLSSDGVLTVNGPR (#124-149) + Na αB: PLTITSSLSSDGVLTVNGPRKQVSGPER (#130-157) αB: SSLSSDGVLTVNGPRKQVSGPER (#135-157) αB: TIPITREEKPAVTAAPK (#158-174) αB: TIPITREEKPAVTAAPK (#158-174) αB: PITREEKPAVTAAPK (#160-174)	βA3: ITIYDQENFQGK (#33-44) βA3: MTIFEKENFIGR (#126-137, Oxid. M) βB2: IILYENPNFTGK (#108-119) βS: VLEGVWIFYELPNYR (#131-145)		
4	αB: Ac-MDIAIHHPWIR (#1-11, Oxid. M) αB: DIAIHHPWIR (#2-11) αB: APSWFDTGLSEMR (#57-69, Oxid. M; PO3H on S59) αB: APSWFDTGLSEMR (#57-69, Oxid. M) αB: APSWFDTGLSEMRLEK (#57-72, Oxid. M) αB: YRIPADVDPLTITSSLSSDGVLTVNGPR (#122-149)	βA3: ITIYDQENFQGK (#33-44) βA3: MEFTSSCPNVSERSFDNVR (#46-64, Oxid. M) βA3: GEYPRWDAWSGSNAYHIER (#91-109) βA3: WDAWSGSNAYHIER (#96-109) βA3: IQSGAWVCYQYPGYR (#163-177) βA4: LTIFEQENFLGK (#107-118) βB1: LVVFELENFQGR (#60-71) βB1: ISLFEGANFK (#150-159) βB1: APSLWVYGFSDR (#170-181) βB1: VSSGTWVGYQYPGYR (#187-201) βB1: GYQYLLEPGDFR (#202-213) βB1: HWNEWGAFQPQMQSLR (#214-229, Oxid. M)		
5	αB: Ac-MDIAIHHPWIR (#1-11, Oxid. M) αB: APSWFDTGLSEMR (#57-69, Oxid. M) αB: APSWFDTGLSEMR (#57-69) αB: APSWFDTGLSEMR (#57-69, Oxid. M; PO3H on S59) αB: APSWFDTGLSEMR (#57-69, PO3H on S59) αB: HFSPEELK (#83-90)	βA3: ITIYDQENFQGK (#33-44) βA3: MEFTSSCPNVSERSFDNVR (#46-64, Oxid. M) βA3: PNVSERSFDNVR (#53-64) βA3: GEYPRWDAWSGSNAYHIER (#91-109) βA3: WDAWSGSNAYHIER (#96-109) βA3: SGSNAYHIER (#100-109) βA3: MTIFEKENFIGR (#126-137, Oxid. M) βA3: MTIFEKENFIGR (#126-137) βA3: FEKENFIGR (#129-137) βA3: QWEISDDYPSLQAMGWFNNEVGSMK (#138-162, Oxid. M) βA3: IQSGAWVCYQYPGYR (#163-177) βA3: EWGSHAQTSQIQSIR (#197-211) βA4: LTIFEQENFLGK (#106-117) βB1: LVVFELENFQGR (#60-71) βB1: GYQYLLEPGDFR (#202-213)		
6		βA3: ITIYDQENFQGK (#33-44) βA3: TIYDQENFQGKR (#34-45) βA3: IYDQENFQGKR (#35-45) βA3: WDAWSGSNAYHIER (#96-109) βA3: AWSGSNAYHIER (#98-109) βA3: MTIFEKENFIGR (#126-137) βA3: MTIFEKENFIGR (#126-137, Oxid. M) βA3: IQSGAWVCYQYPGYR (#163-177) βA4: VLSGAWVGFEHAGF (#48-61) βA4: LTIFEQENFLGK (#106-117)		

TABLE 2. CONTINUED.

Spot number	α-Crystallin	α- and α-Crystallin	Filensin	Vimentin
7	αA: TVLDSGISEVR (#55-65) αB: HFSPEELK (#83-90)	βA3: ITIYDQENFQGK (#33-44) βA3: ITIYDQENFQGKR (#33-45) βA3: TIYDQENFQGKR (#34-45) βA3: IYDQENFQGKR (#35-45) βA3: RMEFTSSCPNVSERSFDNVR (#45-64, Oxid. M) βA3: MEFTSSCPNVSERSFDNVR (#46-64, Oxid. M) βA3: MEFTSSCPNVSERSFDNVR (#46-64) βA3: GEYPRWDAWSGSNAYHIER (#91-109) βA3: WDAWSGSNAYHIER (#96-109) βA3: MTIFEKENFIGR (#126-137, Oxid. M) βA3: EWGSHAQTSQIQSIR (#197-211) βA4: MVVWDEDGFQGR (#13-24) βA4: VLSGAWVGFEHAGFQGQQYILER (#48-70) βA4: LTIFEQENFLGK (#108-118) βA4: IFEQENFLGKK (#109-119) βB1: LVVFELENFQGR (#60-71) βB1: ISLFEGANFK (#150-159) βB1: VSSGTWVGYQYPGYR (#187-201) βB1: GYQYLLEPGDFR (#202-213)	Filensin: LGELAGPEDALAR (#78-90)	
8		βB1: LVVFELENFQGR (#60-71) βB1: ISLFEGANFK (#150-159) βB1: GYQYLLEPGDFR (#202-213) βB1: HWNEWGAFQPQMQSLR (#214-229, Oxid. M) βB1: DKQWHLEGSFPVLATEPPK (#233-251) βB2:ASDHQTQAGKPQSLNPK (#1-17) βB2: AGSVLVQAGPWVGYEQANCKGEQFVFEKGEYPR (#48-80) βB2: WDSWTSSR (#81-88) βB2: TDSLSSLRPIKVDSQEHK (#90-107) βB2: SSLRPIKVDSQEHK (#94-107) βB2: IILYENPNFTGK (#108-119) βB2: LYENPNFTGKK (#110-120) βB2: KMEIIDDDVPSFHAHGYQEK (#120-139) βB2: MEIIDDDVPSFHAHGYQEK (#121-139) βB2: MEIIDDDVPSFHAHGYQEK (#121-139, Ox. M) βB2: PSFHAHGYQEK (#129-139) βB2: VQSGTWVGYQYPGYR (#145-159) βB2: VQSGTWVGYQYPGYR (#145-159, OH on W) βB2: GLQYLLEKGDYKDSSDFGAPHPQVQSVR (#160-187) βB2: QYLLEKGDYKDSSDFGAPHPQVQSVR (#162-187) βB2: GAPHPQVQSVR (#177-187)		
9		βA3: ITIYDQENFQGK (#33-44) βB1: LVVFELENFQGR (#60-71) βB1: AEFSGECSNLADRGFDR (#73-89) βB1: GEMFILEKGEYPR (#110-122, Ox. M) βB1: ISLFEGANFK (#150-159) βB1: VSSGTWVGYQYPGYR (#187-201) βB1: GYQYLLEPGDFR (#202-213) βB1: HWNEWGAFQPQMQSLR (#214-229, Oxid. M) βB1: DKQWHLEGSFPVLATEPPK (#233-251) βB2: WDSWTSSR (#81-88) βB2: TDSLSSLRPIK (#90-100) βB2: TDSLSSLRPIKVDSQEHK (#90-107) βB2: SSLRPIKVDSQEHK (#94-107) βB2: IILYENPNFTGK (#108-119) βB2: LYENPNFTGKK (#110-120) βB2: KMEIIDDDVPSFHAHGYQEK (#120-139, Ox. M) βB2: PSFHAHGYQEK (#129-139) βB2: VQSGTWVGYQYPGYR (#145-159) βB2: GLQYLLEK (#160-167) βB2: FGAPHPQVQSVR (#176-187) βB2: GAPHPQVQSVR (#177-187)		
10		βB1: LVVFELENFQGR (#61-72) βB1: ISLFEGANFK (#151-160) βB1: VSSGTWVGYQYPGYR (#188-202) βB1: GYQYLLEPGDFR (#203-214) βB1: HWNEWGAFQPQMQSLR (#215-230, Oxid. M) βB2: IILYENPNFTGK (#108-119)		
11		βB1: LVVFELENFQGR (#60-71) βB1: VFELENFQGRR (#62-72) βB1: ISLFEGANFK (#150-159) βB1: VSSGTWVGYQYPGYR (#187-201) βB1: GYQYLLEPGDFR (#202-213) βB1: HWNEWGAFQPQMQSLR (#214-229, Oxid. M) βB1: QWHLEGSFPVLATEPPK (#235-251) βB2: IILYENPNFTGK (#108-119)	Filensin: LGELAGPEDALAR (#78-90)	
12	αB: Ac-MDIAIHHPWIR (#1-11, Oxid. M) αB: APSWFDTGLSEMR (#57-69, Oxid. M) αB: HFSPEELK (#83-90)	βA4: MVVWDEDGFQGR (#13-24, Ox. M) βB1: AAELPPGNYR (#50-59) βB1: LVVFELENFQGR (#60-71) βB1: LVVFELENFQGRR (#60-72) βB1: AEFSGECSNLADR (#73-85) βB1: LMSFRPIK (#135-142, Ox. M) βB1: ISLFEGANFK (#150-159) βB1: VSSGTWVGYQYPGYR (#187-201) βB1: GYQYLLEPGDFR (#202-213) βB1: HWNEWGAFQPQMQSLR (#214-229, Oxid. M) βB1: DKQWHLEGSFPVLATEPPK (#233-251)	Filensin: LGELAGPEDALAR (#78-90) Filensin: LNKEADEALLHNLR (#144-157) Filensin: LQLEAQFLQDDISAAKDR (#158-175)	
13			Filensin: LGELAGPEDALAR (#78-90) Filensin: VRDLEAER (#99-106)	
14	αA: TVLDSGISEVR (#55-65)	βB1: LVVFELENFQGR (#60-71) βB1: ISLFEGANFK (#150-159)	Filensin: LGELAGPEDALAR (#78-90)	Vimentin: SLYASSPGGVYATR (#50-63) Vimentin: ILLAELEQLKGQGK (#129-142) Vimentin: MALDIEIATYR (#390-400, Oxid. M) Vimentin: ISLPLPNFSSLNLR (#410-423)
15			Filensin: LGELAGPEDALAR (#78-90) Filensin: VRDLEAER (#99-106)	

Amino acid sequences of tryptic peptides recovered from protein spots present in two-dimensional gel electrophoretic profiles of water insoluble-urea soluble protein fraction isolated from the normal lens of a 68-year-old donor. The spot numbers in the table correspond to those shown in Figure 1A in the WI-US fraction of a normal lens. The amino acid sequences reported are those of tryptic fragments from individual spots.

methionine-121, 129-139, 145-159, with oxidized tryptophan-150, 160-187, 162-187, and 177-187) crystallins. Spot 9 contained βA3-, βB1-, and βB2-crystallins and had the following tryptic peptide sequences: βA3- (residue 33-44), βB1- (residue 60-71, 73-89, 110-122, with oxidized methionine-113, 150-159, 187-201, 202-213, 214-229, with oxidized methionine-226, 233-251), and βB2- (residue 81-88, 90-100, 90-107, 94-107,108-119, 110-120, 120-139, with oxidized methionine-121, 129-139, 145-159, 160-167, 176-187, and 177-187) crystallins. Spot 10, a mixture of βB1- and βB2-crystallins,

Table 3. Identification of crystallins present in protein spots of a 2D-gel of the WI-US protein fraction of a cataractous lens by the ES-MS/MS method

Spot No.	α-Crystallin	β- and γ-Crystallin	Filensin	Vimentin
3		βA3: ITIYDQENFQGK (#33-44) βA3: IYDQENFQGKR (#35-45) βA3: MEFTSSCPNVSERSFDNVR (#46-64, Oxid. M) βA3: WDAWSGSNAYHIER (#96-109)		
4		βA3: ITIYDQENFQGK (#33-44) βA3: TIYDQENFQGKR (#34-45) βA3: IYDQENFQGKR (#35-45) βA3: YDQENFQGKR (#36-45) βA3: WDAWSGSNAYHIER (#96-109) βA3: SGSNAYHIER (#100-109) βA3: MTIFEKENFIGR (#126-137, Oxid. M)		
5	αB: APSWFDTGLSEMR (#57-69, Oxid. M)	βA3: ITIYDQENFQGK (#33-44) βA3: MEFTSSCPNVSERSFDNVR (#46-64) βA3: MEFTSSCPNVSERSFDNVR (#46-64, Oxid. M) βA3: PNVSERSFDNVR (#53-64) βA3: GEYPRWDAWSGSNAYHIER (#91-109) βA3: WDAWSGSNAYHIER (#96-109) βA3: MTIFEKENFIGR (#126-137, Oxid. M) βA3: IQSGAWVCYQYPGYR (#163-177) βA3: GYQYILECDHHGGDYK (#178-193) βA3: EWGSHAQTSQIQSIR (#197-211) βA4: LTIFEQENFLGK (#106-117) βB1: LVVFELENFQGR (#60-71) βB1: ISLFEGANFK (#150-159) βB1: GYQYLLEPGDFR (#202-213) βB2: IILYENPNFTGK (#108-119)		
6		βA3: ITIYDQENFQGK (#33-44) βA3: WDAWSGSNAYHIER (#96-109)		
8		βA3: ITIYDQENFQGK (#33-44) βA4: LTIFEQENFLGK (#106-117) βB1: LVVFELENFQGR (#60-71)		
11		βB1: LVVFELENFQGR (#60-71) βB1: ISLFEGANFK (#150-159) βB1: GYQYLLEPGDFR (#202-213)		
12		βB1: LVVFELENFQGR (#60-71) βB1: LVVFELENFQGRR (#60-72) βB1: VFELENFQGRR (#62-72) βB1: FELENFQGRR (#63-72) βB1: AEFSGECSNLADRGFDRVR (#73-91) βB1: FSGECSNLADRGFDRVR (#75-91) βB1: ISLFEGANFK (#150-159) βB1: VSSGTWVGYQYPGYR (#187-201) βB1: GYQYLLEPGDFR (#202-213) βB1: HWNEWGAFQPQMQSLR (#214-229, Oxid. M) βB1: DKQWHLEGSFPVLA (#233-246) βB2: IILYENPNFTGK (#108-119)		
13		βB1: LVVFELENFQGR (#60-71) βB1: ISLFEGANFK (#150-159) βB1: APSLWVYGFSDR (#170-181) βB1: VSSGTWVGYQYPGYR (#187-201) βB1: GYQYLLEPGDFR (#202-213) βB1: HWNEWGAFQPQMQSLR (#214-229, Oxid. M)		
15		βB1: LVVFELENFQGR (#60-71) βB1: GYQYLLEPGDFR (#202-213)		

Amino acid sequence of tryptic peptides recovered from protein spots present in two-dimensional gel electrophoretic profiles of water insoluble-urea soluble protein fraction from a 61-year-old cataractous human lens. The spot numbers from a cataractous lens correspond to those shown in Figure 1B. The amino acid sequences reported are those of tryptic fragments from individual spots.

showed the following tryptic fragment sequences: βB1- (residue 61-72, 151-160, 188-202, 203-214, 215-230, with oxidized methionine-226), and βB2- (residue 108-119) crystallins. Spot 11 also contained βB1- and βB2-crystallins, and filensin and showed the following tryptic peptide sequences: βB1- (residue 60-71, 62-72, 150-159, 187-201, 202-213, 214-229, with oxidized methionine-226, 235-251), βB2- (residue 108-119) crystallins, and filensin (residue 78-90). Spot 12, which contained αB-, βA4-, and βB1-crystallins and filensin, showed the following tryptic peptide sequences: αB- (residue 1-11, with oxidized methionine-1, 57-69, with oxidized methionine-68, and 83-90), βA4- (residue 13-24, with oxidized methionine-14), βB1- (residue 50-59, 60-71, 60-72, 73-85,135-142, with oxidized methionine-137, 150-159, 187-201, 202-213, 214-229, with oxidized methionine-216, and 233-251) crystallins, and filensin (residue 78-90, 144-157, 158-175). Spots 13 and 15 were of filensin, and spot 14 was a mixture of αA- and βB1-crystallins, filensin, and vimentin. It showed the following tryptic peptide sequences: αA- (residue 55-65), βB1- (residue 60-71 and 150-159) crystallin, filensin (residue 78-90), and vimentin (residue 50-63, 129-142, 390-400, oxidized methionine-391, and 410-423).

As shown in Table 1 and Table 3, the ES-MS/MS analyses identified only nine of the 15 spots in the WI-US proteins of the cataractous lens. Spot 3 showed the following tryptic peptide sequences of βA3-crystallin: residue 33-44, 35-45, 46-64, with oxidized methionine-46, and 96-109. Spot 4 also contained βA3/A1-crystallin and showed the following tryptic peptide sequences: residue numbers 33-44, 34-45, 35-45, 36-45, 96-109, 100-109, and 126-137, with oxidized methionine-126. Spot 5 contained a mixture of αB-, βA3-, βA4-, and βB1-crystallins, and showed the following peptide sequences: αB- (residue 57-69, with oxidized methionine-68), βA3- (residue 33-44, 46-64, with oxidized methionine-46, 53-64, 91-109, 96-109, 126-137, with oxidized methionine-126, 163-177, 178-193, and 197-211), βA4- (residue 106-117), βB1- (residue 60-71, 150-159, and 202-213), and βB2- (residue 108-119) crystallins. Spot 6 was of βA3-crystallin and showed the tryptic peptide sequences with residue numbers 33-44 and 96-109. Spot 8 contained a mixture of βA4-, βA3-, and βB1-crystallins and showed the following tryptic peptide sequences: βA3- (residue 33-44), βA4- (residue 106-117), and βB1- (residue 60-71) crystallins. Spots 11, 13, and 15 were identified as of βB1-crystallin. Spot 11 showed tryptic fragments of βB1-

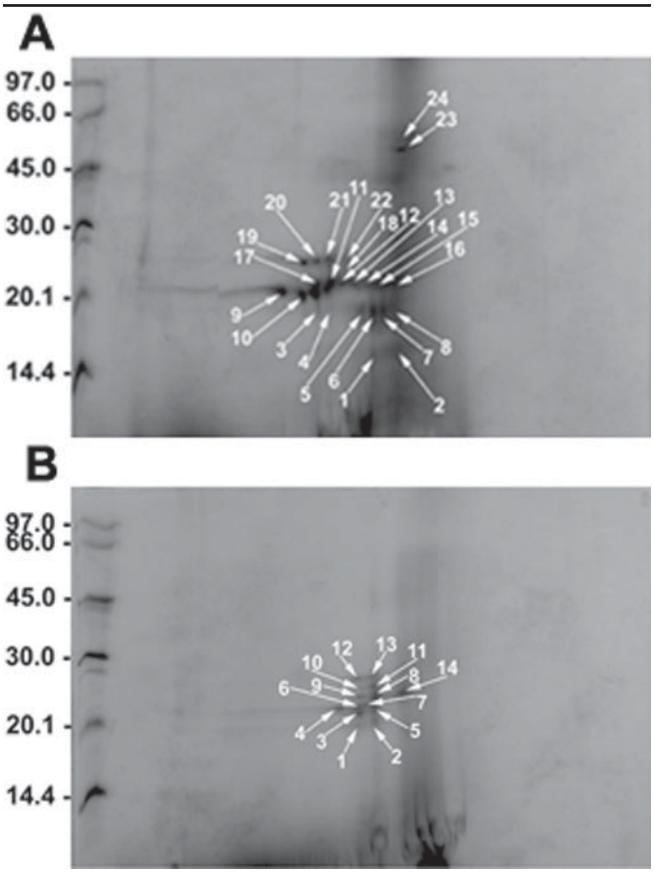

Figure 2. Two-dimensional gel electrophoretic separation of individual protein spots present in WI-UI protein fractions of a normal lens and a cataractous lens. Two-dimensional gel electrophoretic profiles of water insoluble-urea insoluble-protein fractions from (**A**) normal lens from a 68-year-old donor, and (**B**) cataractous lens with nuclear opacity from a 61-year-old donor. The numbers with arrows point to individual protein spots (starting from the lowest to the highest molecular weights) of a normal lens and a cataractous lens.

Table 4. Majority of spots on a 2D-gel contained a mixture of crystallins in the WI-UI proteins of a normal lens and a cataractous lens

SPOT NUMBER 68-yo-Normal WI-UI	COMPONENTS
1	αA
9	αB
10	αB
12	βA3
17	αB, βA3, βA4, βB1, βB2, and γS
18	βA4, βB1, βB2, γS, and filensin
19	βB1 and βB2
20	βB1 and βB2
21	βB1 and βB2
23	αA, βB1, filensin, and vimentin
61-yo-Cataractous WI-UI	
3	αB
7	αB
8	γS
11	βB1, βB2, and γS
12	βB2
13	βB1 and βB2
14	βA3, βA4, and βB1

Summary of the components identified in the two-dimensional gel separated spots in the water insoluble-urea insoluble-proteins of a 68-year-old normal human lens and a 61-year-old cataractous human lens. The spot numbers in the WI-UI protein fractions of a normal lens and a cataractous lens are identified in Figure 2. The crystallins present in each spot were identified based on the amino acid sequences of tryptic fragments as shown in Table 5 and Table 6.

TABLE 5. MAJORITY OF SPOTS ON A 2D-GEL CONTAINED A MIXTURE OF CRYSTALLINS OF IN THE WI-UI PROTEIN FRACTION OF A NORMAL LENS

Spot No.	α-Crystallin	β- and γ-Crystallin	Filensin	Vimentin
1	αA: Ac-MDVTIQHPWFK (#1-11, Oxid. M)			
9	αB: Ac-MDIAIHHPWIR (#1-11, Oxid. M) αB: Ac-MDIAIHHPWIR (#1-11, Oxid. M; OH on W) αB: Ac-MDIAIHHPWIR (#1-11, Oxid. M; 2 OH groups on W) αB: Ac-MDIAIHHPWIR (#1-11, 2 Oxid. M) αB: DIAIHHPWIR (#2-11) αB: DIAIHHPWIR (#2-11, OH on W) αB: DIAIHHPWIR (#2-11, 2 OH groups on W) αB: AIHHPWIR (#4-11) αB: APSWFDTGLSEMR (#57-69, Oxid. M) αB: APSWFDTGLSEMR (#57-69, Oxid. M) αB: APSWFDTGLSEMRLEKDR (#57-74, Oxid. M) αB: LEKDRFSVNLDVK (#70-82) αB: DRFSVNLDVK (#73-82) αB: GDVIEVHGK (#95-103) αB: EVHGKHEERQDEHGFISR (#99-116) αB: HGKHEERQDEHGFISR (#101-116) αB: PITREEKPAVTAAPK (#160-174)			
10	αB: TIPITREEKPAVTAAPK (#158-174) αB: PITREEKPAVTAAPK (#160-174)			
12		βA3: ITIYDQENFQGK (#33-44) βA3: IFEKENFIGR (#128-137) βA3: FEKENFIGR (#129-137)		
17	αB: APSWFDTGLSEMR (#57-69, Oxid. M)	βA3: ITIYDQENFQGK (#33-44) βA3: TIYDQENFQGKR (#34-45) βA3: IYDQENFQGKR (#35-45) βA3: YDQENFQGKR (#36-45) βA3: MEFTSSCPNVSERSFDNVR (#46-64, Oxid. M) βA3: MTIFEKENFIGR (#126-137, Oxid. M) βA3: IFEKENFIGR (#128-137) βA3: FEKENFIGR (#129-137) βA4: LTIFEQENFLGK (#106-117) βB1: LVVFELENFQGR (#60-71) βB1: GYQYLLEPGDFR (#202-213) βB2: IILYENPNFTGK (#108-119) γS: TFYEDKNFQGR (#8-18) γS: FYEDKNFQGR (#9-18) γS: YEDKNFQGR (#10-18)		
18		βA4: LTIFEQENFLGK (#107-118) βB1: LVVFELENFQGR (#60-71) βB1: ISLFEGANFK (#150-159) βB1: VSSGTWVGYQYPGYR (#187-201) βB1: GYQYLLEPGDFR (#202-213) βB2: IILYENPNFTGK (#108-119) γS: ITFYEDKNFQGR (#7-18) γS: FYEDKNFQGR (#9-18) γS: KPIDWGAASPAVQSFR (#158-173)	Filensin: LGELAGPEDALAR (#78-90)	
19		βB1: LVVFELENFQGR (#60-71) βB1: ISLFEGANFK (#150-159) βB1: GYQYLLEPGDFR (#202-213) βB2: AC-ASDHQTQAGKPQSLNPK (1-17) βB2: IILYENPNFTGK (#108-119) βB2: IILYENPNFTGKK (#108-120) βB2: LYENPNFTGKK (#110-120) βB2: PSFHAHGYQEK (#129-139) βB2: GLQYLLEK (#160-167) βB2: QYLLEKGDYK (#162-171) βB2: YLLEKGDYK (#163-171) βB2: SDFGAPHPQVQSVR (#174-187) βB2: GAPHPQVQSVR (#177-187)		
20		βB1: LVVFELENFQGR (#60-71) βB1: LVVFELENFQGR (#60-71, OH on F69) βB1: ISLFEGANFK (#150-159) βB1: GYQYLLEPGDFR (#202-213) βB2: AC-ASDHQTQAGKPQSLNPK (1-17) βB2: AC-ASDHQTQAGKPQSLNPK (1-17) βB2: IILYENPNFTGK (#108-119) βB2: LYENPNFTGKK (#110-120) βB2: PSFHAHGYQEK (#129-139) βB2: GLQYLLEKGDYK (#160-171)		
21		βB1: LVVFELENFQGR (#60-71) βB1: ISLFEGANFK (#150-159) βB1: VSSGTWVGYQYPGYR (#187-201) βB1: GYQYLLEPGDFR (#202-213) βB2: IILYENPNFTGK (#108-119) βB2: VQSGTWVGYQYPGYR (#145-159)		
23	αA: TVLDSGISEVR (#55-65) αA: LDSGISEVR (#57-65)	βB1: LVVFELENFQGR (#60-71) βB1: ISLFEGANFK (#150-159) βB1: GYQYLLEPGDFR (#202-213)	Filensin: LGELAGPEDALAR (#78-90)	Vimentin: TYSLGSALRPSTSR (#36-49) Vimentin: FANYIDKVR (#113-121) Vimentin: ILLAELEQLK (#129-138) Vimentin: ILLAELEQLKGQGK (#129-142) Vimentin: LGDLYEEEMRELR (#145-157, Oxid. M) Vimentin: KVESLQEEIAFLK (#222-234) Vimentin: FADLSEAANRNNDALR (#294-309) Vimentin: HLREYQDLLNVK (#378-389) Vimentin: EYQDLLNVK (#381-389) Vimentin: MALDIEIATYR (#390-400, Oxid. M) Vimentin: ISLPLPNFSSLNLR (#410-423) Vimentin: PLPNFSSLNLR (#413-423) Vimentin: PNFSSLNLR (#415-423) Vimentin: ETNLDSLPLVDTHSK (#424-438) Vimentin: PLVDTHSKR (#431-439)

Amino acid sequences of tryptic peptides recovered from protein spots present in two-dimensional gel electrophoretic profiles of water insoluble-urea insoluble protein fraction from a 68-year-old normal human lens. The spot numbers in the WI-UI protein fraction of a normal lens are identified in Figure 2A. The crystallins present in each spot were identified based on the amino acid sequences of tryptic fragments.

crystallin with sequences of residue 60-71, 150-159, and 202-213. Spot 13 contained βB1-crystallin, and showed sequences of peptides with residue 60-71, 150-159, 170-181, 187-201, 202-213, and 214-229, with oxidized methionine-226. Similarly, spot 15 of βB1-crystallin showed peptide sequences with residue 60-71 and 202-213.

Taken together, the aforedescribed comparative analyses showed that mostly β-crystallins became water insoluble in the cataractous lens compared to the normal aging lens.

ES-MS/MS analyses of water insoluble-urea insoluble protein spots from two-dimensional gels of cataractous and normal lenses: The WI-UI proteins on 2D gel analysis showed 24 major spots in the normal lens from a 68-year-old donor (Figure 2A) and 14 major spots in the cataractous lens from a 61-year-old donor (Figure 2B). The identities of the species present in each spot from normal and cataractous lenses are shown in Table 4. The detailed amino acid sequences of the tryptic peptides of species in each spot of the normal and the cataractous lenses are shown in Table 5 and Table 6, respectively. Because of the lack of adequate quantities for ES-MS/MS analyses, the tryptic peptide sequences of only ten out of 24 spots of the normal lens were successfully analyzed (Table 4 and Table 5). Among these, except for the four spots (1, 9, 10, and 12), the remaining six spots (17, 18, 19, 20, and 21) contained multiple crystallins, whereas spot 23 contained crystallins plus filensin and vimentin (Table 4 and Table 5). Among the WI-UI protein spots of the cataractous lens, four spots (3, 7, 8, and 12) were of individual crystallins and three spots (11, 13, and 14) were mixtures of different β-crystallins (Table 4 and Table 6).

The amino acid sequences of tryptic peptides from each spot are described in Table 5 and Table 6 and the description to follow identifies these species by their residue numbers corresponding to their location within a crystallin. In the normal lens (Table 5), spot 1 was of αA-crystallin as it contained the crystallin tryptic peptide with residue 1-11 (with an oxidized

Table 6. Majority of spots on a 2D-gel contained a mixture of crystallins in the WI-UI proteins of a cataractous lens

Spot No.	α-Crystallin	β- and γ-Crystallin	Filensin	Vimentin
3	αB: MDIAIHHPWIR (#1-11, Oxid. M) αB: DIAIHHPWIR (#2-11) αB: APSWFDTGLSEMR (#57-69, Oxid. M) αB: APSWFDTGLSEMR (#57-69) αB: TIPITREEKPAVTAAPK (#158-174) αB: PITREEKPAVTAAPK (#160-174) αB: EEKPAVTAAPK (#164-174)			
7	αB: DIAIHHPWIR (#2-11) αB: APSWFDTGLSEMR (#57-69, Oxid. M) αB: HFSPEELK (#83-90) αB: GDVIEVHGKHEERQDEHGFISR (#95-116)			
8		γS: FYEDKNFQGR (#9-18)		
11		βB1: ISLFEGANFK (#150-159) βB2: IILYENPNFTGK (#108-119) γS: TFYEDKNFQGR (#8-18) γS: FYEDKNFQGR (#9-18)		
12		βB2: AC-ASDHQTQAGKPQSLNPK (#1-17) βB2: IILYENPNFTGK (#108-119) βB2: VQSGTWVGYQYPGYR (#145-159) βB2: GLQYLLEKGDYKDSSDFGAPHPQVQSV R (#160-187)		
13		βB1: LVVFELENFQGR (#60-71) βB1: ISLFEGANFK (#150-159) βB1: VSSGTWVGYQYPGYR (#187-201) βB1: GYQYLLEPGDFR (#202-213) βB2: AC-ASDHQTQAGKPQSLNPK (#1-17) βB2: IILYENPNFTGK (#108-119) βB2: VQSGTWVGYQYPGYR (#145-159)		
14		βA3: MEFTSSCPNVSERSFDNVR (#46-64) βA3: WDAWSGSNAYHIER (#96-109) βA4: LTIFEQENFLGK (#106-117) βB1: LVVFELENFQGR (#60-71)		

Amino acid sequences of tryptic peptides recovered from protein spots present in two-dimensional gel electrophoretic profiles of water insoluble-urea insoluble-protein fraction from a 61-year-old human cataractous lens. The spot numbers in the WI-UI protein fractions of a cataractous lens are identified in Figure 2B. The crystallins present in each spot were identified based on the amino acid sequences of tryptic fragments.

methionine-1 residue). Spot 9 was of αB-crystallin and exhibited the entire sequence of the crystallin, i.e., residue 1-11, (with an oxidized methionine-1 and tryptophan-9—both residues with one or two oxygen), 2-11, with an oxidized tryptophan-9 with one or two oxygen molecules, 4-11, 57-69 (with oxidized methionine), 57-74 (with an oxidized methionine-68), 70-82, 73-82, 95-103, 99-116, 101-116, and 160-174. Spot 10 of the normal lens was also of αB-crystallin as it showed tryptic peptide sequences with residue number 158-174 and 160-174. Spot 12 was of βA3-crystallin, and it exhibited the tryptic peptide sequences with residue numbers 33-44, 128-137, and 129-137. Spot 17 was identified as a mixture of six crystallin (i.e., αB-, βA3-, βA4-, βB1-, βB2-, and γS-crystallins), and showed the following tryptic peptide sequences: αB- (residue 57-69, with oxidized methionine-68), βA3- (residue numbers 33-44, 34-45, 35-45, 36-45, 46-64, with oxidized methionine-46, 126-137, with oxidized methionine-126, 128-137, and 129-137), βA4- (residue 106-117), βB1- (residue 60-71 and 202-213), βB2- (residue 108-119), and γS- (residue 8-18,9-18, and 10-18) crystallins. Spot 18 was also a mixture of four crystallins (βA4-, βB1-, βB2-, and γS-crystallins) and filensin, and it showed the following tryptic peptide sequences: peptides representing partial amino acid sequences of βA4- (residue 106-117), βB1- (residue 60-71, 150-159, 187-201, and 202-213), βB2- (residue 108-119), γS- (residue 7-18, 9-18, and 158-173) crystallins and filensin (residue 78-90). Spots 19, 20, and 21 contained βB1- and βB2-crystallins and showed the following tryptic fragments sequences: βB1- (residue 60-71, 150-159, and 202-213) and βB2- (residue 1-17, 108-119, 108-120, 110-120, 129-139, 160-167, 162-171, 163-171, 174-187, and 177-187) crystallins. Similarly, spot 20, a mixture of βB1 and βB2-crystallins, showed the following amino acid sequences: βB1- (residue 60-71, 60-71, OH on phenylalamine-69, 150-159, and 202-213), and βB2- (residue numbers 1-17, 108-119, 110-120, 129-139, and 160-171) crystallins. Spot 21 contained βB1- and βB2-crystallins and showed the following tryptic peptide sequences: βB1- (residue 60-71, 150-159, 187-201, and 202-213) and βB2- (residue 108-119 and 145-159) crystallins. Spot 23 was a unique mixture of αA- and βB1-crystallins and filensin and vimentin and showed the following tryptic peptide sequences: αA- (residue 55-65 and 57-65), βB1- (residue 60-71, 150-159, and 202-213) crystallins, filensin (residue 78-90), and vimentin (residue 36-49, 113-121, 129-138, 129-142, 145-157, oxidized methionine-154, 222-234, 294-309, 378-389, 381-389, 390-400, with oxidized methionine-391, 410-423, 413-423, 415-423, 424-438, and 431-439).

As stated, only seven of the 13 spots of WI-UI proteins of the cataractous lens could be identified by the ES-MS/MS method (Table 6). Spots 3 and 7 were of αB-crystallin, and spot 3 showed the following tryptic peptide sequences of αB-crystallin: residue 1-11, with oxidized methionine-1, 2-11, 57-69, with oxidized M-68, 158-174, 160-174, and 164-174. Spot 8 was of γS-crystallin and showed only a tryptic fragment with residue 9-18. Spot 11 was a mixture of βB1-, βB2-, and γS-crystallins that showed the following tryptic peptide sequences: βB1- (residue 150-159), βB2- (residue 108-119) and γS- (residue 8-18 and 9-18) crystallins. Spot 12 was of βB2-crystallin and showed tryptic peptide sequences with residue numbers 1-17, 108-119, 145-159, and 160-187. Spot 13 contained βB1- and βB2-crystallins and exhibited the following tryptic peptide sequences: βB1- (residue 60-71, 150-159, 187-201, and 202-213), and βB2- (residue 1-17, 108-119, and 145-159) crystallins. This spot showed similar composition as spots 18, 19, and 20 of the WI-UI protein fraction of normal human lenses. Spot 14 was a mixture of βA3-, βA4-, and βB1-crystallins, and was unique to the cataractous lens because of its absence in the WI-UI proteins of the normal lens. This spot showed the following tryptic peptide sequences: βA3-crystallin (residue 46-64 and 96-109), βA4-crystallin (residue 107-118), and βB1-crystallin (residue 60-71).

DISCUSSION

This study was an extension of our two previous studies [18,19], which were focused on characterization of crystallin complexes in vivo in aging and cataractous human lenses. Our first study [19] showed the existence of two types of covalent multimers in the WI proteins found in 25- and 41-year-old normal human lenses, i.e., one was composed of fragments of eight different crystallins (αA-, αB-, βA3-, βA4-, βB1-, βB2-, γS-, and γD-crystallin), and the second composed of fragments of α-, β-, and γ-crystallins and two beaded filament proteins (phakinin and filensin). The αA-crystallin fragments in these complexes showed four major posttranslational modifications (truncation of crystallins, oxidation of methionine and tryptophan residues, conversion of serine to dehydroalanine, and formylation of histidine residue), which might be responsible for the aggregation/covalent cross-linking among crystallins in the human lens. Our second study [18] showed that the crystallin species of the WS-HMW and WI protein fractions of cataractous lenses differed from that of normal lenses, and the former lenses showed selective insolubilization of fragments of βA3/A1- and βB1-crystallins. Additionally, crystallin species showed relatively greater truncation, deamidation of asparagine to aspartic acid residue, and oxidation of tryptophan residues in cataractous lenses compared to aging lenses.

In the present study, we comparatively analyzed the 2D gel electrophoretically separated spots from WI-US and WI-UI proteins of normal and cataractous lenses. The whole lens extracts from one normal and one cataractous lens were used to isolate WI-US and WI-UI protein fractions. No distinction was made in this study regarding changes between the cortical and nuclear regions of these lenses. It was also not determined whether ultrasound, heat and oxygenation that the cataractous lens underwent during the surgical removal affected protein solubility and structure. The major findings of the comparative study of WI-US protein species of normal and cataractous lenses were as follows: (1) Although the majority of WI-US protein spots in both the 68-year-old normal lens and the 61-year-old cataractous lens showed M_r between 20 to 30 kDa on a SDS-gel, their amino acid sequence analyses showed that they contained a mixture of αA-, αB-, and β-crystallins and filensin as well as vimentin; (2) in the normal lens, the

relative number of spots containing αB-crystallin and βA3-, βA4-, βB1-, or βB2-crystallins were greater compared to spots with αA-crystallin; (3) the absence of αA- but not of αB-crystallin in the protein spots of the cataractous lens but not of the normal lens was observed; (4) the cataractous lens showed 2D gel spots that contained mostly β-crystallins, suggesting their preferential insolubilization during cataractogenesis. These spots had either a mixture of five crystallins (αB-, βA3-, βA4-, βB1-, and βB2-crystallins), three crystallins (βA3-, βA4-, and βB1-crystallin) or two crystallins (βB1- and βB2-crystallins), and the spot with five crystallins was uniquely present only in the cataractous lens; and (5) the major modifications in the water insolubilized species were truncation, phosphorylation of serine-66, and oxidation of methionine and tryptophan residues in αB-crystallin. The deamidation of these species as a modification was not examined. Taken together, the aforedescribed findings suggested that certain crystallins with the posttranslational modifications might undergo insolubilization in both normal and cataractous lenses.

The comparative study of WI-UI protein species of normal and cataractous lenses showed the following: (1) the normal lens contained spots with individual αA-, αB-, and βA3/A1-crystallins, but a few spots also had a mixture of: (i) βB1- and βB2-crystallins, (ii) βA4-, βB1-, βB2-, βS-crystallins and filensin, (iii) αA- and βB1-crystallins, plus filensin and vimentin, and (iv) αB-, βA3-, βA4-, βB1-, βB2-, and βS-crystallins. In contrast, the cataractous lenses showed spots containing individual αB-, βB2-, and γS-crystallins, and a mixture of β-crystallins that contained (i) βB1-, βB2-, and γS-crystallins, (ii) βB1- and βB2-crystallins, and (iii) βA3-, βA4-, and βB1-crystallins; and (2) in contrast to the normal lens, the WI-UI protein spots of the cataractous lens contained αB-crystallin while αA-crystallin was absent, suggesting a major role of αB-crystallin in the insolubilization process.

The aforedescribed findings of the existence of cataract-specific insoluble species that differed from those present in a normal aging lens were novel and different from previous reports in the literature. The findings might be the result of our extensive amino acid sequence analyses of tryptic peptides of species present in 2D gel separated spots. It was intriguing that although the 2D gel separated spots had M_r of 20 to 30 kDa, the majority of them contained multiple crystallins. Because during the 2D gel electrophoresis, the protein were treated with 10 mM DTT and 10 mM iodoacetamide after the first dimension (IEF) and prior to the second dimensional SDS-PAGE, the disulfide bondings among the protein species present with individual spots were minimized. Although our previous findings [19] and results of the present study suggested the presence of multiple crystallin might be due to their association by aggregation, this was not further investigated in this study.

Several past studies have suggested that modified crystallin could aggregate and cross-link in human lenses during aging and cataractogenesis [22-28]. However, the major challenge has been to identify cataract-specific complexes, and elucidate their modifications as potential mechanisms for cross-linking including identification of participating amino acids in covalent bonding. The unique presence of only αB- without αA-crystallin with βA3-, βA4-, βB1-, and βB2-crystallins in the WI-US protein spots of the cataractous lens compared to the presence of both αA- and αB-crystallins with other crystallins in the normal lens was significant. Further, in contrast to the normal lens, the WI-UI proteins of the cataractous lens showed mostly β-crystallins (βB1-, βB2-, βA3-, and βA4-crystallins) as insoluble species. These species could not separated as individual spots even after urea treatment, suggesting their potential cataract-specific complex nature. The results further warrant investigations whether mainly acidic and basic β-crystallins interact with αB-crystallin, and exist as complexes in vivo.

Although partial amino acid sequences of various crystallins were observed, whether they existed in truncated form is difficult to determine from ES-MS/MS data. However, several past studies have implicated a potential role for crystallin fragments in lens protein aggregation and cross-linking: (1) the crystallin fragments were present in the opaque but not in the clear portion of a human brunescent cataractous lens [29]; (2) the COOH-terminally truncated bovine αA-crystallin species formed oligomeric complexes of much higher molecular weights than those formed by the native species [30]; (3) the in vitro proteolysis of rat lens soluble proteins by calpain resulted in a rapid increase in turbidity that was inhibited by E-64, an inhibitor of calpain-type cysteine proteinases [31,32]; (4) human cataract-specific HMW aggregates contained a heterogeneous 10 kDa breakdown product, in addition to 20 and 43 kDa components [33]; (5) a human lens 10 kDa polypeptide was found to be glycated and might play a role in protein aggregation and insolubilization [34]; (6) we have reported covalent multimers of >90 kDa in aging human lenses; the multimers were made of either crystallin fragments or crystallin fragments and filensin and phakinin (two beaded filament proteins) [19]; and (7) our results showed age-related aggregation (i.e., an increase in the levels of crystallin fragments in both WS-HMW proteins: 5-6% of total protein in 16- to 19-year-old lenses compared to 27% in the 60- to 80-year-old lenses) [35], and in the WI proteins (up to 20% of total protein) [36].

Although the present literature suggests that β-crystallin oligomers exist and play a critical role in maintenance of lens transparency [35], the exact nature of interactions among acidic and basic β-crystallins in the oligomeric state is unclear.. A previous study [37] identified three β-crystallin oligomers in human lenses: β1- (150 kDa), β2- (92 kDa), and β3-crystallin (46 kDa). The β1-crystallin oligomer contained βA3/A1-, βA4-, βB1-, and βB2-crystallins, the β2-crystallin oligomer contained βA3/A1-, βA4-, βB1-, and βB2-crystallins, and the β3-crystallin oligomer contained βB1- and βB2-crystallins. The study concluded that the major differences in the oligomers were the presence of βA3/A1- and βA4-crystallins in the β1- and β2-crystallin oligomers and their absence in the β3-crystallin oligomer, and the aggregate sizes correlated with the length of the NH_2-terminal extension of βB1-crystallin. While this study identified species that interact to form the three different β-crystallin oligomers in human lenses, it also suggested

that the NH_2-terminal arm of βB1-crystallins might be involved in the higher order oligomerization. Previous studies have shown that the deletion of NH_2- and COOH-terminal extensions of βB1- and βB2-crystallin had little effect on stability of structures of βB1- and βB2-crystallins [38,39]. However, the effects of truncation of NH_2- and COOH-terminal regions in motifs in the two domains β-crystallins are still unknown. Our recent study involving deletion mutants (missing one of the four motifs at a time) of βA3/A1-crystallin showed that deletion of NH_2-terminal extension plus motif I, or the NH_2 extension plus motif I and II, NH_2 extension plus motifs I, II, and connecting peptide or only of motif IV resulted in the insolubilization of the crystallin and its appearance in the inclusion bodies [40]. We are presently studying the effect of these deletions on the solubility of βA3/A1-crystallin.

The βA3/A1-crystallin and βB2-crystallin showed spontaneous oligomerization into tetramer species in vitro. The NMR studies revealed that the NH_2-terminal extension of βA3-crystallin was water exposed, whereas both NH_2- and COOH-terminal extensions of βB2-crystallin were involved in the protein-protein interactions [41]. The data further suggested an interaction between βA3- and βB2-crystallins, and the COOH-terminal region of βA3-crystallin and both NH_2- and COOH-terminal regions of βB2-crystallin participated in the oligomer formation. Therefore, if either βA3- or βB2-crystallin were truncated, it would disrupt their interactions and might also lead to insolubility.

Our study also identified specific modifications in crystallins; however, their potential roles could only be speculated. The major modifications in the crystallins present as insoluble species were phosphorylation of serine-66, and oxidation of methionine and tryptophan residues in αB-crystallin. A previous study showed that αB-crystallin, in response to various stress, was phosphorylated at serine-19, serine-45, and serine-59 [42]. We identified an additional phosphorylation site at serine-66 in αB-crystallin that existed in the WI-US proteins of normal lenses (Table 3). A previous study has suggested that the phosphorylation of serine-59 in αB-crystallin protects the apoptosis of cardiac myocytes under physiological stress such as hypoxia. Therefore, the observed phosphorylation of αB-crystallin in our study might also be related to stress and possibly to its chaperone function.

A recent report indicated that deamidation—not truncation—decreased the urea stability of βB1-crystallin during examination at a concentration when βB1-crystallin would mainly exist as a monomer or a dimer [10]. The report showed that truncation of up to 47 residues at the NH_2-terminal and five residues at the COOH-terminal region did not affect the stability of βB1-crystallin. Because the βB1-crystallin existed alone without other companion β-crystallins as reported in β1-, β2-, and β3-crystallin oligomers [37], the effect of truncation and deamidation of βB1-crystallin on its stability under native conditions might be different. The crystal structure of a truncated βB1-crystallin (lacking 41 NH_2-terminal amino acids) has been published [43]. In contrast to βB2-crystallin, the homodimer of βB1-crystallin showed that its domains were paired intramolecularly, and thus more distinctly related to monomeric γ-crystallin. According to the study, the dimeric βB1-crystallin structure was extremely suited to form higher order lattice structure using its hydrophobic patches, linker regions and sequence extensions.

Our study also showed the presence of several oxidized residues in crystallin fragments of WI-US as well as WI-UI proteins of both normal and cataractous lenses. Four examples of such oxidized residues in tryptic fragments are: an αB-crystallin fragment (residue 1-11, MDIAIHHPWIR, oxidized methionine-1 and tryptophan-9), two βA3-crystallin fragments (residue 46-64, MEFTSSCPNVSERSFDNVR, oxidized methionine-46, and residue 126-137, MTIFEKENFIGR, oxidized methionine-126), and βB1-crystallin fragment (residue 215-230, HWNEWGAFQPQMQSLR, oxidized methionine-226). Human and animal studies have shown a strong correlation between oxidative insult of crystallins and cataract development. The tryptophan oxidation products of αA- and αB-crystallins have been identified in a previous study [44]. Tryptophan (molecular weight 186), on oxidation acquires one oxygen and becomes hydroxyltryptophan (HRTP; molecular weight 202), and on acquiring two oxygen produces N-formylkynurenine (NFK; molecular weight 218) [44]. As shown in our study, αA-crystallin species contained modified tryptophan with either one or two oxygen, suggesting the conversion of the residue to HRTP and NFK. Because the oxidation is believed to play a major role in the development of senile cataract [3], both tryptophan oxidation products (HRTP and TFK) might act as a photosensitizer, capable of producing reactive oxygen species [45].

Based on our observations and the present literature, it is clear many factors play a role in the formation of aggregated and cross-linked crystallin species during cataract development. However, their relative roles remain unclear. It could be that the age-related senile cataract development is the result of cumulative effects of variety of factors such as truncation, phosphorylation, and oxidative insults of crystallins. In turn, these might together overwhelm changes in crystallins, causing them to aggregate, cross-link, and become water insoluble.

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