Amino Acids (2006) 30: 369–389
DOI 10.1007/s00726-005-0283-9

Amino Acids
Printed in Austria

Eye lens proteomics

Review Article

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Received August 17, 2005
Accepted September 1, 2005
Published online April 4, 2006;

Summary. The eye lens is a fascinating organ as it is in essence living transparent matter. Lenticular transparency is achieved through the peculiarities of lens morphology, a semi-apoptotic process where cells elongate and loose their organelles and the precise molecular arrangement of the bulk of soluble lenticular proteins, the crystallins. The 16 crystallins ubiquitous in mammals and their modifications have been extensively characterized by 2-DE, liquid chromatography, mass spectrometry and other protein analysis techniques. The various solubility dependant fractions as well as subproteomes of lenticular morphological sections have also been explored in detail. Extensive post translational modification of the crystallins is encountered throughout the lens as a result of ageing and disease resulting in a vast number of protein species. Proteomics methodology is therefore ideal to further comprehensive understanding of this organ and the factors involved in cataractogenesis.

Keywords: Eye lens – Proteomics – Crystallins – 2-DE – Liquid chromatography – Mass spectrometry

Introduction

Proteomics research on the eye lens has a long history. The lens is an avascular cellular conglomerate whose function is to guarantee proper light refraction and visual acuity (Bloemendal, 1977). Following the development of the lens nucleus from the posterior epithelial cells of the lens vesicle, lenticular cells cease mitosis with the exception of the cuboidal cells of the anterior epithelium. These cells are eventually passed from the central region of the anterior epithelium through the germinative region to the lens equator, where they undergo differentiation into fiber cells. The cuboidal cells elongate and synthesize large amounts of proteins of the crystallin protein super family (Mörner, 1894) and at the terminal stage of differentiation loose their organelles in a process reminiscent of apoptosis (Papaconstantinou, 1967; Bassnett, 2002). These so called secondary fiber cells are layered onto the lens nucleus and successively onto each other forming the lens cortex. Lenticular development is essentially complete a few days after birth but continues at a slowed rate throughout life.

The lack of organelles, high protein concentrations and the short range order of the soluble proteins are necessary for lens transparency (Delaye and Tardieu, 1983). Naturally, aspects of lenticular biology vary greatly between species, reflecting optical specialization and environmental demands. The human and fish lens represent two extremes, the former, like diurnal animals having a softer more uniform mould, while the latter is clearly divided into a hard nucleus and a much softer cortical region (Koretz and Handelmann, 1988; Fernald and Wright, 1983). The rodent lens is very hard with evidence from Raman spectroscopy suggesting the proteins in its core are denatured (Yu et al., 1985). Concurrently, refractive indices also vary greatly between species (Kröger et al., 1994).

Thirty to thirty-five percent of bovine lens wet weight is protein the other 65% is virtually water. The crystallin protein super family comprises 90% of lenticular protein mass (Bloemendal, 1977). The 16 crystallins ubiquitous in mammals undergo extensive post translational modification so that a vast number of protein species are in constant flux in various degrees of dynamic interaction both physically and physiologically (Bhat, 2004) maintaining the soluble basis for lenticular transparency. Serious perturbations of this balance leads to improper protein association,

aggregation and insolubilization. Incident light is diffracted by the protein aggregates resulting in lens opacification and a more or less grievous loss of visual acuity clinically termed cataract (Lambert, 1994).

Proteomics evidence characterizing various aspects of lens proteins has accumulated over the years. The term proteome was initially introduced in 1995 (Wasinger et al., 1995) and defines a samples full protein complement. One must bear in mind that the proteome is highly dynamic as transcription, translation and protein modification activity to name just three primary cellular processes are constantly changing. While methods such as *in situ* labeling that realize the protein constituents at a precise point in an organism's life are available, the core proteomics techniques, 2-DE and LC for protein and/or peptide separation and MS for identification, applied by themselves realize a samples history up to the point of analysis. Both applications are valid and should be considered for the problem of interest. Also, many prefractionation techniques can be implemented reducing proteome complexity or for the enrichment of specific subproteomes (Jungblut and Klose, 1985). Most importantly, advances in proteomics have shown the high degree of modification undergone by many primary translation products (Scheler et al., 1997) as well as individual heterogeneity (Klose et al., 2002) thus demanding an increased consciousness for the protein species concept (Jungblut et al., 1996; Jungblut and Thiede, 1997), i.e. the understanding of the proteome on a molecular level.

The eye lens, consisting almost exclusively of protein and water is ideal for proteomics research, which in turn has proven invaluable for characterizing the healthy lenticular protein constituents both of the whole lens and various morphological sections in various species and in the elucidation of some of the complex factors involved in cataractogenesis. In the course of this review with a few important exceptions we will limit ourselves to discussing work based on 2-DE, LC and MS arbitrarily drawing the line between proteomics and protein analysis and techniques such as SDS-PAGE, Edman degradation and Western blot.

The lenticular proteome

The crystallins are the primary soluble lenticular proteins in all vertebrate species comprising about 90% of total lenticular protein mass. They are divided into three major classes, alpha (α), beta (β) and gamma (γ) crystallins. Evolutionary constraints have imparted a high degree of stability on this protein super family, as lenticular cells devoid of cellular machinery are unable to turnover proteins.

Native alpha crystallin is an oligomer with a Mw in excess of 300 kDa consisting of up to three types of monomeric subunits in dynamic exchange (van der Ouderaa et al., 1973; Puri et al., 1983; van den Oetelaar et al., 1990; Gesierich and Pfeil, 1996; Bova et al., 1997). The two major alpha crystallin monomers are the products of two genes, alpha A and alpha B and share this terminology. They are 60% identical in primary structure (Schoenmakers et al., 1969; Bloemendal and de Jong, 1991). The third alpha crystallin monomer is an alternative splicing product of the alpha A crystallin gene and is termed alpha A insert crystallin (Hendriks et al., 1990). The alpha A chain has a Mw around 20 kDa, alpha B around 22 kDa and alpha A insert around 24 kDa. The monomers have a global, hydrophobic N-terminal domain characterized by α-helical structure function regions (Smith et al., 1996; Pasta et al., 2003), a β-sheet structured C-terminal region containing the so called ''alpha crystallin domain'' (Caspers et al., 1995) and a C-terminal extension. The alpha crystallin domain is implicit to all small heat shock proteins and it was shown that alpha B crystallin is indeed a member of this family (Klemenz et al., 1991) and that the alpha A crystallin chain can function as a molecular chaperone (Horwitz, 1992).

Both native and recombinant alpha crystallin has not been successfully crystallized due to its polydisperse nature, however models of the monomers tertiary structures (Farnsworth et al., 1997) and the functional oligomers quaternary structure have been proposed (Tardieu et al., 1986; Groenen et al., 1994; Haley et al., 1998; Horwitz et al., 1998; Abgar et al., 2000). In a typical mammalian lens alpha crystallin constitutes about 35% of proteins and is synthesized throughout lens development and is thus evenly distributed throughout the organ (Aarts et al., 1989) with trace amounts being detected in other tissues (Iwaki et al., 1990; Srinivasan et al., 1992). Apparently alpha crystallin is not only a major structural component of transparent lenticular architecture but its properties as a molecular chaperone are an important factor in maintaining lenticular homeostasis.

The beta and gamma crystallins are organized in two structural domains with a short connecting peptide. The domains are in turn made up of two consecutive greek key motifs each consisting of four β-strands which intercalate and arrange the domain in anti-parallel β-sheet conformation (Driessen et al., 1981; Wistow et al., 1981; Wistow and Piatigorsky, 1988; Slingsby et al., 1996; Carver, 1999). Native beta crystallins assemble in homo- or heterodimers

which can dissociate into monomers or associate in higher Mw oligomers probably up to octamers while gamma crystallins generally do not associate (Berbers et al., 1984; Bateman et al., 2001; Hejtmancik et al., 1997, 2004; Slingsby and Bateman, 1990; Zarina et al., 1994). These similarities in protein secondary and tertiary structure suggest a common ancestral gene encoding a single motif and a series of ancient gene duplication and recruitment events resulting in the genetic diversification of beta and gamma crystallins in today's vertebrate species (Wistow and Piatigorsky, 1988; Lubsen et al., 1988; Norledge et al., 1996; Clout et al., 2000).

There are six mammalian beta crystallin genes and similar genes detected in amphibian and fish expressing three basic (B1–B3) and four acidic (A1–A4) beta crystallin proteins. Beta A1 and A3 crystallin are encoded by the same gene from distinct transcription start codons, the beta A3 start codon being upstream to A1. The beta crystallin monomers have molecular weights ranging from 22 kDa (beta A4) to 28 kDa (beta B1) (Berbers et al., 1984). They are characterized by N- and C-terminal extensions reaching beyond the molecular domains and a short connecting peptide between the domains. It is an accepted hypothesis that these extensions and the connecting peptide are instrumental in dimer- and oligomerization of beta crystallins (Carver et al., 1993; Cooper et al., 1993a, 1993b) initiated by 3D domain swapping (Bennett et al., 1994, 1995) with the extensions promoting higher order association beyond the dimer (Lampi et al., 2001; Bateman et al., 2001, 2003). The beta B1 and B3 crystallin genes are expressed early in lenticular development, consequently their proteins are found primarily in the lens nucleus. Beta B2 is often seen as the main beta crystallin and the acidic beta crystallins are found throughout the lens in the nucleus and cortex (Aarts et al., 1989; Chambers and Russell, 1991; Lampi et al., 1998; Ueda et al., 2002a).

The six gamma crystallin proteins common to mammals, gamma crystallins A through F, are linked to a gene cluster of highly homologous genes (Shinohara et al., 1982; Breitman et al., 1984; Lok et al., 1984; Goring et al., 1992; Graw et al., 1993). They are among the earliest gene products in lenticular development in fiber cells and like the beta crystallins they are fiber cell specific. It has been suggested that the mammalian gamma crystallins constitute the tightest molecular arrangement among crystallins and exclude water to the highest degree being constituents of the harder lens nucleus especially in rodents (Slingsby, 1985). Gamma S crystallin, the only gamma crystallin detected in all vertebrate species and present in numerous tissues outside the lens is particularly suited to water rich tissue regions and can thus be regarded as a separate branch in vertebrate gamma crystallin gene evolution (van Rens et al., 1989). Not all beta and gamma crystallin polypeptides are detected in all mammalian species reflecting the proteins diverse nature and ideal suitability to environmental demands.

Crystallins are extensively modified in the course of healthy lenticular development beginning at an early onset. Unhealthy protein modification eventually results in an organism developing a cataract or other disease phenotype if a certain threshold is surpassed and perpetuated. The concept is emerging that the crystallins in high concentration are more than physical structural lens components, but that their protein species possess additional manifold catalytical or regulatory functions also essential for lens transparency (Bhat, 2004). Thus the dynamics of this protein super family and indeed of the lens itself are only beginning to be understood. A standard and two newer comprehensive crystallin reviews are cited for further reading (Graw, 1997; Bhat, 2003; Bloemendal et al., 2004).

Another central concern in guaranteeing light transmission without diffraction is proper lens architecture (Kuszak et al., 1994). Fiber cell shape is maintained by an extensive cytoskeleton whose protein components are synthesized and finalized prior to terminal differentiation (Kuwabara, 1975; FitzGerald, 1988). Actin, synthesized in the epithelial cells is polymerized from globular actin (Ramaekers et al., 1981) while the intermediate filament protein vimentin also synthesized throughout the epithelium and the elongating fiber cells is eliminated (Bradley et al., 1979; Sandilands et al., 1995). The major component of the differentiated fiber cell cytoskeleton, the lens unique beaded filament structure is established last (Maisel and Perry, 1972). The beaded filament is made up of two proteins, phakinin and filensin also termed CP49 and CP95, and has a 5–7 nm backbone with 20 nm beads evenly spaced throughout. It associates with alpha crystallin and is itself membrane associated and has a primary function in lens architecture (Ireland and Maisel, 1984, 1989; Carter, 1995).

Membrane proteins such as gap junctions constituents also contribute to cellular stability and arrangement. The maintenance of homeostasis of the interior fiber cells in the lens is attributed to a network connecting the cells to each other, to the differentiating cells at the lens equator and to the mitotically active cells of the anterior epithelium (Robinson and Patterson, 1982–1983; Mathias et al., 1997). Gap junctions consisting of two hemichannels each

made up of six connexin proteins are a large part of this network (Goodenough, 1992; Rae et al., 1996). The α1, α3 and α8 connexin proteins have been detected in the lens and so naturally contribute to stability and proper cellular arrangement (Gong et al., 1997).

Sample treatment and prefractionation

Proper sample handling and sensible prefractionation are prerequisites for any successful proteomics experiment. Following excision the lens can be decapsulated either removing the mitotically active cells of the anterior epithelium (Toyofuku and Bentley, 1970) or leaving them attached to the underlying cortical fibers (Dewey et al., 1995). The decapsulated organ can be dissected further into the major morphological regions termed the lens cortex and nucleus which in turn can arbitrarily be subdivided into distinct zones such as various cortical layers and the embryonic-, fetal- or adult nucleus (David and Shearer, 1984; Garland et al., 1996). These regions can be analyzed individually or the organ can be analyzed in its entirety foregoing dissection.

Prior to protein extraction lenticular material is often frozen in liquid nitrogen and ground to a powder or homogenized directly. The material can then be resuspended in distilled water or an aqueous buffer yielding the water-soluble fraction (WS) upon excitation and centrifugation (Clark et al., 1969; Kibbelaar and Bloemendal, 1975). The resulting pellet can be solubilized by sonication in aqueous buffer, pH manipulation or most common in buffer containing 6 M urea yielding the water-insoluble fraction (WI) in the supernatant upon further centrifugation (Ortwerth et al., 1986; Ortwerth and Olesen 1989, 1992; Kibbelaar and Bloemendal, 1975). The remaining pellet is termed the urea-insoluble fraction (UI) and can be solubilized in buffer containing high SDS concentrations (Kibbelaar and Bloemendal, 1975). Alternatively, the starting material can be resuspended directly in a buffer containing 6 M urea resulting in the urea-soluble fraction (US) in the supernatant and the urea-insoluble fraction in the pellet upon centrifugation. The urea-soluble fraction termed here constitutes the water-soluble and water-insoluble protein fractions described by Kibbelaar and Bloemendal (Kibbelaar and Bloemendal, 1975).

In many cases size exclusion chromatography is employed for the further fractionation of the raw protein extracts. The water-soluble (WS) protein fraction of the total lens or of morphological sections is generally separated by size exclusion chromatography into the high molecular weight (HMW), alpha (α), beta high (β_H) and beta low (β_L), and gamma (γ) fractions (Kibbelaar and Bloemendal, 1975; Berbers et al., 1982; Ringens et al., 1982; Kleiman et al., 1988; Kelley et al., 1993) with slight variations depending on the material and age. The HMW fraction consists of protein aggregates considered to be on the verge of insolubilization and precipitation (Jedziniak et al., 1978) and contains mainly alpha crystallin (Messmer and Chakrabarti, 1988; Chiou and Azari, 1989) and is isolated in the chromatographic void volume. The α fraction contains the native alpha crystallin oligomers with a Mw in excess of 200 kDa. The β_H fraction contains all seven beta crystallin polypeptides (David et al., 1993a) in diverse oligomeric arrangement probably consisting of 7 or 8 subunits (Bindels et al., 1981) with Mw around 160 kDa whereas the β_L fraction contains dimers and trimers (Bindels et al., 1981) with a Mw around 40 kDa. The beta fractions can be separated further into six or more β_H and two β_L fractions, β_{L1} and β_{L2} (Asselbergs et al., 1979), as well as a monomeric crystallin containing fraction, βs, today termed γs (Bindels et al., 1982; van Rens et al., 1989). The γ fraction with a molecular weight around 20 kDa contains the gamma crystallin monomers and can be subdivided into two fractions (Bindels et al., 1982).

The water-insoluble (WI) raw protein fraction can also be subjected to size exclusion chromatography on a column equilibrated with 6 M urea and was shown to result in four distinct chromatographic peaks. Fraction I contained actin while both cytoskeletal proteins actin and vimentin were recovered from fraction II. Fraction III contained the monomeric alpha crystallin subunits while fraction IV contained two low molecular weight proteins (Kibbelaar and Bloemendal, 1979). However other investigations separated the adult human WI fraction into three and two chromatographic peaks (Lund et al., 1996; Hanson et al., 2000).

The size exclusion chromatography fractions or the unfractionated water-soluble (WS), water-insoluble (WI) and urea-soluble (US) fractions can subsequently be applied to 2-DE or liquid chromatography steps for further separation and analysis prior to protein identification. Finally, the urea-insoluble (UI) raw protein fraction was applied to SDS-PAGE and shown to contain the membrane protein MP26 (Kibbelaar and Bloemendal, 1979). As a final note, the results of the described fractionation procedures apply to the healthy adult mammalian lens and may vary depending on species and age as well as on the analysis of whole lens or morphological sections (Li et al., 1986; Bours et al., 1990). An overview of the procedures is given in Fig. 1.

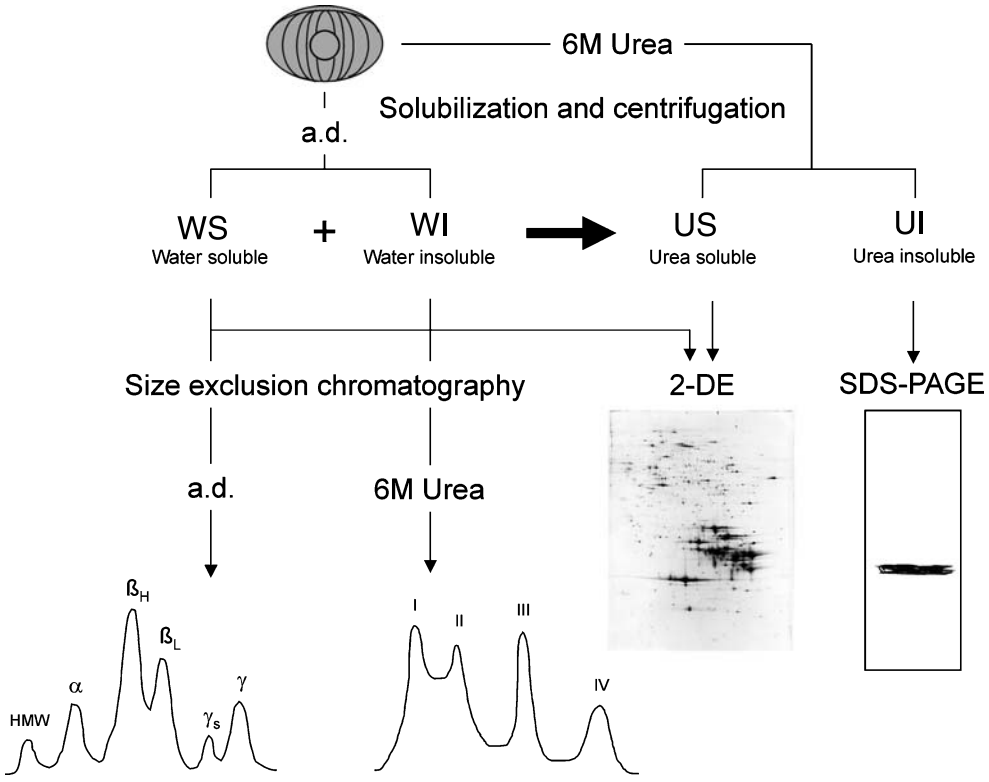

Fig. 1. Overview of lens solubilization and prefractionation procedures and common separation steps in a proteomics study

Two-dimensional electrophoresis and lenticular proteomics: a long relationship

Two-dimensional electrophoresis (2-DE) is probably the core proteomics technique for protein separation. It was developed independently by O'Farrell and Klose in 1975 and combines SDS-PAGE and isoelectric focusing for protein separation in two dimensions (O'Farrell, 1975; Klose, 1975). Proteins migrate to positions termed spots on the 2-DE gel as dictated by the relation of their chemical parameters to the applied 2-DE parameters. Generally the proteins are then stained with a dye and the spots excised from the gel. The proteins are digested in the gel spots with enzymes or reagents and the resulting peptides are analyzed by mass spectrometry, the essential proteomics technique for polypeptide characterization measuring the masses of ionized molecules or in the past by Edman degradation (Edman, 1949). The key improvement in mass spectrometry for the analysis of peptides and proteins was the introduction of soft ionization techniques such as matrix assisted laser ionization/desorption (MALDI) (Karas and Hillenkamp, 1988; Tanaka et al., 1988) and electrospray ionization (ESI) (Fenn et al., 1989). Today's generation of mass spectrometers yield both peptide masses and *de facto* peptide sequence information by fragmenting analyzed peptides in the mass spectrometer and applying a second round of mass spectrometry to the fragments. This data can then be used to identify peptides and proteins. Alternatively, proteins can be analyzed directly by mass spectrometry without prior digestion yielding masses of the entire polypeptide (Mann et al., 2001).

High quality 2-DE has remarkable resolution capability separating and concomitantly visualizing up to 10000 sample constituents distinguished by a single amino acid or PTM (Klose and Kobalz, 1995). The ability to detect protein modifications by themselves is offered by a number of techniques, the visualization of the polypeptides however is a premier advantage of 2-DE as it allows the correct assignment of these modifications to the respective protein species. At this analytical level modified and unmodified polypeptides should not be termed protein but protein species as this describes translation products at the covalent molecular level (Jungblut and Thiede, 1997). An experienced user can thus readily pinpoint differences between proteomes and truly characterize and understand them in detail expediting detection of physiologically or clinically relevant molecules such as genetic

variants or disease markers (Klose et al., 2002; Krah et al., 2004).

Two-dimensional electrophoresis and lens proteomics go together from the outset. Already in 1975 Kibbelaar and Bloemendal applied whole water soluble, size exclusion chromatography α, β_H, β_L and γ fractions and water insoluble as well as the urea solubilized pellet from calf lens to urea PAGE combined with SDS-PAGE producing a first rudimentary spot pattern of the major lenticular protein components, the unmodified crystallins and suggested alpha crystallin interaction with membrane components (Kibbelaar and Bloemendal, 1975). With the improvement of 2-DE a definitive pattern or reference map for the crystallins and other lenticular components was achieved in 1982 for the water soluble proteins of the bovine lens cortex with a final nomenclature for beta crystallins added in 1984 (Berbers et al., 1982, 1984). Similar investigations were performed in chicken, mouse, rat and human yielding comparable crystallin reference patterns and alpha crystallin characterization with nomenclature as well as demonstrating the presence of vimentin, actin and GAPDH in the urea soluble fraction presumably from the anterior epithelial cell layer and outer cortex (Garadi et al., 1983; Garber et al., 1984; de Vries et al., 1991; Datiles et al., 1992; David and Shearer, 1993b). Morphological regions of human lenses were analyzed

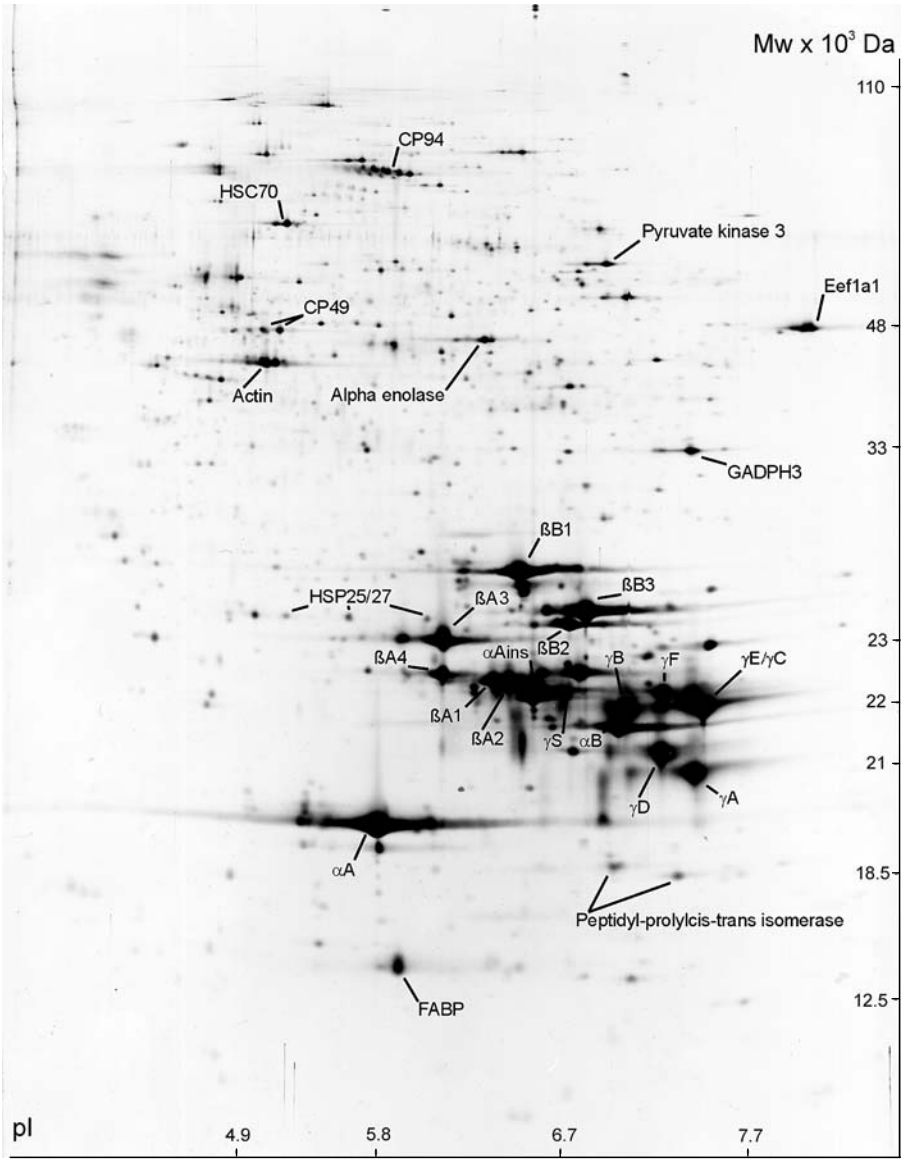

Fig. 2. 2-DE protein pattern of whole urea soluble mouse C57BL/6J 10 day old eye lens fraction with some identified proteins. The crystallins are named as token only

separately following dissection revealing a nuclear 2-DE pattern which is quite distinct from the previously described cortical or whole lens pattern (Garland et al., 1996). This implies a high degree of post translational modification as the nuclear protein species must be derived from the primary crystallin proteins as well as highlighting the differences in protein complement between lenticular regions. 2-DE patterns of human epithelial lens cells were also published recently (Wang-Su et al., 2003; Paron et al., 2004).

Newer investigations have become increasingly comprehensive, often further characterizing primary crystallin proteins, confirming amino acid sequences predicted from cDNA or detecting discrepancies and annotating sequences and creating higher quality reference maps as well as detecting numerous non crystallin lenticular proteins for various species and ages (Shih et al., 1998; Lampi et al., 2002; Wang-Su et al., 2003; Wilmarth et al., 2004; Hoehenwarter et al., 2005). A detailed reference map of the entire adult murine lenticular proteome using high resolution large scale 2-DE which separated the urea soluble fraction into 1940 spots was produced (Jungblut et al., 1998) ultimately followed by an in depth analysis of the water soluble and insoluble protein fractions at different ages (Ueda et al., 2002a). A 2-DE image of the young urea soluble whole lens murine lenticular proteome with the characteristic crystallin 2-DE pattern produced in our laboratory is shown in Fig. 2. An accessible repository for mouse lens and other proteomics data is urgently required and under preparation in the form of our 2-DE database at 2-DPAGE at http://www.mpiib-berlin.mpg.de/2D-PAGE/.

Endeavors towards an inclusive map and database of the water soluble proteins of the human lens culminated in the attainment of the infant, young, mature and old whole human lenticular proteomes as well as of the α, β_H, β_L and γ size exclusion chromatography fractions (Datiles et al., 1992; David et al., 1996; Bloemendal et al., 1997; Lampi et al., 1997, 1998). Considering 90% of the lenticular proteome is made up of twelve primary translation products these investigations have impressively demonstrated the extent of post translational modification in the lens beginning in the prenatal organism and increasing with age.

Recent advances in technology have firmly established 2-DE in the analytical laboratory allowing directed implementation as part of larger experimental designs. The water soluble and insoluble fractions of the lens nucleus and cortex have been comparatively analyzed by 2-DE with emphasis on the beta crystallins (Werten et al., 1999), the beaded filament constituents phakinin and filensin (Carter et al., 1995) and covalent crystallin multimers in the water insoluble fraction (Srivastava et al., 2004). The water soluble size exclusion chromatography fractions HMW and α (Spector et al., 1985; Harrington et al., 2004), β_H (Srivastava et al., 1999; Kamei et al., 2003), β_L (Kleiman et al., 1988) and γ (Srivastava and Srivastava, 1998) as well as the α crystallin A and B chains in the urea soluble fractions (Colvis et al., 2000; Colvis and Garland, 2002; Schaefer et al., 2003) have been analyzed independently with specific questions in mind.

In conclusion, 2-DE has not only been a pillar in the exploration of the lenticular proteome from the beginning, shedding light on the primary constituents, the unmodified crystallins and cell architecture components and marking changes as a result of aging and characterizing morphological sections but has also seen specialized applications. Most importantly the technique has been invaluable in the elucidation of post translational modifications in the healthy and cataractous lens which will be discussed below. Lenticular proteomics and 2-DE can thus be seen as a vanguard for functional proteomics at the protein species level.

Liquid chromatography and mass spectrometry for lens proteome analysis

Liquid chromatography is one of the oldest and best techniques for molecular separation in biochemical analysis earning Archer Martin and Richard Synge the Nobel Prize for Chemistry in 1952. It is based on the individual behavior of soluble analytes in a mobile phase passed over and interacting differentially with a solid phase or matrix. Various combinations of liquid and solid phases featuring distinct molecular interaction and separation properties have been developed and tried over the years leading to several popular liquid chromatography approaches, affinity chromatography, ion exchange chromatography, reverse phase chromatography and size exclusion chromatography to name a few.

Initially reverse phase chromatography was the method of choice for separating proteins and peptides according to hydrophobic interaction with C4, C8 or C18 alkyl chains in many proteomics experiments. By means of a connecting apparatus the molecules were eluted directly into an ESI mass spectrometer for analysis of proteins by exact measurement of protein mass and peptides by MS/MS analysis and measurement of fragment masses. This marriage of reverse phase liquid chromatography and ESI mass spectrometry proved ideal as the organic

composition and liquid state of aggregation of the reverse phase eluent is highly compatible with the electrospray ionization process (Dole et al., 1968; Yamashita and Fenn, 1984; Fenn et al., 1989) and was originally termed shotgun proteomics (McCormack et al., 1997; Yates 3rd, 1998).

Early experiments quickly showed that one chromatographic step alone could not adequately separate complex protein or peptide mixtures for reasonable detection and characterization by mass spectrometry due to molecular abundance in chromatographic retention time exceeding the mass spectrometers specifity. Therefore a second chromatographic step, in many cases on- or offline ion exchange chromatography was added to improve separation and alleviate this problem. This procedure became known as multidimensional protein identification technology or MudPIT (Washburn et al., 2001). The primary advantage offered by liquid chromatography techniques coupled to mass spectrometers is the relative speed at which complex protein mixtures can be characterized at the protein level. However, should more directed applications or the detection of post translational modifications be aspired to, higher degrees of separation and consequently often more than two chromatographic steps are required. Ultimately, true high-end multidimensional chromatography for the analysis of complex proteomes includes up to 15 or more chromatography steps with reverse phase chromatography directly preceding mass spectrometry as most other chromatographic buffers and solutions are incompatible with ionization (Link et al., 1999; Washburn et al., 2001; Wolters et al., 2001). Experiments of this type with peptides generate between 10 and 100 thousand distinct mass spectra or scans, making comprehensive evaluation equally laborious as the complete analysis of spots from 2-DE (Hoehenwarter et al., 2006; Swanson and Washburn, 2005).

Recently MALDI mass spectrometry has also become available for shotgun proteomics experiments with automated spotting techniques depositioning liquid chromatography eluents onto MALDI templates (Fung et al., 2004; Nagele and Vollmer, 2004; Zhen et al., 2004).

Major multi-dimensional liquid chromatography investigations have characterized the infant to adult water soluble and the adult water insoluble human lens proteome. Following size exclusion chromatography water soluble α, β_H, β_L and γ fractions and two water insoluble fractions were applied to reverse phase chromatography on C4 columns for further fractionation. Whole proteins were then analyzed offline with ESI mass spectrometry. The 11 human crystallin proteins known then, alpha A and B, beta B1, B2 and B3, A1, A3 and A4 and gamma C, D and S were detected in the water soluble fraction and changes in their abundance as well as post translational modifications with age were characterized (Ma et al., 1998). The adult water insoluble fraction contained little beta B2 crystallin, a major component of the water soluble fraction and numerous crystallin degradation products. Nevertheless, the major components were shown to be intact crystallins (Hanson et al., 2000).

Another investigation subjected the water insoluble monomeric alpha crystallin subunit containing size exclusion chromatography fraction to cation exchange chromatography before reverse phase chromatography and offline ESI mass spectrometry. A high degree of separation with three liquid chromatography steps was achieved and it was shown that alpha crystallin is the major component of the water insoluble fraction constituting about half of protein abundance and numerous alpha crystallin protein species were identified and distinguished from the water soluble fraction (Lund et al., 1996). Also, a high end MudPit analysis of young human cataractous lens combining 18 chromatographic steps was carried out and in addition to detecting the 11 human crystallin proteins and modifications detected 270 non-crystallin proteins, an impressive number by any means (MacCoss et al., 2002).

As mentioned above a first step in lenticular proteomics is often size exclusion chromatography. Water soluble high molecular weight and α fractions were analyzed by reverse phase chromatography and MALDI-MS as well as further separated into alpha A and alpha B crystallin subunit containing fractions using ion exchange chromatography prior to mass spectrometry, implicating conformational changes effected by spontaneous asparagine racemization and isomerization in alpha crystallin aggregation and insolubilization (Fujii et al., 2003, 2004). A reverse phase chromatography ESI-MS analysis of the HMW and α fractions in conjunction with other data suggested that alpha crystallin subunit C-terminal truncation and phosphorylation do not adversely affect protein chaperone activity (Carver et al., 1996). All of the investigations confirmed alpha crystallin as the main component of the HMW size exclusion chromatography fraction while another investigation identified chaperone sites by analyzing hydrogen-deuterium exchange in the presence of ATP with ESI-MS (Hasan et al., 2002). Major C-terminal truncation sites on the alpha A crystallin chain were characterized using MALDI-MS (Takemoto, 1995) and cleavage in the N-terminal region was shown using fast atom bombardment (FAB) MS (Kamei et al., 1997). Finally, a novel modification on the C-terminal lysine of the alpha B

crystallin chain was detected by ESI/TOF-MS after purification of the alpha B crystallin subunit from the α size exclusion chromatography fraction with reverse phase LC (Lin et al., 1997).

The human beta crystallins were purified using anion exchange chromatography and reverse phase chromatography following size exclusion chromatography and analyzed with ESI-MS resulting in the detection of numerous post translational modifications and of beta A2 crystallin in humans (Ajaz et al., 1997; Lapko et al., 2003a). The solubility properties of beta B2 crystallin, the major beta crystallin throughout the lens, were also analyzed with reverse phase LC and ESI-MS (Feng et al., 2000).

The human size exclusion γ fraction was analyzed in detail corroborating the known sequences for the human gamma crystallins, gamma C, D and S as well as identifying deamidation, disulfide bonding, methylation and carbamylation sites. Truncated low molecular mass fragments were also analyzed (Hanson et al., 1998; Abbasi et al., 1998; Lapko et al., 2003b) and the bovine gamma E crystallin sequence was realized following additional purification of the γ fraction with cation exchange chromatography (Kilby et al., 1997).

A smaller LMW fraction containing proteins under 10 kDa was produced by 2 subsequent size exclusion chromatography steps and was shown to contain ubiquitin by MALDI-MS as well as its C-terminal degradation products in cataractous lenses (Stiuso et al., 2002). Finally, the major intrinsic membrane protein (MIP) comprising 50% of membrane protein content was extracted with urea and analyzed with reverse phase ESI- and MALDI-MS (Schey et al., 2000).

In conclusion, liquid chromatography techniques coupled with mass spectrometry for protein separation and analysis have characterized most of the lenticular proteome and detected large numbers of post translational modifications. For a low complexity sample such as the lens where the individual components can be purified to a high degree using several chromatography steps the protein species level can be investigated and has been extensively realized.

Crystallin post-translational modifications: important molecules in lenticular ageing and implicated in cataractogenesis

It is a known fact that the primary crystallin translation products are extensively modified beginning at an early age. Also, evidence is abundant that crystallin post translational modifications increase as the lens ages both in the appearance of new modification types and in the continued increase of earlier onset modifications. This is illustrated in Fig. 3 which shows the crystallin area of 2-DE gels prepared in our laboratory of the urea soluble fraction of the whole mouse lens proteome at different ages.

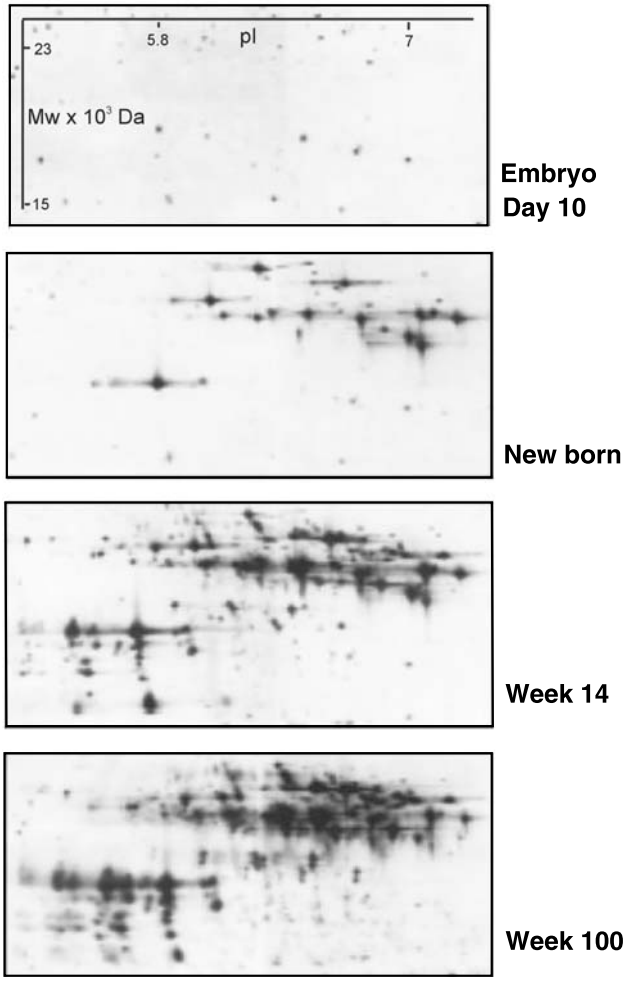

Fig. 3. The crystallin protein super family. Post translational modifications and the effects of development and ageing

Probably the most common post translational modification is truncation, primarily at the alpha crystallin C-terminal as well as N- and C-terminal beta crystallin extensions. It seems alpha crystallin C-terminal truncation is a result of calpain protease activity (Ueda et al., 2001, 2002b). C-terminal cleavage by m-calpain adversely effects alpha crystallin chaperone activity as well as subunit assembly while there is evidence that cleavage at the major C-terminal lens specific protease Lp82 sites does not (Carver et al., 1996; Thampi and Abraham, 2003). Beta crystallin N- and C-terminal truncation may be a result of calpain activity and effect subunit organization and higher order assembly (David et al., 1993a, 1996;

Table 1. Crystallin modifications in the lens detected by proteomics techniques

Protein	Modification[a]	Lenticular region[b]	Species[c]	Detection method[d]	Citation
alpha A crystallin	acetylation K70	WL: WS, α	H, H*	P LC/ESI-MS p LC/ESI-MS/MS p MudPIT/ESI-MS/MS	Lin et al., 1998 MacCoss et al., 2002
alpha A crystallin	acetylation K78	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
alpha A crystallin	acetylation K88	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
alpha A crystallin	acetylation K145	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
alpha A crystallin	acetylation M1	WL: WS; WI	B, C, H	P LC/ESI-MS p 2-DE ESI-MS/MS	Smith et al., 1991 Srivastava et al., 2004 Wilmarth et al., 2004
alpha A crystallin	deamidation N101	WL: WS; WI, mF	H	p LC/ESI-MS p LC/ESI-MS/MS p LC/FAB-MS	Lund et al., 1996 Miesbauer et al., 1994
alpha A crystallin	deamidation Q6	WL: WS; WI, mF	H	P LC/ESI-MS p LC/ESI-MS p LC/ESI-MS/MS p LC/FAB-MS	Lund et al., 1996 Hanson et al., 2000 Miesbauer et al.,1994
alpha A crystallin	deamidation Q50	WL: WS; WI, mF	H	p LC/ESI-MS p LC/ESI-MS/MS p LC/FAB-MS	Lund et al., 1996 Miesbauer et al., 1994
alpha A crystallin	deamidation Q90	WL: WI, mF	H	p LC/ESI-MS p LC/ESI-MS/MS	Hanson et al., 2000 Lund et al., 1996
alpha A crystallin	deamidation Q104	WL: WI	H	p LC/ESI-MS p LC/ESI-MS/MS	Hanson et al., 2000
alpha A crystallin	deamidation Q147	WL: WS; WI, mF	H	P LC/ESI-MS p LC/ESI-MS p LC/ESI-MS/MS p LC/FAB-MS	Lund et al., 1996 Hanson et al., 2000 Miesbauer et al., 1994
alpha A crystallin	disulfide bond C131–142	WL: WS; WI, mF	H	p LC/ESI-MS p LC/ESI-MS/MS p LC/FAB-MS	Lund et al., 1996 Miesbauer et al., 1994
alpha A crystallin	formylation H79	WL: WI	H	p 2-DE ESI-MS/MS	Srivastava et al., 2004
alpha A crystallin	methylation K88	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
alpha A crystallin	methylation R21	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
alpha A crystallin	oxidation M1	WL: WI, mF	H	p LC/ESI-MS p LC/ESI-MS/MS p 2-DE ESI-MS/MS	Hanson et al., 2000 Lund et al., 1996 Srivastava et al., 2004
alpha A crystallin	oxidation M138	WL: WI, mF	H	p LC/ESI-MS p LC/ESI-MS/MS	Hanson et al., 2000 Lund et al., 1996
alpha A crystallin	oxidation W9	WL: WI	H	p 2-DE ESI-MS/MS	Srivastava et al., 2004
alpha A crystallin	oxidation Y18	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
alpha A crystallin	oxidation Y34	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
alpha A crystallin	phosphorylation S45	WL: WS; WI, mF	H, H*	P LC/ESI-MS p LC/ESI-MS/MS p MudPIT/ESI-MS/MS	Lund et al., 1996 MacCoss et al., 2002
alpha A crystallin	phosphorylation S122	WL: WS; WI, mF; US	B, H, H*	P LC/ESI-MS p LC/ESI-MS p LC/ESI-MS/MS p MudPIT/ESI-MS/MS p LC/FAB-MS p LC/FAB-MS/MS p LC/MALDI-MS	Lund et al., 1996 MacCoss et al., 2002 Miesbauer et al., 1994 Schaefer et al., 2003 Smith et al., 1991 Takemoto, 1996
alpha A crystallin	phosphorylation S148	WL: US	M	p LC/ESI-MS/MS	Schaefer et al., 2003
alpha A crystallin	phosphorylation T13	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
alpha A crystallin	phosphorylation T140	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
alpha A crystallin	racemization D151	WL: WS, HMW, α	H, R, R*	p LC/MALDI-MS p LC Edman	Fujii et al., 1994, 1999, 2003, 2004
alpha A crystallin	racemization D58	WL: WS, HMW, α	H, R, R*	p LC/MALDI-MS p LC Edman	Fujii et al., 2001, 2003, 2004
alpha A crystallin	S59 to dehydroalanine	WL: WI	H	p 2-DE ESI-MS/MS	Srivastava et al., 2004
alpha A crystallin	truncation 3/4	WL: WS	H	p LC/FAB-MS p LC Edman	Kamei et al., 1997
alpha A crystallin	truncation 41/42	WL: US	M	p 2-DE ESI-MS/MS	Hoehenwarter et al., 2006
alpha A crystallin	truncation 50/51	WL: WI, mF	H	P LC/ESI-MS p LC/ESI-MS/MS	Lund et al., 1996
alpha A crystallin	truncation 65/66	WL: WI	H	P LC/ESI-MS p LC/ESI-MS/MS	Hanson et al., 2000

(*continued*)

Table 1 (*continued*)

Protein	Modification[a]	Lenticular region[b]	Species[c]	Detection method[d]	Citation
alpha A crystallin	truncation 80/81	WL: WI	H	P LC/ESI-MS p LC/ESI-MS/MS	Hanson et al., 2000
alpha A crystallin	truncation 101/102	WL: WS, HMW; WI, mF	B, H	P LC/ESI-MS p LC/ESI-MS/MS	Carver et al., 1996 Kilby, 1995a Lund et al., 1996
alpha A crystallin	truncation 118/119	N: WI	R*	P LC/ESI-MS	Ueda et al., 2002a
alpha A crystallin	truncation 119/120	N: WI	R, R*	P LC/ESI-MS	Ueda et al., 2002a
alpha A crystallin	truncation 126/127	N: WI	R, R*	P LC/ESI-MS	Ueda et al., 2002a
alpha A crystallin	truncation 130/131	N: WI	R, R*	P LC/ESI-MS	Ueda et al., 2002a
alpha A crystallin	truncation 147/148	N: WI	R, R*	P LC/ESI-MS	Ueda et al., 2002a
alpha A crystallin	truncation 151/152	WL: WS, HMW, α; WI N: WI	B, M, R, R+, R*	P LC/ESI-MS P 2-DE ESI-MS p 2-DE ESI-MS/MS	Smith et al., 1991 Thampi et al., 2002 Ueda et al., 2002a, 2002b
alpha A crystallin	truncation 154/155	WL: WS, α	B	P LC/ESI-MS	Smith et al., 1991
alpha A crystallin	truncation 156/157	N: WI	R, R*	P LC/ESI-MS	Ueda et al., 2002a
alpha A crystallin	truncation 157/158	WL: WS, HMW, α N: WI	R, R+, R*	P LC/ESI-MS	Thampi et al., 2002 Ueda et al., 2002a
alpha A crystallin	truncation 162/163	WL: WS, HMW, α; US N: WI	H, R, R+	P LC/ESI-MS p 2-DE MALDI-MS	Colvis et al., 2002 Thampi et al., 2002 Ueda et al., 2002a
alpha A crystallin	truncation 163/164	WL: WS, HMW, α N: WI	R, R+, R*	P LC/ESI-MS	Thampi et al., 2002 Ueda et al., 2002a
alpha A crystallin	truncation 165/166	N: WI	R, R*	P LC/ESI-MS	Ueda et al., 2002a
alpha A crystallin	truncation 168/169	WL: WS, HMW, α; WI, mF; US N: WI	B, H, M, R, R+, R*	P LC/ESI-MS P LC/MALDI-MS P 2-DE ESI-MS p LC/ESI-MS/MS p 2-DE ESI-MS/MS	Lund et al., 1996 Takemoto, 1995 Thampi et al., 2002 Ueda et al., 2001 Ueda et al., 2002a, 2002b
alpha A crystallin	truncation 172/173	WL: WS, HMW, α; WI, mF; US	B, H, R+	P LC/ESI-MS p LC/ESI-MS/MS P LC/MALDI-MS p 2-DE MALDI-MS	Carver et al., 1996 Colvis et al., 2002 Emmons and Takemoto, 1992 Hanson et al., 2000 Kilby et al., 1995a Lund et al., 1996 Miesbauer et al., 1994 Smith et al., 1991 Takemoto, 1995 Thampi et al., 2002 Ueda et al., 2001
alpha B crystallin	acetylation K92	WL: WS, α	H*	P LC/ESI-MS p LC/ESI-MS/MS p MudPIT/ESI-MS/MS	Lapko et al., 2001 MacCoss et al., 2002
alpha B crystallin	acetylation M1	WL: WS	C	p 2-DE ESI-MS/MS	Wilmarth et al., 2004
alpha B crystallin	carbamylation K92	WL: WS, α	H*	P LC/ESI-MS p LC/ESI-MS/MS	Lapko et al., 2001
alpha B crystallin	deamidation N78	WL: WI	H	p LC/ESI-MS p LC/ESI-MS/MS	Hanson et al., 2000
alpha B crystallin	deamidation N146	WL: WI, mF	H	p LC/ESI-MS p LC/ESI-MS/MS	Lund et al., 1996
alpha B crystallin	deamidation Q108	WL: WI, mF	H	p LC/ESI-MS p LC/ESI-MS/MS	Hanson et al., 2000 Lund et al., 1996
alpha B crystallin	methylation R22	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
alpha B crystallin	methylation R50	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
alpha B crystallin	novel modification K175	WL: WS C: US	H, HSr	P LC/ESI-MS p LC/ESI-MS/MS p 2-DE MALDI-MS	Colvis et al., 2000 Lin et al., 1997
alpha B crystallin	oxidation M1	WL: WI, mF	H	p LC/ESI-MS p LC/ESI-MS/MS	Hanson et al., 2000 Lund et al., 1996
alpha B crystallin	oxidation M68	WL: WS; WI	H, H*	p 2-DE ESI-MS/MS p MudPIT/ESI-MS/MS	MacCoss, et al., 2002 Srivastava et al., 2004
alpha B crystallin	oxidation M168	WL: WI, mF	H	p LC/ESI-MS p LC/ESI-MS/MS	Hanson et al., 2000 Lund et al., 1996
alpha B crystallin	oxidation W60	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
alpha B crystallin	oxidation Y48	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002

(*continued*)

Table 1 (*continued*)

Protein	Modification[a]	Lenticular region[b]	Species[c]	Detection method[d]	Citation
alpha B crystallin	phosphorylation S19	WL: WS, α; WI, mF C: US	B, H, H*, HSr	P LC/ESI-MS p LC/ESI-MS/MS p MudPIT/ESI-MS/MS p LC/FAB-MS p 2-DE MALDI-MS p LC Edman	Colvis et al., 2000 Lund et al., 1996 MacCoss et al., 2002 Miesbauer et al., 1994 Smith et al., 1992 Voorter et al., 1989
alpha B crystallin	phosphorylation S21	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
alpha B crystallin	phosphorylation S43	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
alpha B crystallin	phosphorylation S45	WL: WS, α; WI, mF	B, H, H*	P LC/ESI-MS p LC/ESI-MS/MS p MudPIT/ESI-MS/MS p LC/FAB-MS p LC Edman	Lund et al., 1996 MacCoss et al., 2002 Miesbauer et al., 1994 Smith et al., 1992 Voorter et al., 1989
alpha B crystallin	phosphorylation S53	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
alpha B crystallin	phosphorylation S59	WL: WS, α; WI, mF	B, H, H*	P LC/ESI-MS p LC/ESI-MS/MS p MudPIT/ESI-MS/MS p LC/FAB-MS	Lund et al., 1996 MacCoss et al., 2002 Miesbauer et al., 1994 Smith et al., 1992
alpha B crystallin	phosphorylation S76	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
alpha B crystallin	truncation 1/2	WL: WS	H	p LC/FAB-MS p LC Edman	Kamei et al., 1997
alpha B crystallin	truncation 6/7	WL: WS	H	p LC/FAB-MS p LC Edman	Kamei et al., 1997
alpha B crystallin	truncation 40/41	C: WS	H, H*	p 2-DE MALDI-MS	Jimenez-Asensio et al., 1999
alpha B crystallin	truncation 42/43	C: WS	H, H*	p 2-DE MALDI-MS	Jimenez-Asensio et al., 1999
alpha B crystallin	truncation 44/45	C: WS	H, H*	p 2-DE MALDI-MS	Jimenez-Asensio et al., 1999
alpha B crystallin	truncation 150/151	C: US	H, HSr	p 2-DE MALDI-MS	Colvis et al., 2000
alpha B crystallin	truncation 151/152	C: US	H, HSr	p 2-DE MALDI-MS	Colvis et al., 2000
alpha B crystallin	truncation 152/153	C: US	H, HSr	p 2-DE MALDI-MS	Colvis et al., 2000
alpha B crystallin	truncation 153/154	C: US	H, HSr	p 2-DE MALDI-MS	Colvis et al., 2000
alpha B crystallin	truncation 154/155	C: US	H, HSr	p 2-DE MALDI-MS	Colvis et al., 2000
alpha B crystallin	truncation 161/162	C: US	HSr	p 2-DE MALDI-MS	Colvis et al., 2000
alpha B crystallin	truncation 162/163	C: US	H, HSr	p 2-DE MALDI-MS	Colvis et al., 2000
alpha B crystallin	truncation 163/164	N: WI	R	P LC/ESI-MS	Ueda et al., 2002a
alpha B crystallin	truncation 170/171	WL: WS, HMW, α. C: US	H, HSr, R, R+, R*	P LC/ESI-MS	Colvis et al., 2000
		N: WI		p 2-DE MALDI-MS	Thampi et al., 2002 Ueda et al., 2002a
alpha B crystallin	truncation 174/175	WL: WI; mF C: WS; US	H, H*, HSr	P LC/ESI-MS p LC/ESI-MS p LC/ESI-MS/MS p 2-DE MALDI-MS	Jimenez-Asensio et al., 1999 Colvis et al., 2000 Lund et al., 1996
beta A1 crystallin	acetylation, M1 removed	WL: WS	C	P LC/ESI-MS p 2-DE ESI-MS/MS	Wilmarth et al., 2004
beta A1/A3 crystallin	acetylation K122	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta A1/A3 crystallin	acetylation K125	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta A1/A3 crystallin	acetylation K131	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta A1/A3 crystallin	deamidation N22	N: WI	R*	p 2-DE Edman	David et al., 1993a
beta A1/A3 crystallin	deamidation Q38	WL: WI	H	p LC/ESI-MS p LC/ESI-MS/MS	Hanson et al., 2000
beta A1/A3 crystallin	deamidation Q42	WL: WI	H	p LC/ESI-MS p LC/ESI-MS/MS	Hanson et al., 2000
beta A1/A3 crystallin	methylation R137	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta A1/A3 crystallin	oxidation M126	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta A1/A3 crystallin	phosphorylation S160	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta A1/A3 crystallin	phosphorylation T34	WL: WS, β_H	B	p 2-DE MALDI-MS	Kamei et al., 2003
beta A1/A3 crystallin	phosphorylation T127	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta A1/A3 crystallin	truncation 20/21	N: WI	R*	p 2-DE Edman	David et al., 1993a
beta A1/A3 crystallin	truncation 21/22	WL: WS; WI N: WI	M*, R*	p 2-DE Edman	David et al., 1993a, 1994
beta A1/A3 crystallin	truncation 22/23	WL: WS, β_H, β_L; WI C: WS, WI N: WS, WI	B, C, H, M	P LC/ESI-MS P 2-DE ESI-MS p 2-DE ESI-MS p 2-DE ESI-MS/MS p 2-DE Edman p 2-DE FAB-MS	Lampi et al., 1997, 1998 Ma et al., 1998 Shih et al., 1998 Ueda et al., 2002b Werten et al., 1999 Wilmarth et al., 2004

(*continued*)

Table 1 (*continued*)

Protein	Modification[a]	Lenticular region[b]	Species[c]	Detection method[d]	Citation
beta A1/A3 crystallin	truncation 26/27	WL: WS	H	p 2-DE ESI-MS p 2-DE Edman	Lampi et al., 1998
beta A1/A3 crystallin	truncation 39/40	WL: WS, β_H	H	P 2-DE Edman	Srivastava et al., 1999
beta A2 crystallin	acetylation, M1 removed	WL: WS	C	P LC/ESI-MS p 2-DE ESI-MS/MS	Wilmarth et al., 2004
beta A2 crystallin	acetylation, M1 removed alternate start codon M5	WL: WS	C	P LC/ESI-MS p 2-DE ESI-MS/MS	Wilmarth et al., 2004
beta A2 crystallin	phosphorylation S30	WL: WS	H	p LC/ESI-MS/MS	Lapko et al., 2003a
beta A3 crystallin	truncation 11/12	WL: WS, β_H; WI	B, M, M*, R, R*	P LC/ESI-MS	David et al., 1993a, 1993b, 1994
		N: WS; WI		P 2-DE ESI-MS p 2-DE ESI-MS/MS p 2-DE Edman	Lampi et al., 2002 Shih et al. 1998 Ueda et al., 2002b Werten et al., 1999
beta A4 crystallin	oxidation M13	WL: WI	H	p 2-DE ESI-MS/MS	Srivastava et al., 2004
beta A4 crystallin	oxidation W149	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta A4 crystallin	phosphorylation S35	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta A4 crystallin	phosphorylation T43	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta A4 crystallin	truncation 18/19	WL: WS; WI N: WI	M*, R*	p 2-DE Edman	David et al., 1993a, 1994
beta B1 crystallin	acetylation K6	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta B1 crystallin	acetylation K160	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta B1 crystallin	deamidation N157	WL: WI	H	p LC/ESI-MS p LC/ESI-MS/MS	Hanson et al., 2000
beta B1 crystallin	methylation K235	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta B1 crystallin	methylation R230	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta B1 crystallin	methylation R231	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta B1 crystallin	oxidation M112	WL: WI	H	p 2-DE ESI-MS/MS	Srivastava et al., 2004
beta B1 crystallin	oxidation M226	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta B1 crystallin	oxidation W126	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta B1 crystallin	phosphorylation S10	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta B1 crystallin	phosphorylation T12	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta B1 crystallin	truncation 4/5	WL: WS	C	p 2-DE Edman	Wilmarth et al., 2004
beta B1 crystallin	truncation 11/12	WL: WS; WI	M*	p 2-DE Edman	David et al., 1994
beta B1 crystallin	truncation 15/16	WL: WS, β_H, β_L	B, H	P LC/ESI-MS p LC ESI MS p LC Edman p 2-DE ESI-MS p 2-DE Edman p 2-DE FAB-MS	Ajaz et al., 1997 David et al., 1996 Lampi et al., 1997, 1998 Ma et al., 1998 Shih et al., 1998
beta B1 crystallin	truncation 26/27	WL: WS; WI	M*	p 2-DE Edman	David et al., 1994
beta B1 crystallin	truncation 27/28	N: WI	R*	p 2-DE Edman	David et al., 1993a
beta B1 crystallin	truncation 34/35	WL: WS, β_H, β_L; WI	H	P LC/ESI-MS p LC/ESI-MS p LC Edman p LC/ESI-MS/MS	Ajaz et al., 1997 David et al., 1996 Ma et al., 1998 Hanson et al., 2000
beta B1 crystallin	truncation 35/36	WL: WS, β_H, β_L	H	P LC/ESI-MS p LC/ESI-MS p LC Edman	Ajaz et al., 1997 David et al., 1996 Ma et al., 1998
beta B1 crystallin	truncation 36/37	WL: WS, β_H, β_L	H	P LC/ESI-MS p LC/ESI-MS p LC Edman	Ajaz et al., 1997 David et al., 1996 Ma et al., 1998
beta B1 crystallin	truncation 39/40	WL: WS, β_H, β_L	H	P LC/ESI-MS p LC/ESI-MS p LC Edman p 2-DE FAB-MS	Ajaz et al., 1997 David et al., 1996 Lampi et al., 1997 Ma et al., 1998
beta B1 crystallin	truncation 40/41	WL: WS, β_H, β_L	H	P LC/ESI-MS p LC/ESI-MS p LC Edman	Ajaz et al., 1997 David et al., 1996 Ma et al., 1998
beta B1 crystallin	truncation 41/42	WL: WS, β_H, β_L	H	P LC/ESI-MS p LC/ESI-MS p LC Edman	Ajaz et al., 1997 David et al., 1996 Ma et al., 1998
beta B1 crystallin	truncation 49/50	WL: WS; WI N: WI	M*, R*	p 2-DE Edman	David et al., 1993a, 1994
beta B1 crystallin	truncation 72/73	WL: WI	H	P LC/ESI-MS p LC/ESI-MS/MS	Hanson et al., 2000
beta B2 crystallin	acetylation K76	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta B2 crystallin	acetylation K121	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002

(*continued*)

Table 1 (*continued*)

Protein	Modification[a]	Lenticular region[b]	Species[c]	Detection method[d]	Citation
beta B2 crystallin	acetylation, M1 removed	WL: WS, β_H	B	p 2-DE MALDI-MS	Kamei et al., 2003
beta B2 crystallin	acetylation, M1 removed alternative splicing	WL: WS	C	p 2-DE ESI-MS/MS	Wilmarth et al., 2004
beta B2 crystallin	disulfide bond C37–66	WL: US	H*	p LC/MALDI-MS p LC Edman	Takemoto, 1997
beta B2 crystallin	glutathione adduct C66	WL: WS	H	P LC/ESI-MS p LC/ESI-MS/MS	Feng et al., 2000
beta B2 crystallin	methylation K42	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta B2 crystallin	methylation K68	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta B2 crystallin	methylation K121	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta B2 crystallin	oxidation M122	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta B2 crystallin	oxidation W59	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta B2 crystallin	oxidation W151	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta B2 crystallin	phosphorylation T118	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta B2 crystallin	truncation 7/8	WL: WS; WI	M	P 2-DE ESI-MS p 2-DE ESI-MS/MS	Ueda et al., 2002b
beta B2 crystallin	truncation 8/9	WL: WS	B	p 2-DE Edman	Shih et al., 1998
beta B2 crystallin	truncation 203/204	WL: WS	B	P LC/ESI-MS p LC/ESI-MS/MS	Kilby et al., 1995b
beta B3 crystallin	acetylation K128	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta B3 crystallin	acetylation, M1 removed	WL: WS, β_H	C	P LC/ESI-MS p 2-DE ESI-MS/MS	Wilmarth et al., 2004
beta B3 crystallin	methylation K128	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta B3 crystallin	oxidation M129	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta B3 crystallin	phosphorylation S6	WL: WS, β_H	B	p 2-DE MALDI-MS	Kamei et al., 2003
beta B3 crystallin	phosphorylation T7	WL: WS, β_H	B	p 2-DE MALDI-MS	Kamei et al., 2003
beta B3 crystallin	phosphorylation Y29	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
beta B3 crystallin	truncation 5/6	WL: WS; WI N: WI	M*, R*	p 2-DE Edman	David et al., 1993a, 1994
beta B3 crystallin	truncation 17/18	WL: WS; WI	M, M*, R*	P 2-DE ESI-MS	David et al., 1993a, 1993b, 1994
		N: WI		p 2-DE ESI-MS/MS p 2-DE Edman	Ueda et al., 2002b
beta B3 crystallin	truncation 22/23	WL: WS	B	p 2-DE Edman	Shih et al., 1998
gamma B crystallin	acetylation, M1 removed	C: WS, γ N: WS, γ	H	P LC/ESI-MS p LC/ESI-MS/MS p LC/MALDI-MS	Lapko et al., 2003b
gamma B crystallin	carbamylation, M1 removed	C: WS, γ N: WS, γ	H	P LC/ESI-MS p LC/ESI-MS/MS p LC/MALDI-MS	Lapko et al., 2003b
gamma B crystallin	kynurenine W158	WL: US	M	p 2-DE MALDI-MS	Jungblut et al., 1998
gamma B crystallin	methylation C22	C: WS, γ N: WS, γ	H	P LC/ESI-MS p LC/ESI-MS/MS p LC/MALDI-MS	Lapko et al., 2003b
gamma B crystallin	methylation C79	C: WS, γ N: WS, γ	H	P LC/ESI-MS p LC/ESI-MS/MS p LC/MALDI-MS	Lapko et al., 2003b
gamma B crystallin	oxidation M70	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
gamma B crystallin	oxidation W69	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
gamma B crystallin	phosphorylation Y63	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
gamma B crystallin	phosphorylation Y66	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
gamma C crystallin	acetylation, M1 removed	C: WS, γ N: WS, γ	H	P LC/ESI-MS p LC/ESI-MS/MS p LC/MALDI-MS	Lapko et al., 2003b
gamma C crystallin	carbamylation, M1 removed	C: WS, γ N: WS, γ	H	P LC/ESI-MS p LC/ESI-MS/MS p LC/MALDI-MS	Lapko et al., 2003b
gamma C crystallin	deamidation N137	WL: WS, γ	H	P LC/ESI-MS	Hanson et al., 1998
gamma C crystallin	disulfide bond C22–32	WL: WS, γ	H	P LC/ESI-MS	Hanson et al., 1998
gamma C crystallin	disulfide bond C78–79	WL: WS, γ	H	P LC/ESI-MS	Hanson et al., 1998
gamma C crystallin	kynurenine W157	WL: US	M	p 2-DE MALDI-MS	Jungblut et al., 1998
gamma C crystallin	methylation C22	C: WS, γ N: WS, γ	H	P LC/ESI-MS p LC/ESI-MS/MS p LC/MALDI-MS	Lapko et al., 2003b
gamma C crystallin	methylation C79	C: WS, γ N: WS, γ	H	P LC/ESI-MS p LC/ESI-MS/MS p LC/MALDI-MS	Lapko et al., 2003b

(*continued*)

Table 1 (*continued*)

Protein	Modification[a]	Lenticular region[b]	Species[c]	Detection method[d]	Citation
gamma C crystallin	oxidation M70	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
gamma C crystallin	oxidation W69	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
gamma C crystallin	oxidation W131	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
gamma C crystallin	oxidation Y56	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
gamma C crystallin	phosphorylation Y63	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
gamma C crystallin	phosphorylation Y66	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
gamma D crystallin	acetylation, M1 removed	C: WS, γ	H	P LC/ESI-MS	Lapko et al., 2003b
		N: WS, γ		p LC/ESI-MS/MS	
				p LC/MALDI-MS	
gamma D crystallin	carbamylation, M1 removed	C: WS, γ	H	P LC/ESI-MS	Lapko et al., 2003b
		N: WS, γ		p LC/ESI-MS/MS	
				p LC/MALDI-MS	
gamma D crystallin	disulfide bond C18–32	WL: WS, γ	H	P LC/ESI-MS	Hanson et al., 1998
gamma D crystallin	disulfide bond C108–110	WL: WS, γ	H	P LC/ESI-MS	Hanson et al., 1998
gamma D crystallin	methylation C110	C: WS, γ	H	P LC/ESI-MS	Lapko et al., 2003b
		N: WS, γ		p LC/ESI-MS/MS	
				p LC/MALDI-MS	
gamma D crystallin	oxidation Y46	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
gamma D crystallin	truncation 73/74	WL: WS, γ	H	P 2-DE Edman	Srivastava et al., 1998
gamma D crystallin	truncation 86/87	WL: WS, γ	H	P LC/ESI-MS	Abbasi et al., 1998
				P 2-DE Edman	Srivastava et al., 1998
gamma D crystallin	truncation 172/173	WL: WS, γ	H	P LC/ESI-MS	Abbasi et al., 1998
		C: WS, γ		p LC/ESI-MS/MS	Lapko et al., 2003b
		N: WS, γ		p LC/MALDI-MS	
gamma S crystallin	deamidation N14	N: WS; WI	H*	p LC/ESI-MS/MS	Lapko et al., 2002
gamma S crystallin	deamidation N37	N: WS; WI	H*	p LC/ESI-MS/MS	Lapko et al., 2002
gamma S crystallin	deamidation N53	N: WS; WI	H*	p LC/ESI-MS/MS	Lapko et al., 2002
gamma S crystallin	deamidation N76	WL: WI	H, H*	P LC/ESI-MS	Hanson et al., 2000
		N: WS; WI		p LC/ESI-MS/MS	Lapko et al., 2002
gamma S crystallin	deamidation N143	N: WS; WI	H*	p LC/ESI-MS/MS	Lapko et al., 2002
gamma S crystallin	deamidation Q16	N: WS; WI	H*	p LC/ESI-MS/MS	Lapko et al., 2002
gamma S crystallin	deamidation Q63	N: WS; WI	H*	p LC/ESI-MS/MS	Lapko et al., 2002
gamma S crystallin	deamidation Q70	N: WS; WI	H*	p LC/ESI-MS/MS	Lapko et al., 2002
gamma S crystallin	deamidation Q92	WL: WS, γ; WI	H, H*	P LC/ESI-MS	Hanson et al., 1998, 2000
		N: WS; WI		p LC/ESI-MS	Lapko et al., 2002
				p LC/ESI-MS/MS	
gamma S crystallin	deamidation Q106	N: WS; WI	H*	p LC/ESI-MS/MS	Lapko et al., 2002
gamma S crystallin	deamidation Q120	N: WS; WI	H*	p LC/ESI-MS/MS	Lapko et al., 2002
gamma S crystallin	deamidation Q148	N: WS; WI	H*	p LC/ESI-MS/MS	Lapko et al., 2002
gamma S crystallin	deamidation Q170	WL: WS, γ; WI	H, H*	P LC/ESI-MS	Hanson et al., 1998, 2000
		N: WS; WI		p LC/ESI-MS	Lapko et al., 2002
				p LC/ESI-MS/MS	
gamma S crystallin	disulfide bond C22–24	WL: WS, γ	H	p LC/ESI-MS	Hanson et al., 1998
gamma S crystallin	disulfide bond C114–129	WL: WS, γ	H	p LC/ESI-MS	Hanson et al., 1998
gamma S crystallin	methylation K6	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
gamma S crystallin	oxidation M41	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
gamma S crystallin	oxidation M73	WL: WI	H	P LC/ESI-MS	Hanson et al., 2000
				p LC/ESI-MS/MS	
gamma S crystallin	oxidation M101	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
gamma S crystallin	oxidation M106	WL: WS	H*	p MudPIT/ESI-MS/MS	MacCoss et al., 2002
gamma S crystallin	truncation 61/62	WL: WS, γ; WI	H	P LC/ESI-MS	Abbasi et al., 1998
				p LC/ESI-MS/MS	Hanson et al., 2000
gamma S crystallin	truncation 83/84	WL: WS, γ	H	P 2-DE Edman	Srivastava et al., 1998
gamma S crystallin	truncation 84/85	WL: WS, γ	H	P 2-DE Edman	Srivastava et al., 1998
gamma S crystallin	truncation 86/87	WL: WS, γ	H	P 2-DE Edman	Srivastava et al., 1998
gamma S crystallin	truncation 89/90	WL: WS, γ	H	P LC/ESI-MS	Abbasi et al., 1998
gamma S crystallin	truncation 90/91	WL: WS, γ	H	P LC/ESI-MS	Abbasi et al., 1998
				P 2-DE Edman	Srivastava et al., 1998
gamma S crystallin	truncation 174/175	WL: WS, γ	H	P LC/ESI-MS	Abbasi et al., 1998
gamma S crystallin	truncation 176/177	WL: WS, γ	H	P LC/ESI-MS	Abbasi et al., 1998

[a] Modification sites are listed as modification type followed by the modified amino acid residue in one-letter code and the residue number

[b] Lenticular regions are listed as morphological sections followed by solubility fractions followed by size exclusion chromatography fractions. Abbreviations are: WL, whole lens; C, cortex; N, nucleus; WS, water-soluble fraction; WI, water-insoluble fraction; US, urea-soluble fraction; HMW, high molecular weight fraction; α, alpha fraction; β_H, beta high fraction; β_L, beta low fraction; γ, gamma fraction; mF, monomeric fraction

[c] Species are abbreviated as follows: B, bovine; C, chicken; H, human; HSr, human Soemmerring's ring; H*, human cataract; M, mouse; M*, mouse cataract; R, rat; R+, rat diabetic; R*, rat cataract

[d] Non-standard detection method abbreviations are: P, protein; p peptide; denoting whether the analysis was carried out on the entire protein or on cleaved peptides

David and Shearer, 1993b; Ajaz et al., 1997). It has also been suggested that beta crystallin truncation leads to a tighter molecular arrangement and therefore heightened water exclusion increasing from cortex to nucleus (Werten et al., 1999). Other important modifications are phosphorylation, deamidation, which has been shown to have an impact on molecular structure and subunit assembly (Lampi et al., 2001; Lapko et al., 2002), methylation, oxidation and tryptophan oxidation to kynurenine (Jungblut et al., 1998) as well as covalent kynurenine attachment (Garner et al., 2000). Numerous post-translational modifications have been detected in the healthy lens and are therefore a part of the natural ageing process (Miesbauer et al., 1994). We have attempted a more or less comprehensive list of post-translational modifications detected by proteomics techniques in Table 1. Many of the major post-translational modifications in the table were previously characterized by techniques such as peptide mapping. These citations are not included as we have limited ourselves to proteomics techniques in the actual sense.

Appearance of post-translational crystallin modification is also correlated to the onset of lenticular opacification or cataract. Cataract is the leading cause of blindness worldwide. Twenty million children under the age of sixteen suffer from congenital cataract appearing early in life (Graw, 2003) and four million people are rendered blind yearly in India alone as a result of increasing lenticular opacification (Minassian and Mehra, 1990). It seems the unfolding, misfolding, insolubilization and eventual aggregation of proteins leads to diffraction of incident light classifying cataract as a conformational disease (Harding, 1972; Carrell and Lomas, 1997). Chemical changes in a molecules primary structure alter its conformation. Therefore many of the protein species resulting from post-translational modification of the crystallins can be implicated in cataractogenesis. Indeed, major proteomics studies on cataractous lenses with varying degrees of opacification have identified large numbers of post translationally modified crystallins using 2-DE (Garber et al., 1984; Li et al., 2002; Calvin et al., 1996; Tumminia et al., 2001; David et al., 1994). For a more complete collection of work on cataractous lenses with the full proteomics methods repertoire see Table 1.

Many of the modifications detected in the healthy lens are also inherent to cataractous lenses. This suggests that while in some cases there may be unique modifications that compulsorily lead to cataract, in many cases the onset of lenticular opacification may be a question of degrees. Protein species that are mandatory for establishing a healthy refractive index and lenticular milieu may be deleterious when their abundance exceeds a certain threshold which can no longer be checked by other factors. For an illustration of some of the pathways involved in cataractogenesis see a review by Harding (Harding, 2002).

Conclusion

The eye lens is a unique tissue whose facile simplicity disguises an exquisite dynamics we are beginning to understand. Proteomics methods are ideal for exploring this tissue as it is composed almost exclusively of proteins, the proteins are mainly the result of 10 to 20 primary translation products and the proteins can be fractionated and purified with relative ease allowing the realization of most of the protein species with today's proteomics technology. A possible equilibrium between healthy and disease states and the evident importance of post translationally modified proteins may prove a valuable model for a deeper understanding of biological concepts. The availability of a simple and complete proteome for both congenital and age related cataract can be valuable in the analysis of congenital disease. The large number of cataract patients gives the work immanent clinical relevance.

Note added in proof

Recently a new crystallin, gamma N crystallin was detected. This seems to be the link in beta and gamma crystallin gene evolution (Wistow et al., 2005).

Acknowledgement

This work was funded by Deutsche Forschungsgemeinschaft within SFB 577: Molecular Basis of Clinical Variability in Mendelian Disorders.

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