Localization of low molecular weight crystallin peptides in the aging human lens using a MALDI mass spectrometry imaging approach

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Abstract
Low molecular weight (LMW) peptides, derived from the breakdown of the major eye lens proteins, the crystallins, accumulate in the human lens with age. These LMW peptides are associated with age-related lens opacity and cataract, with some shown to inhibit the chaperone activity of α-crystallin. However, the mechanism(s) giving rise to the production of these peptides, as well as their distribution within the lens, are not well understood. In this study, we have mapped the distribution of these crystallin-derived peptides present in human lenses of different ages using matrix-assisted laser desorption/ionization-imaging mass spectrometry (MALDI-IMS). Our data showed that most of these LMW peptides emerge in the lens at early middle-age, with peptides greater than 1778 Da in mass being confined to the water insoluble fractions, and to a lesser extent the water soluble fractions of older lenses. MALDI-IMS analyses showed that four peptides, derived from αA-, αB- and γS crystallins, were confined to the lens nuclear fibre cells upon emergence during early middle-age, but were present in both the cortex and nucleus of old lenses. In contrast, another major peptide, derived from the C-terminal breakdown of βA3-crystallin, was present in the cortical and nuclear regions of both young and old lenses. A comparison between age-matched cataractous and non-cataractous lenses showed no distinct differences in LMW peptide profiles, indicating that although cataract may be a potential consequence caused by the emergence of these peptides, it does not contribute directly to the peptide-generating process.

Keywords
lens, aging, cataract, proteolysis, endogenous peptides, imaging mass spectrometry, anti-chaperone, CMMB

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Abstract
Low molecular weight (LMW) peptides, derived from the breakdown of the major eye lens proteins, the crystallins, accumulate in the human lens with age. These LMW peptides are associated with age-related lens opacity and cataract, with some shown to inhibit the chaperone activity of α-crystallin. However, the mechanism(s) giving rise to the production of these peptides, as well as their distribution within the lens, are not well understood. In this study, we have mapped the distribution of these crystallin-derived peptides present in human lenses of different ages using matrix-assisted laser desorption/ionization-imaging mass spectrometry (MALDI-IMS). Our data showed that most of these LMW peptides emerge in the lens at early middle-age, with peptides greater than 1778 Da in mass being confined to the water insoluble fractions, and to a lesser extent the water soluble fractions of older lenses. MALDI-IMS analyses showed that four peptides, derived from αA-, αB- and γS-crystallins, were confined to the lens nuclear fibre cells upon emergence during early middle-age, but were present in both the cortex and nucleus of old lenses. In contrast, another major peptide, derived from the C-terminal breakdown of βA3-crystallin, was present in the cortical and nuclear regions of both young and old lenses. A comparison between age-matched cataractous and non-cataractous lenses showed no distinct differences in LMW peptide profiles, indicating that although cataract may be a potential consequence caused by the emergence of these peptides, it does not contribute directly to the peptide-generating process.

Keywords: lens, aging, cataract, proteolysis, endogenous peptides, imaging mass spectrometry, anti-chaperone
1. Introduction

Human lens fibre cells contain high concentrations of structural proteins known as α-, β- and γ-crystallins. α-Crystallin, which is composed of the subunits of αA- and αB-crystallin, also acts as a molecular chaperone to suppress protein unfolding in the lens (Horwitz, 1992; Rao et al., 1995). During aging, the lens undergoes negligible protein turnover and, consequently, the crystallins become increasingly modified. These age-related modifications include truncation, oxidation, glycation, deamidation and phosphorylation, amongst others (Hanson et al., 2000; Aquilina and Truscott, 2002; Harrington et al., 2004, 2007; Srivastava et al., 2004; Truscott, 2005; Wilmarth et al., 2006). Whilst certain structural changes appear to be associated with the normal maturation process of the lens, improper or unregulated modifications have been associated with lens opacity (Truscott et al., 1990; Truscott, 2005).

Crystallin breakdown increases significantly in aged lenses and with the onset of cataract (Harrington et al., 2004, 2007; Srivastava et al., 2004). For example, crystallin fragments (peptides) have been found in both water soluble (WS) and water insoluble (WI) fractions of the lens, with the prevalence of these fragments in the WI fraction increasing with age (Srivastava et al., 1996; Lampi et al., 1998; Harrington et al., 2007). Recently, low molecular weight (LMW) peptides derived from the breakdown of crystallins have been identified in young, old and cataractous lenses (Santhoshkumar et al., 2008). Interestingly, a number of these endogenous LMW peptides appeared to be confined to a certain age group (young or old lenses), whilst some were present exclusively in cataractous lenses. As there is very little protease activity in older lenses (David and Shearer, 1989), the mechanism(s) underlying this crystallin breakdown remain unclear.

The emergence of LMW peptides is potentially a key event in lens aging and may be responsible for the increase in protein aggregation and WI proteins in aged and cataractous lenses. For example, the LMW peptides present in aged lenses, αB1-18 and βA3/A159-74, have been shown to diminish the chaperone activity of α-crystallin (Santhoshkumar et al., 2008). In the lens, this can lead to increased protein aggregation and light scattering - key events in the development of age-related nuclear cataract (Rao et al., 1995; Truscott, 2005; Harrington et al., 2007). Furthermore, the cleavage mechanism(s) responsible for these LMW peptides are likely to compromise crystallin structure, further contributing to protein aggregation and a reduction in the chaperone efficacy of α-crystallin (Sharma et al., 2000; Bhattacharyya et al., 2006).
Despite recent reports on the presence of LMW peptides in the human lens (Santhoshkumar et al., 2008; Sharma and Santhoshkumar, 2009), the emergence and distribution of these peptides during the aging process have not been investigated. Matrix-assisted laser desorption/ionization-imaging mass spectrometry (MALDI-IMS) has proven to be a reliable technique for studying the distribution of proteins/peptides in tissue sections (Caprioli et al., 1997). Recent studies using MALDI-IMS on lens tissue have demonstrated that mapping and quantification of native and modified crystallins in the lens is possible (Han and Schey, 2006; Grey and Schey, 2009; Sharma and Santhoshkumar, 2009). In the present study, we have examined the localization of LMW crystallin peptides in the WS and WI fractions of both the cortical and nuclear regions of human lenses of different ages. Furthermore, we have used MALDI-IMS to map the emergence and distribution with age of the major LMW crystallin peptides in the human lens.
2. Material and methods

2.1 Lens protein extraction and isolation of LMW crystallin peptides

Human lenses were obtained from donors at the Lions New South Wales Eye Bank (Sydney, Australia) and stored immediately at -80 °C. Lenses were dissected into cortical and nuclear regions using a 5 mm trephine. Tissue from respective regions was separately homogenized in buffer A (20 mM Tris-HCl, 20 mM NaCl, 1 mM EDTA, pH 7.4) with a hand-held tissue homogenizer, using 1 ml of buffer per 100 mg tissue (wet weight), and incubated at room temperature for 15 min. The homogenates were centrifuged at 10,000 g for 30 min at 4 °C, and the pellets were re-homogenized with Buffer A and centrifuged at 10,000 g. The supernatants from both extraction steps were pooled together as WS protein extracts. Remaining pellets were re-homogenized in buffer B (8 M urea in buffer A), incubated at room temperature for 15 min, then centrifuged at 10,000 g for 30 min at 4 °C. The supernatants were collected to give the water insoluble-urea soluble (WI-US) protein extracts. The urea concentration in all samples was adjusted to 4 M using buffer B for WS extracts and buffer A for WI-US extracts. β-Mercaptoethanol (0.5% v/v final concentration) was added and the samples were incubated at room temperature for 30 min, before being filtered through a 10 kDa molecular weight cut-off YM-10 Microcon centrifugal filter unit (Millipore, Billerica, Massachusetts, USA) by centrifugation at 8,000 g, 4 °C. Protease inhibitor cocktail (P2714, Sigma-Aldrich, St. Louis, Missouri, USA) was prepared as per the manufacturers specifications and used in all buffers.

2.2 MALDI-MS and nanoLC-ESI-MS/MS peptide identification

Peptides present in the WS and WI-US protein extracts were concentrated using C18 Ziptips (Millipore), and spotted directly onto a MALDI plate by eluting the peptides with α-cyano-4-hydroxycinnamic acid matrix. MALDI-MS analysis was performed using a Shimadzu Axima TOF2 MALDI mass spectrometer (Save Sight Institute, Sydney, Australia). The LMW peptides present in the lens extracts were identified using nanoflow liquid chromatography electrospray-ionization (nanoLC-ESI) MS/MS analysis at the Australian Proteome Analysis Facility (Macquarie University, Sydney, Australia). Urea was removed from the extracts using C4 ZipTips (Omix, Redwood City, California, USA) prior to freeze-drying and loading onto an Agilent 1100 nanoLC system (Agilent Technologies, Forest Hill, Australia) fitted with a peptide Captraps (Michrom Bioresources, Auburn, California, USA) and a 150 μm x 100 mm, 300 Å C18 Protecol column (SGE Analytical Science, Ringwood, Australia), coupled to a Qstar XL ESI mass spectrometer (AB Sciex, Mt Waverly, Australia). Peptides were separated using gradient elution from 5% solvent B (90% acetonitrile, 0.1% formic acid) to 40% solvent B in 40 minutes, at a flow-rate of 500 nl/min (solvent A,
0.1% formic acid). Peptides eluting from the column were analysed directly in nanoLC-ESI-MS/MS IDA scan mode. MS/MS results were searched against protein sequences published on Swissprot through the Mascot search engine (peptide scores > 37 considered significant).

2.3 Tissue preparation and MALDI tissue imaging
Frozen lenses were sectioned equatorially into 20 μm thick tissue sections with disposable blades at -20 °C, in a Shandon Cryotome E Cryostat (Thermo Electron Corporation, Waltham, Massachusetts, USA). Lens sections were immediately thaw-mounted onto a Shimadzu MALDI-plate (TO-487, Shimadzu, Kyoto, Japan). Lens sections were air-dried at room temperature, before being washed consecutively with 30%, 50% and 70% ethanol for a period of 60 seconds each, allowing air-drying in between washes. This method of ethanol-washing removes physiological salts and lipids that would otherwise mask the ion signals of the peptides of interest. Plates were then sprayed with a solution of 20 mg/ml sinapic acid (Fluka, St. Louis, Missouri, USA) in 50% acetonitrile. Hand spraying was performed in an open-faced box using a Preval Spray compressed gas device (Precision Valve Corp., New York, USA). Sections were coated 8 times in a dual pass process from a distance of 40 cm, and aiming the spray plume 15 cm above the sample plates. A total volume of 30 ml of matrix solution was used in the coating process. Air-drying between coats was allowed to occur for a period of 5 min. MALDI-IMS was performed in linear mode on a Shimadzu Confidence MALDI-TOF mass spectrometer (Australian Institute for Innovative Materials, University of Wollongong). Effective laser power of 80 V was used for all imaging with a TV raster function (50 shots per raster position) set at 200 μm intervals across a 100 mm² area, sufficient to cover entire sections. Total number of data points per tissue section was 2601. Data was exported into BioMAP 3.7.5.5 functional image analysis software (Novartis Institutes for Biomedical Research, Basel, Switzerland) for processing and image optimization.
3. Results

3.1 Distribution of LMW crystallin peptides in WS and WI-US extracts of human lenses of different ages

We have examined a broad age range of human lenses in an attempt to determine at what stage of life, and in which lens regions, the crystallin-derived LMW peptides are distributed. Figure 1 illustrates the MALDI-MS spectra obtained for each of the extracts from a 65 year old lens. Peptides which gave rise to most of the major peaks observed in figure 1 were separated, sequenced and identified using a nanoLC-ESI-MS/MS approach in conjunction with Mascot database searching (Supplementary Fig. S1-S4; Table S1-S4). Species of primary interest in this study were those that have previously been shown to possess anti-chaperone properties (Santhoshkumar et al., 2008), were found to increase in ion intensity with age, or were major peptides in the WI-US fractions. The WS fractions of a 65 year old lens (Fig. 1A and B) were found to have peptides derived predominantly from αA- and αB-crystallin, along with an N-terminal peptide from γS-crystallin (residues 2-22), a βB1 peptide (residues 238-247) and two βB2 peptides (residues 169-180 and 91-102). Peptides extracted using urea included two N-terminal peptides of αB-crystallin (residues 1(2)-18) and a trio of βA3 peptides (Fig. 1C).

Table 1 presents a summary of the distribution of LMW crystallin peptides detected in the WS and WI-US extracts from the cortical and nuclear regions of lenses ranging in age from 20 to 86 years. Peptides up to 1730 Da in mass were, on the whole, found in both the WS and WI-US extracts of a broad lens age range, with some being confined exclusively to the WS extracts. Interestingly, the γS_{167-178} and αA_{37-49} peptides were found across a broad age range in the nucleus water soluble (NWS) and also in the cortex and nucleus WI-US extracts of some age groups. The absence of these peptides in the cortex water soluble (CWS) fraction suggests that the molecular environments of the cortex and nucleus are sufficiently distinct in the human lens such that the nucleus renders this peptide more soluble than the cortex. Peptides greater than 1778 Da in mass were confined predominantly to the WI-US lens extracts and, to a lesser extent, the WS of old lenses. The N-terminal peptides of αB-crystallin (residues 1(2)-18) and C-terminal peptides of βA3-crystallin were isolated exclusively from the WI-US fractions. These peptides were found to be present in the WI-US fractions of lenses above 35 years, but not in the 20 year lens. Interestingly, even though deamidation has been mapped to residues on peptides αA_{2-12(13)}, γS_{2-22}, and the βA3 C-terminus peptides (Wilmarth et al., 2006), no such modification was observed.
3.2 MALDI-IMS mapping of LMW crystallin peptides across tissue sections of human lenses of different ages

Having established that crystallin-derived peptides vary greatly in solubility and gross distribution with age, we attempted to use MALDI-IMS to more accurately map the distributions of anti-chaperone peptides, peptides that were found to increase in ion intensity and/or area of distribution with age, and those present in the WI-US fractions. Lens sections were thoroughly washed in order to remove lipids and salts, as well as the WS proteins and peptides, in an effort to identify peptides in the WI fractions. Figure 2 represents the summed mass spectra, acquired at 200 μm intervals across lens tissue sections, for a 37 year (Fig. 2A) and an 80 year (Fig. 2B) old lens. In the younger lens, the major species were the C-terminal βA3 peptides at m/z3197, m/z3253 and m/z3390, suggesting that these peptides are the first to accumulate in the WI-US component with age. This is in agreement with the detection of these peptides in the cortex water insoluble-urea soluble (CWI-US) and nucleus water insoluble-urea soluble (NWI-US) fractions of lenses older than 20 years (Table 1). In contrast to this, MALDI-IMS analysis of an 80 year lens section revealed further significant peaks at m/z2187, m/z2359 and m/z2598, corresponding to N-terminal peptides of αB- and γS-crystallins. A fourth, small peak, which matched our lens extract data corresponding to the αA66-80-H2O peptide was noted at m/z1848. Along with the major βA3 peptide at m/z3253, these ions, indicated by an asterisk (Fig. 2B), were mapped in MALDI-IMS experiments performed across a broad lens age range (Fig. 3).

Images from a 37 year lens revealed that the βA3189-215 peptide was the only significant ion detected at this age (Fig. 3). At 42 years, this ion was equally abundant, and additionally, signal arising from the αA, αB and γS peptides were shown to be emergent in the nuclear region of the lens. By 58 years, a high abundance of the αB2-18 peptide was evident in the nuclear region of the lens, with further less dramatic increases of the other peptides. By age 80, all peptides were clearly detected in both cortex and nucleus. The N-terminal αB peptides displayed a dramatic expansion into the cortical region in this transition to old age, a pattern that was also evident for the αA66-80 and γS2-22 peptides. The βA3189-215 peptide appeared to be distributed throughout the lens cross-section at ages 37 years and above. The oldest lens analysed was 86 years, which also was the only lens to display nuclear opacification consistent with age-related cataract. This lens had a significant increase in the prevalence of the αA66-80-H2O peptide compared with the other peptides, which showed only incremental expansion towards the periphery of this larger lens. Another feature of this lens was the apparent bias in the distribution of all peptides towards one hemisphere of the equatorial section, however, as the superior-inferior orientation of
lenses was not noted prior to sectioning, it is difficult to make any assumptions regarding this bias.

3.3 Determining the effect of cataract on the genesis and abundance of LMW crystallin peptides

In order to assess the impact of cataractogenesis upon the apparent abundance and distribution of LMW peptides, spectra were acquired across the sections from a 69 year healthy lens (Fig. 4A) and a 62 year lens which displayed opacity consistent with age-related nuclear cataract (Fig. 4B). The healthy lens contained each of the peptides previously described and are labelled accordingly. Interestingly, there were no major distinctions in the species of peptides present in the lenses. Taken together with the imaging data (Fig. 3), we conclude that cataract per se is not symptomatic of dramatic changes in the abundance of these LMW peptides.
4. Discussion

In this study we have examined the LMW peptides in the WS and WI-US extracts of human lens cortices and nuclei. Our results complement those of a recent study by Grey and Schey (2009), who reported the distributions of α-crystallin subunits and N-terminal peptides with m/z values greater than 4000 in human lenses over a broad age range. In the present study, peptides greater than m/z4000 were not detected, indicating that our use of lower percentage ethanol solutions to wash the tissue sections may have removed these larger peptides. The trend observed by Grey and Schey (2009) that crystallin truncation increases with age, in particular during the middle-age, and that the products are degraded progressively to low mass peptides is in good agreement with our findings.

Our results showed that peptides greater than 1778 Da were isolated predominantly from the WI-US extracts of lenses 35 years and older. This implies that either the solubility of these peptides in aqueous buffer is low, or that they are bound to WI protein complexes, cytoskeleton or lens fibre cell membranes. These peptides were found to originate from the chaperone binding region of αA-crystallin (Sharma et al., 2000), the N-terminus of αB- and γS-crystallins and the C-terminus of βA3-crystallin. The N-terminal peptides of αB-crystallin have been shown to significantly reduce the molecular chaperone function of α-crystallin oligomers (Santhoshkumar et al., 2008). In situ mapping of the αB peptides revealed that αB2-18 was densely localized to the nucleus of a 58 year lens, with αB1-18 showing similar distribution but lower ion density. This dramatic increase in the αB1(2)-18 peptides in the nucleus during middle-age and their subsequent emergence in the cortex of old lenses may well facilitate aggregation of crystallins in vivo, particularly if their in vitro demonstrated anti-chaperone properties apply to the crowded environment of the lens.

It has been suggested that the interaction of lenticular α-crystallin with anti-chaperone peptides could be a key event in protein aggregation and the development of cataract during aging (Santhoshkumar et al., 2008). The extended periods that these LMW peptides remain in the lens indicate that the lens may be unable to efficiently degrade them, which can lead to unregulated accumulation of these peptides with age. These peptides emerge in the lens (~35-42 years in our study) during a period which also sees a drastic increase in WI proteins and crystallin truncation (Harrington et al., 2004; Srivastava et al., 2004), an increase in membrane associated lens proteins (Friedrich and Truscott, 2009) and the onset of a barrier to diffusion at the nucleo-cortical interface in the lens (Sweeney and Truscott, 1998). Taken together, the early middle-age is potentially an important period in lens aging, during which crystallins
in the lens nucleus become extensively structurally modified and truncated. The structural damage arising from the cleavage of these proteins may also result in the loss of the proteins’ normal function, including the molecular chaperone function of α-crystallin, particularly if the cleavages occur at the chaperone site sequence (e.g. the αA_{66(67)-75}, αA_{66-80} and αA_{69-80} peptides).

In addition, we have investigated the possibility that the production of these LMW peptides in the lens may be a direct causative factor in cataractogenesis by comparing age-matched healthy and cataractous lenses. Our data revealed no significant differences in the abundance of LMW crystallin peptides, indicating that the changes to the lens environment caused by cataract formation do not contribute directly to the peptide-generating process. This result corresponds to a similar observation on the effect of cataract formation had on crystallin truncation (Grey and Schey, 2009).

Although we have clearly demonstrated that these LMW peptides are present in human lenses, and that their distributions are dependent on age and solubility, the mechanisms leading to peptide cleavage from the intact crystallins is not understood. Interestingly, when examining the peptide sequences, we noticed that the majority of these LMW peptides began with an Asp (αA_{67-75(80)}), αA_{69-80}, αA_{2-12(13)}, αB_{2-18}, Ser (γS_{2-22}), or even a Ser-Asp (αA_{66-75(80)}), Ser-Pro (αB_{45-54(57)}), γS_{167-178} combination. Moreover, the sequences of a number of peptides were followed immediately by an Asp (αA_{66(67)-75}) or Asp-Ser (βB_{291-102}), Ser-Pro (αA_{66(67)-69-80}), αB_{45-57}) combination. Also, the last residue of the γS_{2-22} peptide is an Asp, the last residue of the αA_{150-160}, αB_{28-39} and βB_{2169-180} peptides is a Pro, and the αA_{43-56} peptide is preceded by a Ser. Spontaneous peptide bond cleavage via imide ring formation at labile Asp residues has previously been reported in bovine α-crystallins (Geiger and Clarke, 1987; Voorter et al., 1988). Asp residues are known to be intrinsically unstable, thus they are common sites of post-translational modifications (Voorter et al., 1988; Robinson and Robinson, 2004). This may explain the large number of LMW crystallin peptides cleaved at Asp residues.

The frequency of Ser, Pro and their dipeptide combinations either at the beginning of the observed peptide sequences, or immediately following in the corresponding crystallin sequence, is also suggestive of a chemical or enzymatic cleavage mechanism at these sites. While it is known that proline-directed kinases favour phosphorylation of Ser-Pro (Aitken et al., 1995) and similar cleavage patterns have been reported from both bovine and human αA-crystallin (Van Kleef et al., 1975; Takemoto, 1995), it is not known if this motif is recognized by proteases. A sequence
pattern also emerges when looking at the penultimate two residues of the αA<sub>66(67)(69)-80</sub> and αB<sub>1(2)-18</sub> peptides and the two residues preceding the βA<sub>3189-215</sub> peptide. The dipeptides are His-Phe, Phe-His, and His-His, respectively, each motif then contains consecutive aromatic residues. As calpains, the primary proteases in the human lens, are known to cleave after consecutive aromatic residues (Cuerrier et al., 2005), this may suggest a role for lens-based calpains in these particular instances. Furthermore, tertiary structural elements rather than primary amino acid sequences are likely responsible for directing the cleavage of protein substrates by calpains (Cuerrier et al., 2005), which may explain the high degree of specificity that we have observed for the αA, αB and βA3 peptides. However, the activity of calpain in the primate lens has been reported to be largely inhibited by high levels of the endogenous calpain inhibitor - calpastatin (David et al., 1989; Nakajima et al., 2006), indicating that other mechanisms may be responsible for these cleavages.

At present, there appears to be no clear evidence linking calpains and other lenticular proteases (Swanson et al., 1981, 1985; David and Shearer, 1989; Wride et al., 2006) with these crystallin cleavages. The absence of significant proteolytic activity in adult lenses suggests that these LMW peptides may be generated through nonenzymatic cleavage of crystallins. Whilst the mechanisms are not fully understood, lens proteins (such as aquaporins) have been shown to undergo chemical cleavage with age (Ball et al., 2004; Korlimbinis et al., 2009). Age-dependent changes in the lens, such as the formation of a barrier at the nucleo-cortical interface (Sweeney and Truscott, 1998), may encourage peptide cleavages, as they induce changes to the chemical environment of the lens nucleus. In addition, lens nuclear fibre cells are older, have a slightly acidic environment (Bassnett and Duncan, 1985) and are more susceptible to oxidative-damage compared to cortex fibre cells (Sweeney and Truscott, 1998; Truscott, 2005) - characteristics that may all favour chemical cleavages.

In conclusion, the data presented in this study is the first to examine the distribution and localization of LMW crystallin peptides in the aging human lens using MALDI-IMS. The timing and the nucleus-to-cortex emergence pattern of these LMW peptides suggests that they may have a significant impact on the later development of the human lens.
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**Figure Legends**

**Figure 1:** MALDI-TOF MS spectra of the LMW crystallin peptides present in the CWS (A), NWS (B), CWI-US (C) and NWI-US (D) extracts of a 65 year old human lens. Peak ions and the peptide sequence origins of the major peaks are labelled.

**Figure 2:** Summed mass spectra, acquired at 200 μm intervals across lens tissue sections, for a 37 year (A) and an 80 year (B) old lens. In the younger lens, the major species were the C-terminal βA3 peptides at $m/z$3197, $m/z$3253 and $m/z$3390. The 80 year lens section revealed further significant peaks at $m/z$2187, $m/z$2359 and $m/z$2598, corresponding to N-terminal peptides of αB- and γS-crystallins. Along with the major βA3 peptide at $m/z$3253 and a smaller peak at $m/z$1848 corresponding to the $αA_{66-80}$-H$_2$O peptide, these ions have been indicated by an asterisk.

**Figure 3:** MALDI-IMS spectra mapping the peptides indicated by an asterisk in figure 2B. Lenses were sectioned equatorially and prepared for analysis by manual spray deposition of sinapic acid. Ages (20-86 years) are noted in the x-axis whereas peptide identities are labelled in the y-axis. Relative peptide intensities are illustrated using a black-red-white scale, white being of highest relative intensity. Dashed oval outlines indicate the edge of the tissue sections. Scale bars, 2 mm.

**Figure 4:** Summed MALDI-MS spectra from imaging experiments performed on sections from a 69 year healthy lens (A) and a 62 year moderately cataractous lens (B). No major distinctions in the species of peptides present were observed, suggesting that cataract formation as such is not causative, nor symptomatic, of the abundance of these LMW peptides.
Table 1: Distribution of LMW crystallin peptides present in the CWS, NWS, CWI-US and NWI-US extracts of human lenses aged between 20 and 86 years. Peptides in the lens extracts were identified using MALDI-MS and nanoLC-ESI-MS/MS analysis. Shaded cells indicate a positive detection (peptide ion intensity greater than 10% of base peak) of the specific peptide in the fraction.

<table>
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<th>Peptide Mass (M+H)</th>
<th>Peptide Source</th>
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<th>CWI-US Extract</th>
<th>NWI-US Extract</th>
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<td></td>
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<td>Lens Age (years)</td>
<td>20 35 43 65 86</td>
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a. Peptide has a loss of H₂O at the D69 residue.

b. Peptide has an acetylated-Met at the N-terminus.

c. Peptide has an acetylated-Ser at the N-terminus.
Figure 1
Figure 2
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Figure 3
Figure 4