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Age-Related Changes in the Spatial Distribution of Human Lens α -Crystallin Products by MALDI Imaging Mass Spectrometry

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Abstract

Purpose—To develop a protocol for MALDI (matrix-assisted laser desorption ionization) imaging mass spectrometry for mapping the distributions of α -crystallin and its modified forms in human lens tissue as a function of lens age and cataract.

Methods—Frozen human lenses were cryosectioned equatorially and axially into 20- μ m-thick sections, and the sections were mounted onto conductive glass slides by methanol soft-landing. An ethanol washing procedure facilitated uniform matrix crystal formation by a two-step matrix deposition procedure to produce high-quality mass spectral data. Molecular images of modified and unmodified α -crystallin subunits were obtained from mass spectral data acquired in 100- μ m steps across normal and cataractous lens sections. Proteins extracted from the lens sections were digested with endoproteinase Glu-C and subjected to mass spectrometric analysis for identification of modifications.

Results—Intact α -crystallin signals were detected primarily in the outer cortical fiber cells in lenses up to 29 years of age. Multiple truncation products were observed for α -crystallin that increased in abundance, both with distance into the lens and with lens age. Phosphorylated α B-crystallin forms were most abundant in the cortical region of older lenses. In axial sections, no significant anterior–posterior pole variation was observed. A previously unreported α A-crystallin mutation was detected in an age-matched cataractous human lens.

Conclusions—A method has been developed to spatially map the age-related changes of human lens α -crystallin by MALDI imaging mass spectrometry including a novel L52F α A-crystallin mutation in a cataractous lens. Application of this spatially resolved proteomic technique to lens biology enhances the understanding of α -crystallin protein processing in aging and diseased human lenses.

The ocular lens consists of an epithelial cell monolayer covering the anterior pole, and highly elongated fiber cells, which make up the bulk of the lens cell mass. Throughout life, lens epithelial cells migrate toward the lens equator where they begin to elongate and differentiate into lens fiber cells. Over time, layers of fiber cells are laid down to create a gradient of cell age in which the oldest cells are located in the lens nucleus and the youngest in the lens cortex. During fiber differentiation, potential light-scattering elements such as cell organelles and nuclei are degraded,^{1,2} which not only removes them from the path of light, but also removes the ability of differentiated lens fiber cells to synthesize new protein. Together with the abundant expression of crystallin proteins in lens fiber cells, which contributes to the gradient

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of refractive index in the lens,^{3,4} these vital lens fiber cell-specific adaptations help maintain lens transparency throughout life.

Three main classes of crystallin proteins that are present in the mammalian lens— α , β , and γ —together constitute up to 90% of total lens protein.⁵ A typical mammalian lens contains 30% to 40% α -crystallin,⁶ and its structure and function have been studied extensively in normal and diseased lens states. A member of the small heat shock protein family, α -crystallin consists of two subunits, α A and α B, which exist as large heterogeneous multimeric complexes with molecular weights ranging from 300,000 to more than 1,000,000.⁶ Generally, the ratio of α A to α B subunits in the mammalian lens is 3:1. It is well established that α -crystallin acts as both a structural protein and a molecular chaperone binding to denatured lens proteins to prevent nonspecific protein aggregation.^{7,8} Since there is no protein turnover in differentiated lens fibers, lens proteins, including α -crystallin, undergo many age-related post-translational modifications that include truncation, deamidation, glycation, and phosphorylation, which are thought to induce functional changes.^{5,9–11} Truncation^{12–14} and phosphorylation^{15–18} are known to alter the chaperone activity of α -crystallin. Furthermore, lens protein modifications accumulate over life and may contribute to lens opacification and cataractogenesis by inducing aggregation and water insolubilization of lens proteins in aged and cataractous tissue, although this process is not fully understood.¹⁹

Early studies of lens crystallins used whole lens homogenates and one-dimensional gel electrophoresis methodologies to investigate structure and function.²⁰ More recently, microdissection techniques producing multiple distinct lens regions,^{21,22} two-dimensional gel electrophoresis (2-DE),^{23,24} and mass spectrometric techniques have been used to identify and localize many posttranslational modifications of α -crystallin.^{25,26} Although immensely powerful, these studies remain limited in the amount of spatial information produced.

Recently, MALDI (matrix-assisted laser desorption ionization) imaging mass spectrometry (MALDI tissue imaging) techniques were developed to map the distribution of α -crystallin products in bovine and rabbit lenses.^{27,28} MALDI tissue imaging is a relatively new proteomic technique that utilizes the molecular specificity of biological mass spectrometry while maintaining spatial information and allows multiple unmodified and modified forms of a protein to be mapped simultaneously, directly from thin tissue sections.^{29,30} With this technology, multiple truncation products and phosphorylated α -crystallin forms have been detected in the bovine and rabbit lens. In the present study, the MALDI imaging technique has been adapted to study posttranslational processing of human α -crystallin as a function of lens age and disease.

Methods

Reagents

Acetonitrile, high-performance liquid chromatography (HPLC)-grade water, formic acid, and sinapinic acid (SA) were purchased from Sigma-Aldrich (St. Louis, MO); indium tin oxide (ITO)-coated conductive glass microscope slides from Bruker Daltonics (Billerica, MA); and tissue freezing medium (TFM) from Triangle Biomedical Sciences, Inc. (Durham, NC). Frozen human lenses were obtained from the National Disease Research Interchange (Philadelphia, PA) and stored at -80°C until further use. Lens tissue was treated in compliance with the Declaration of Helsinki. Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich.

Tissue Preparation for MALDI Analysis

Frozen human lenses were attached to cold specimen chucks with application of a small amount of TFM at the base of the tissue only. Lenses were sectioned equatorially or axially at -20°C into $20\text{-}\mu\text{m}$ -thick tissue sections using a disposable blade stage-equipped cryostat (model HM 550; Microm, Walldorf, Germany). For collection of frozen sections, a thin uniform layer of methanol (room temperature, RT) was applied to the conductive glass slides (RT), and cryosections thaw mounted by touching the glass slide to the tissue section before evaporation of methanol. This procedure helped to maintain tissue integrity and to attach the tissue section to the glass slide. After air drying, the tissue sections were bath washed successively for 60 seconds each in 70%, 95%, and 100% ethanol, which facilitated uniform matrix crystal formation across the entire lens section by fixing the tissue and washing away physiological salts and lipids.

Matrix Deposition

Human lens tissue sections were first seeded with several coats of an acid-free matrix solution of 15 mg/mL SA freshly prepared in acetonitrile-water (50:50, vol/vol) using a TLC sprayer (Kimble/Kontes, Vineland, NJ). After the sections were dried, a solution of 15 mg/mL SA freshly prepared in acetonitrile-water-formic acid (50:40:10, vol/vol/vol) was sprayed evenly on them. Repeated cycles of matrix solution spraying were applied when tissue sections appeared mostly dry, approximately 45 to 60 seconds after previous spray application. Typically, a total volume of 15 mL was used to obtain an even matrix coating generating good-quality test MALDI mass spectra.

MALDI Imaging

Mass spectrometric analyses were performed in the linear positive mode at $+20\text{ kV}$ accelerating potential on a time-of-flight mass spectrometer (Autoflex III Linear; Bruker Daltonik, Bremen, Germany), which was equipped with a laser (Smartbeam; Bruker Daltonik) capable of operating at a repetition rate of 200 Hz with optimized delayed extraction time. Using a protein standard (Protein Standard 1, containing bovine insulin, equine cytochrome *c*, bovine ubiquitin I, and equine myoglobin; Bruker Daltonik), a linear external calibration was applied to the instrument before data collection. Mass spectral data sets were acquired over whole human lens sections (flexImaging software; Bruker Daltonik) in the mass range of m/z 3,000 to 30,000, with a raster step size of $100\text{ }\mu\text{m}$ and 250 laser shots per spectrum. After data acquisition, molecular images were reconstituted with the software. Each data set consisted of approximately 8000 individual sampling locations, each representing one pixel in the resultant image. Data were normalized to total ion current in the software, and each m/z signal was plotted ± 6 mass units. For display purposes, the data were interpolated and pixel intensities were normalized to the maximum intensity for each m/z displayed in the software to use the entire dynamic range. Assignments of protein identifications were made based on previous tandem mass spectrometry (LC-MS/MS) identifications in the bovine lens²⁸ by matching observed m/z values to predicted m/z values of abundant human lens crystallin proteins (Table 1).

Gel Electrophoresis and Western Blot Analysis

Young (7 years) and old (68 years) human lenses were manually dissected on ice into cortical and nuclear regions, and the tissue was homogenized in buffer containing 300 mM ammonium bicarbonate, 5 mM EDTA, and 10 mM sodium fluoride. Samples were centrifuged at $90,000g$ (average) for 20 minutes at 4°C with a rotor (model TLA45; Optima TL Ultracentrifuge; Beckman Coulter, Inc., Fullerton, CA) to separate lens membranes from cytosolic proteins. After determination of protein concentration by BCA protein assay (Thermo Fisher Scientific, Rockford, IL), $3\text{ }\mu\text{g}$ of cytosolic lens protein from cortical and nuclear regions

of young and old lenses was loaded onto a 12% Bis-Tris gel (Invitrogen, Carlsbad, CA). Gel electrophoresis was performed (Novex XCell II Mini Cell; Novex, San Diego, CA) with MES SDS running buffer (Invitrogen). After electrophoresis, the proteins were visualized in the gel (SYPRO Ruby Protein Gel Stain; Invitrogen), according to the manufacturer's instructions. For Western blot analysis, proteins were transferred onto PVDF membrane (Immun-Blot; Bio-Rad Laboratories, Inc., Hercules, CA) for 1 hour at room temperature with a blotter apparatus (Criterion Blotter apparatus; Bio-Rad Laboratories, Inc.). The membrane was blocked for 1 hour in 5% milk and then labeled with anti- α A-crystallin antibody (1:1000 in 5% milk; Abcam, Inc., Cambridge, MA) overnight at 4°C. After six washes of 5 minutes each in phosphate-buffered saline Tween-20 (PBST), the membrane was incubated in horseradish peroxidase-conjugated secondary antibody (1:10,000 in 5% milk; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Finally, the membrane was washed six times for 5 minutes each in PBST and labeling was detected by chemiluminescence (ECL Western Blot Detection reagent; Amersham Biosciences, Bucks, UK).

MALDI Mass Spectrometric Comparison of Normal and Cataractous α A-Crystallin

Sister sections of the normal (51 year) and cataractous (54 year) lenses used for MALDI imaging were divided into cortical (full-length α A-crystallin detected) and nuclear (full-length α A-crystallin not detected) regions based on the MALDI images, by separating and scraping the two regions from the conductive glass slides with a razor blade. Proteins were extracted from dissected regions with acetonitrile-water-formic acid (50:40:10, vol/vol/vol), the samples were centrifuged at 12,000g for 20 minutes at 4°C (model 5415C centrifuge; Eppendorf AG, Hamburg, Germany) to remove cellular debris, and the supernatant was removed and concentrated (Speed-Vac; Labconco, Kansas City, MO). For MALDI analysis, the samples were spotted onto a MALDI plate with 20 mg/mL SA in acetonitrile-water-formic acid (50:40:10, vol/vol/vol) and analyzed in linear positive ion mode on a time-of-flight mass spectrometer (Autoflex III Linear; Bruker Daltonik).

Identification of α A-Crystallin Mutation in Cataractous Lens

One whole equatorial section (20 μ m thick) from the cataractous 54-year-old lens, previously mounted on a conductive glass slide, was removed with a razor blade and placed in a microcentrifuge tube. Proteins were extracted using a solution containing 300 mM ammonium bicarbonate and 5 mM EDTA. After vortexing, cell membranes and debris were removed by centrifugation at 12,000g for 20 minutes at 4°C (model 5415C centrifuge; Eppendorf AG). The supernatant containing soluble lens proteins was removed and digested with endoproteinase Glu-C for 18 hours at 37°C. The digested sample was desalted with a C18 zip tip (Millipore, Billerica, MA) and bound peptides eluted using 90% acetonitrile. The solution containing eluted peptides was dried (Speed-Vac; Labconco), resuspended in 0.1% FA, and injected onto a C18 column that was coupled to a mass spectrometer equipped with a nanospray ion source (LTQ Orbitrap; Thermo Fisher Scientific). Peptides were separated by gradient elution with mobile phases A (0.1% FA) and B (acetonitrile, 0.1% FA) at a flow rate of 500 nL/min using a gradient of 2% B to 25% B over 35 minutes and 25% B to 90% B over 15 minutes. Mass spectra were acquired in the range m/z 400 to 2000 using the mass analyzer (Orbitrap; Thermo Fisher Scientific) at a resolution of 60,000 for high mass accuracy with the dynamic exclusion function enabled. The five most intense peaks in each full MS scan were selected for MS/MS sequence analysis in the ion trap. Protein identification was accomplished using the Sequest algorithm (Bioworks ver. 3.2; Thermo Fisher, San Jose, CA) using a custom database containing human α -crystallin. Peptide assignments were confirmed by manual interpretation.

Results

The age-related modification of lens crystallin proteins has been studied extensively, typically by traditional biochemical methods such as microdissection and gel electrophoresis. This approach has shown that α -crystallin truncation increases with both fiber cell age^{21,23,25} and lens age.^{31,32} While gel electrophoresis combined with Western blot analysis allows investigation of lens crystallin truncation as a function of fiber cell and lens age, these techniques are limited by the spatial resolution of microdissection techniques and the inability to determine the exact sites of modification. In addition, low-molecular-weight truncation products are difficult to detect using standard protein gel staining procedures.

MALDI imaging mass spectrometry is a relatively new spatially resolved proteomic technique that provides simultaneous detection of multiple proteins and their modified forms across thin tissue sections. In the MALDI mass spectrometer, the laser is scanned in a raster pattern across the matrix-coated tissue and a mass spectrum collected at each sampling location. Spatial resolution is set by the distance in x and y between sampling locations. To interpret the data, the intensity of any m/z ratio is plotted as a function of sampling location, creating a 2-dimensional ion image, or MALDI tissue image. MALDI imaging data sets can also be represented in the mass spectral domain by a summary mass spectrum, which displays an average mass spectrum of all mass spectra collected across the entire tissue and contained in the MALDI imaging data set. Figure 1 shows summary mass spectra for α -crystallin products detected in normal young and old human lenses. In young human lenses (7-year-old; Fig. 1A), signals at m/z 19,956 and m/z 9,984 correspond to intact α A-crystallin (predicted $[M+H]^+ = 19,952$; $[M+2H]^{2+} = 9,978$), while signals at m/z 20,207 and m/z 10,109 correspond to intact α B-crystallin (predicted $[M+H]^+ = 20,202$; $[M+2H]^{2+} = 10,102$) and were most abundant. Lower abundance signals for major truncation products were also evident. In older human lenses (29-year-old, Fig. 1B), abundant ion signals for intact α A- and α B-crystallin are evident. In addition, the abundances of predominant α A-crystallin truncation products were increased compared with those observed in the young human lens sections. In particular, signals for α A-crystallin 1–101 at m/z 11,996 (predicted $[M+H]^+ = 11,995$), 1–65 at m/z 7,758 (predicted $[M+H]^+ = 7,757$), 1–58 at m/z 7,030 (predicted $[M+H]^+ = 7,028$), 1–54 at m/z 6,097 (predicted $[M+H]^+ = 6,096$), and 1–40 at m/z 4,913 (predicted $[M+H]^+ = 4,913$) were most prevalent. In 51-year-old human lenses (Fig. 1C), signals for intact α A- or α B-crystallin were further decreased and the aforementioned major α A-crystallin truncation products were abundant. Finally, in 75-year-old human lenses (Fig. 1D), very little intact α -crystallin was detected. Of interest, the signal for intact α B-crystallin appeared more intense than for α A-crystallin. Many α A-crystallin truncation products were detected, although α B-crystallin truncation products 1–40 at m/z 4,928 (predicted $[M+H]^+ = 4,926$) and 1–34 at m/z 4,266 (predicted $[M+H]^+ = 4,265$) became more prevalent in the 75-year-old lens section. Since laser power settings were similar during collection of each MALDI tissue imaging data set, the observation of multiple crystallin truncation products in older lenses is most likely due to biological processing rather than laser-induced dissociation of intact proteins, which is not typically observed in MALDI mass spectrometry.

The posttranslational truncation of α A-crystallin that progresses with both lens fiber cell age and lens age is revealed in MALDI tissue images (Fig. 2). Intact α A-crystallin and the three truncation products 1–101, 1–58, and 1–40 are shown at four lens ages (7, 29, 51, and 75 years). At each age, intact α A-crystallin was most abundant in the lens periphery. Intact α A-crystallin persisted in the nucleus of 7- and 29-year-old lenses and was present only in the very outer cortex in older lenses. These results are consistent with Western blot analysis results (Fig. 3), but what is striking is the sharp transition zone within 100 to 200 μ m where intact α A-crystallin is completely degraded. The major α A-crystallin truncation product 1–101, previously identified as a major truncation product in bovine and rabbit lenses,^{27,28} increased in abundance

in the nucleus of the 7-year-old lens. However, in all other older lenses, signal for α A-crystallin 1–101 was most intense in the lens mid-cortical region forming a ringed image. The observation that α A-crystallin 1–101 decreased in intensity in the lens nucleus is attributed to further truncation of the peptide into smaller truncation products. Indeed, α A-crystallin 1–58 signal increased in intensity in the nucleus of 7-, 29-, and 51-year-old lenses, but was most intense in a mid-cortex ring in 75-year-old lenses. In contrast, α A-crystallin 1–40 was consistently most intense in the nuclear region of each lens, although the area of highest signal intensity increased with lens age. α A-Crystallin truncation products smaller than the 1–40 peptide were also observed in the lens nucleus (data not shown) suggesting colocalization of multiple low-molecular-weight α A-crystallin forms. Note that fewer truncation products of α B-crystallin were observed.

To confirm the age-related processing of α A-crystallin revealed by MALDI tissue imaging, gel electrophoresis separation, and Western blot analysis of water-soluble cytoplasmic proteins from the cortex and nucleus of young (7-year-old) and old (68-year-old) human lenses was performed (Fig. 3). The strongly stained bands in the 20- to 30-kDa range of all gel lanes represent the composition of water-soluble lens crystallin proteins in the two regions of the young and old lenses (Fig. 3A). Likely truncation of the crystallin proteins is evident in the older nuclear regions of both ages of lens, in particular the old human lens, through the detection of a protein band at approximately 10 kDa. In Western blot analysis with an anti- α A-crystallin antibody full-length α A-crystallin was not detected in the nuclear region of old human lenses, suggesting that α A-crystallin is degraded and/or becomes insoluble in older lens nuclear regions (Fig. 3B). In contrast, full-length α A-crystallin was detected in both cortical and nuclear regions of young human lenses. This suggests that the spatial distributions of α -crystallin products obtained by MALDI tissue imaging are, at the very least, accurate for the soluble protein fraction.

Phosphorylation of α -crystallin is known to alter its chaperone function^{15–18} and phosphorylated forms (singly and doubly, respectively) of intact α B-crystallin were detected in the 7-year-old lens at m/z 10,144 and m/z 10,184, in the 29-year-old lens at m/z 20,279 and m/z 20,359, in the 51-year-old lens at m/z 20,269 and m/z 20,349, and in the 75-year-old lens at m/z 20,282 and m/z 20,362 (singly phosphorylated predicted $[M+H]^+ = 20,282$, $[M+2H]^{2+} = 10,142$, doubly phosphorylated predicted $[M+H]^+ = 20,362$, $[M+2H]^{2+} = 10,182$). Phosphorylated α A-crystallin has previously been detected in human lenses,^{33,34} but was not detected consistently in the MALDI tissue imaging experiments, probably due to the extensive processing of α A-crystallin with age. The spatial distributions of unmodified α B-crystallin and its phosphorylated forms are plotted in Figure 4. In the 7-year-old lens, unphosphorylated, intact α B-crystallin was detected throughout the lens. Of interest, in contrast to intact α A-crystallin, the signal intensity for α B-crystallin increased in the mid-cortex and nucleus. However, in older lenses, unphosphorylated intact α B-crystallin distribution was most intense at the lens periphery and decreased in the lens nucleus, which was similar to the spatial distribution of intact α A-crystallin. Previously, phosphorylated forms of intact α B-crystallin in bovine and rabbit lenses were most intense in the lens cortex, forming a ring of increased phosphorylation in the inner cortical region.²⁷ Similarly, in older human lenses (29-, 51-, and 75-year-old), both singly and doubly phosphorylated forms of α B-crystallin were most abundant in a mid-cortical ring. Although α B-crystallin persisted in the nucleus of the 29-year-old lens, albeit at a lower intensity to the mid-cortical ring, phosphorylated α B-crystallin was not detected in the nucleus of 51- and 75-year-old lenses. However, in the 7-year-old lens, phosphorylated forms of α B-crystallin were most intense in the nuclear lens regions. Although this distribution was clearly different from that in older lenses, phosphorylated α B-crystallin was always detected in deeper lying lens fiber cells.

To investigate whether distributions of full-length, truncated or phosphorylated forms of α -crystallin changed in an anteroposterior manner, MALDI tissue images of a 48-year-old human lens sectioned in the axial orientation were generated (Fig. 5). To determine lens polarity, a sister section was stained with hematoxylin and eosin, and cell nuclei present in the epithelial cell monolayer observed as a marker for the anterior pole (data not shown). The spatial distributions of intact and truncated α A-crystallin and unphosphorylated and phosphorylated α B-crystallin were consistent with those acquired in the equatorial orientation and showed no anteroposterior differences. The mid-cortical ring of α B-crystallin phosphorylation was readily apparent.

Alterations to the crystallin profile are known to be involved in lens cataract formation.^{11,25,35–37} As an initial step to investigate potential cataract-related modification to the lens crystallins, water-soluble proteins were extracted from the cortical region of 51-year-old normal and 54-year-old cataractous human lenses, and MALDI mass spectrometry performed without enzymatic digestion (Fig. 6). As expected, the main signals in the mass spectrum from the 51-year-old normal lens (Fig. 6A) correspond to full-length α A-crystallin (observed m/z 19,954) and commonly observed truncation products: α A 1–101 (observed m/z 11,996), α A 1–80 (observed m/z 9,605), α A 1–65 (observed m/z 7,756), α A 1–58 (observed m/z 7,027), α A 1–50 (observed m/z 6,095), and α A 1–40 (observed m/z 4,911). However, the mass spectrum of equivalent α A-crystallin truncation products in the 54-year-old cataractous lens included signals that were shifted approximately +34 mass units (Fig. 6B). A zoomed region between m/z 5800 and 8000 (Fig. 6, insets) showed single peaks for each truncation product in the normal lens and doublet peaks for truncation products α A 1–54 and longer in the cataractous lens. However, shifted signals for α A 1–50 and shorter peptides were not detected in the cataractous lens. This indicated that the putative +34 modification in the cataractous lens was located on residues 51–54 (S-L-F-R).

To investigate the spatial distribution patterns of wild-type and modified α A-crystallin truncation products in the 54-year-old cataractous human lens, MALDI tissue images of a lens section collected from the equatorial region were generated (Fig. 7). For each α A-crystallin truncation product detected, both wild-type and modified forms showed the same spatial distribution within the cataractous lens. These data are consistent with an amino acid substitution in α A-crystallin residues 51–54, assuming that this individual was heterozygous for α A-crystallin and that neither allele is dominant.

To localize and identify the modified α A-crystallin residue in the cataractous lens, the extracted soluble protein mixture was digested with endoproteinase Glu-C, and resultant peptides were subjected to LC-MS/MS analysis (Fig. 8). High-resolution accurate mass spectra were obtained throughout the chromatographic separation and a variety of peptides containing α A-crystallin residues 51–54 were detected. Doubly charged ions of the normal and modified α A-crystallin peptide 52–58 were detected, and their isotopic distributions are shown in Figures 8A and 8B, respectively. All detected isotopic peaks for normal α A-crystallin 52–58 (L-F-R-T-V-L-D) were within 1.6 ppm (parts per million) of predicted values. For modified α A-crystallin 52–58, all detected isotopic peaks were within 2.5 ppm of predicted values when phenylalanine was substituted for leucine at amino acid residue 52 (F-F-R-T-V-L-D), suggesting that this cataractous lens contained a mutated form of α A-crystallin. Sequence information obtained from tandem mass spectra of wild-type and modified α A-crystallin 52–58 confirmed modification at amino acid residue 52 since y-series ions were identical for the wild-type and modified peptide, while b-series ions were shifted +34 mass units in the modified peptide (Figs. 8C, 8D, respectively). Although a novel posttranslational modification to L⁵² that is isobaric with an L52F mutation cannot be ruled out, taken together, the accurate mass spectral and tandem mass spectral data strongly suggest an L52F mutation in α A-crystallin from this cataractous human lens.

Discussion

Age-related truncation of the lens crystallin proteins is a well-established phenomenon that has been investigated by using gel electrophoresis techniques.^{20,38} More recently, mass spectrometric approaches have been used to more fully characterize the posttranslational modifications that occur to lens crystallins with age and disease.^{25,26,39,40} However, in previous studies, relatively crude spatial information based on manual microdissection was used. Recently, MALDI tissue imaging was used to investigate the distribution of α -crystallin in bovine and rabbit lenses.^{27,28} The combination of molecularly specific information and spatial resolution make MALDI tissue imaging unparalleled in its ability to map the major posttranslational modifications of lens crystallin proteins that occur with age and disease. In this study, the spatial distributions of multiple truncation products of both human lens α -crystallin subunits have been mapped, and the region of elevated α B-crystallin phosphorylation observed previously in the cortex of bovine and rabbit lenses²⁷ confirmed in human tissue. Moreover, a mutated form of α A-crystallin containing an L52F substitution in a cataractous lens has been identified.

In the MALDI tissue images, full-length α -crystallin was detected in the nucleus of younger, but not older, human lenses. The presence of α A-crystallin was confirmed by Western blot analysis of the water-soluble protein fraction, indicating the efficacy of the MALDI tissue imaging technique. This suggests that the α -crystallin products detected by MALDI tissue imaging are water soluble, non-cross-linked α -crystallin products. Previous 2-DE and mass spectrometric analysis of aging and cataractous human lens crystallin content indicated that C-terminal truncation of α A- and α B-crystallin occurred in young human lenses, whereas increased N-terminal, and both N- and C-terminal truncation occurred in older lenses.²⁴ Using MALDI tissue imaging, the predominant truncation species detected in both young and old human lenses were identified as C-terminally truncated α A- and α B-crystallin, many of which have been reported previously.^{33,39} However, not all signals observed in the MALDI tissue imaging data were identified, and the detection of other N- and C-terminally truncated species in this data is conceivable. Extensive truncation of α B-crystallin was also observed in older human lenses, although fewer truncation products were observed compared with α A-crystallin, which could be due to greater stability of the α B-crystallin subunit in the aging lens or to the selectivity of the matrix/solvent system used. The use of an alternative matrix, solvent system, or matrix application technique may expand the number and subset of crystallin species detected in a single MALDI tissue imaging experiment.

Whereas truncation of both intact α -crystallin subunits was gradual in young (7- and 29-year-old) lenses, it occurred in a well-defined region over approximately 1000 μ m in 51-year-old and 650 μ m in 75-year-old lenses. In addition, with age, truncation of both intact α -crystallin subunits occurred in fiber cells in progressively earlier stages of fiber cell differentiation. Both nonenzymatic and enzymatic mechanisms are thought to play a role in the age-related truncation of α -crystallin. In the bovine lens, truncation of α A-crystallin at N¹⁰¹ occurs via imide ring formation,⁴¹ whereas α B-crystallin is cleaved at T¹⁷⁰ in vitro by a variety of endopeptidases.⁴² Irrespective of the mechanism of truncation, it is a striking change, particularly in older lenses, that is likely to have functional consequences.

Several α -crystallin truncation products have been identified as having residual or enhanced chaperone function. For example, enhanced chaperone activity was detected for α A-crystallin 1–172⁴³ and α B-crystallin 1–151.⁴⁴ Whereas α B-crystallin 1–151 was not observed in the present study, α A-crystallin 1–172 was detected in 7-, 29-, and 51-year-old human lenses. In 7-year-old lenses, α A-crystallin 1–172 increased in intensity in the lens nucleus, while in 29- and 51-year-old lenses, α A-crystallin 1–172 distribution was most intense in the lens cortex, and decreased in the lens nucleus (data not shown).

However, this form of α A-crystallin was not detected in 75-year-old lenses, although it has previously been detected in homogenized lens regions.^{23,32} Takemoto³² observed the most rapid formation of α A-crystallin 1–172 in the first ~12 years of life. It is possible that in MALDI tissue imaging, this protein form was resolved in mass spectra of young lenses and was not detected in older lenses because of the extensive modification of α A-crystallin that occurs with age.

Evidence of the effect of α A-crystallin C-terminal truncation on chaperone function is divided. The extent of α A-crystallin C-terminal truncation appears an important determinant in the chaperone activity of the truncated form.^{44,45} However, some studies indicate that the C-terminal region is essential for chaperone function,^{13,46} whereas others suggest that this region is ineffective in preventing aggregation of denatured proteins.⁴⁷ A 19-amino-acid peptide corresponding to residues 70–88 of α A-crystallin exhibits antiaggregatory properties.⁴⁷ However, the major α A-crystallin truncation products observed in older human lenses by MALDI tissue imaging contain neither the C-terminal region nor residues 70–88, suggesting that chaperone function of the detected truncation fragments may be perturbed. Furthermore, interaction of low-molecular-mass (<3.5 kDa) α - and β -crystallin-derived peptides with intact β - and γ -crystallins may be involved in age-related protein aggregation in the lens and cataractogenesis.³⁶ Therefore, knowledge of the spatial distribution of β - and γ -crystallin products in normal and diseased human lenses, combined with the results presented in this study, would further enhance our understanding of lens biology and cataractogenesis.

Phosphorylation of α -crystallin is an important functional modulator in the lens and plays a role in protein translocation^{48,49} and interaction with cytoskeletal proteins.^{50–52} Specifically, phosphorylation of α B-crystallin may play a role in stabilization of the cytoskeleton under stress conditions,^{51,53} and in other tissues confers resistance to apoptotic cell death primarily through inhibition of caspase-3 processing.^{54,55} Rings of elevated phosphorylated forms of both α -crystallin subunits were observed previously in the cortex of bovine and rabbit lenses.^{27,28} In human tissue, singly and doubly phosphorylated α B-crystallin were consistently detected in all ages of lens. Of note, phosphorylated forms of α B-crystallin were most abundant in the nucleus of the 7-year-old lens and the mid-cortex of the 29-, 51-, and 75-year-old lenses. Phosphorylation of α B-crystallin has been detected in the normal human lens on residues S¹⁹, S⁴⁵, and S⁵⁹.^{23,33,34} Phosphorylation of α B-crystallin alters its stability and chaperone function.^{15–18}

Phosphorylation-induced perturbation of α B-crystallin chaperone function, together with increased truncation of α A- and α B-crystallin which begins in a similar lens mid-cortical region and fiber cell age, may be precursive to cataract formation.

Many studies report enhanced truncation of α -crystallin in cataractous lenses.^{24,37} Furthermore, it has been suggested that age-related self-aggregation of degraded α -crystallin polypeptides into high-molecular-weight protein aggregates leads to insolubilization and lens diseases.^{20,44} In this study, a novel mutation to α A-crystallin was detected in a single cataractous lens and identified via high resolution and tandem mass spectrometry as L52F, a mutation that requires only a single nucleotide change. Residues 50 to 54 in α A-crystallin are known to bind the fluorescent probe bis-ANS, indicating they are part of an extended hydrophobic region of the protein that may be involved in chaperone function.⁵⁶ Leucine and phenylalanine are both neutral, nonpolar amino acids; however, their hydrophobicities differ. Although it is not clear whether this mutation is causative of cataract, alteration of amino acid hydrophobicity in this region may alter protein structure and/or binding efficiency, which could affect the chaperone activity of α A-crystallin. The origin, prevalence, functional consequence, and possible involvement in cataractogenesis of the L52F mutation requires further investigation.

In summary, a method for MALDI tissue imaging of the spatial distribution of α -crystallin products has been developed and applied to human lens tissue. Multiple truncation and phosphorylation products were detected that altered in intensity and distribution with lens cell age and tissue age. In addition, a novel mutation to α A-crystallin was observed in a single cataractous lens. These observations add to the growing body of knowledge of α -crystallin processing in aging and diseased human lenses.

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Figure 1.

Summary spectra of MALDI tissue imaging data sets in normal human lenses. Each spectrum represents one whole MALDI tissue imaging data set from equatorial sections of (A) 7-, (B) 29-, (C) 51-, and (D) 75-year-old human lenses. In young lenses (A), intact α -crystallin was the most abundant signal. With age (B–D), truncation of intact α -crystallin increased and high mass truncation products were further degraded to low mass truncation products.

Figure 2. MALDI tissue images of major α A-crystallin truncation products with age in equatorial lens sections. MALDI tissue images of intact α A-crystallin (1–173) and the abundant truncation products 1–101, 1–58, and 1–40 in 7-, 29-, 51-, and 75-year-old normal human lenses. At each lens age, intact α A-crystallin was most abundant in the periphery and persisted in the nucleus of 7- and 29-year-old lenses. In 51- and 75-year-old lenses, intact α A-crystallin was not detected in the lens nucleus. Scale bars, 2 mm.

Figure 3. Detection of α A-crystallin in dissected lens regions by SDS-PAGE and Western blot analysis. Water-soluble fractions of cortical and nuclear lens regions of 7- and 68-year normal human lenses were separated by SDS-PAGE (**A**). The crystallin protein content of each lens fraction was evident, with darkly-stained bands at ~20 kDa. Full-length α A-crystallin was detected in the cortical and nuclear fractions of the 7-year-old lens by Western blot analysis with an anti- α A-crystallin antibody (**B**). However, full-length α A-crystallin was not detected in the nuclear fraction of the 68-year-old lens.

Figure 4.

Phosphorylation of α B-crystallin with age in equatorial lens sections. MALDI tissue images of intact α B-crystallin and its singly (Ph) and doubly (2Ph) phosphorylated forms in 7-, 29-, 51-, and 75-year-old normal human lenses. In 7-year-old lenses, intact α B-crystallin was most abundant in the cortex and nucleus. With age, nuclear α B-crystallin was truncated and was detected only in the periphery of older lenses. Similarly, singly and doubly phosphorylated forms of α B-crystallin were most abundant in the nucleus of the 7-year-old lens. In older lenses, both phosphorylated forms of α B-crystallin were most abundant in the lens cortex. Scale bars, 2 mm.

Figure 5.

α -Crystallin distribution in a 48-year-old axial human lens section. Intact α A-crystallin was most abundant in the periphery of the lens. In contrast, α A-crystallin 1–101 was most abundant in the lens cortex, α A-crystallin 1–58 was most abundant in the lens middle cortex, and α A-crystallin 1–40 was most abundant in the lens nucleus. α B-crystallin was distributed in the periphery of the lens, whereas both singly (Ph) and doubly (2Ph) phosphorylated forms of α B-crystallin were most abundant in the lens cortex. No significant anterior pole (A) to posterior pole (P) variation was observed for these protein forms. Scale bar, 2 mm.

Figure 6.

Comparison of α A-crystallin forms in cortical regions of normal and cataractous lens. MALDI mass spectra of soluble lens proteins extracted from cortical regions of (A) 51-year-old normal and (B) 54-year-old cataractous human lenses. *Boxed* regions are zoomed mass spectra from m/z 5800 to 8000. Unmodified full-length and truncated α A-crystallin was detected in normal human lenses, whereas unmodified and modified (*) α A-crystallin was detected in cataractous lenses. The modification was located on residues 51–54, as only forms of α A-crystallin containing these residues were shifted +34 mass units.

Figure 7. Spatial distribution of wild-type and mutated α A-crystallin truncation products in cataractous human lens. No difference was observed in the distribution of any of the main wild-type (WT) and modified (Mod) α A-crystallin truncation products. Scale bar, 2 mm.

Figure 8.

Identification of α A-crystallin mutation in cataractous lens. Isotopic distribution detected in mass spectra for accurate mass measurement of the doubly charged ions of (A) wild-type and (B) modified α A-crystallin peptide 52–58, which were <2.5 ppm from predicted m/z values. These ions were fragmented in an ion trap to produce MS/MS fragmentation spectra of the (C) wild-type and (D) modified α A-crystallin peptide 52–58. The y-series ions shared the same masses (C, D), whereas the b-series ions were shifted approximately +34 mass units (D, arrows), indicating that the modification was localized to amino acid 52.

Table 1
Predicted and Observed m/z Ratios in Human Lens MALDI Tissue Imaging Data Sets

Crystallin Protein Form	Predicted <i>m/z</i>	Observed <i>m/z</i>					
		7 y	29 y	48 y	51 y	54 y	75 y
<i>αA</i> 1–173	19952	19956	19947	19946	19946	19981*	19955
<i>αA</i> 1–173 ²⁺	9978	9984	9978	9978	9974	9992*	9980
<i>αA</i> 1–101	11995	12001	11996	11996	11991	11999	11999
<i>αA</i> 1–80	9605	9611	9607	9605	9606	9608	9609
<i>αA</i> 1–65	7757	7760	7758	7756	7756	7758	7760
<i>αA</i> 1–58	7028	7033	7030	7030	7029	7030	7031
<i>αA</i> 1–54	6600	6604	6601	6601	6601	6602	6602
<i>αA</i> 1–50	6096	6100	6097	6098	6098	6099	6098
<i>αA</i> 1–41	5000	5003	5001	5002	5002	5001	5002
<i>αA</i> 1–40	4913	4914	4914	4913	4914	4913	4913
<i>αB</i> 1–175 2Ph	20362	NR	20359	20356	20349	20359	20362
<i>αB</i> 1–175 2Ph ²⁺	10182	10184	10183	10182	10178	10183	10184
<i>αB</i> 1–175 Ph	20282	NR	20279	20277	20269	20279	20282
<i>αB</i> 1–175 Ph ²⁺	10142	10144	10143	10142	10137	10143	10144
<i>αB</i> 1–175	20202	20207	20199	20199	20189	20199	20202
<i>αB</i> 1–175 ²⁺	10102	10108	10103	10102	10103	10103	10105
<i>αB</i> 1–44	5314	5317	5316	5316	5316	5316	5316
<i>αB</i> 1–42	5114	5117	5117	5115	5115	5117	5116
<i>αB</i> 1–40	4926	4929	4926	4926	4928	4928	4927
<i>αB</i> 1–34	4265	4266	4263	4265	4266	4266	4266

NR, not resolved.

* Mutated form only (+34) detected.