Susceptibility of Ovine Lens Crystallins to Proteolytic Cleavage during Formation of Hereditary Cataract

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Purpose. To produce two-dimensional electrophoresis (2-DE) maps for ovine crystallins and examine changes in ovine crystallins during cataract formation.

Methods. Soluble and insoluble fractions were isolated from normal, whole lenses of 26-week-old sheep, the proteins separated by 2-DE, and the spots digested with trypsin and subjected to tandem mass spectral analysis. Spot identifications were made by using mass spectrometry data from each spot digestion and data from 2-DE maps of proteins from soluble and insoluble cortices of 10-month-old ovine lens. Ovine αA-, αB-, and βB3-crystallin cDNAs were sequenced, whereas other ovine crystallins were identified by using bovine sequences. Proteins were then isolated from whole lenses of 26-week-old lambs with mature hereditary cataracts, and the changes in the crystallins were determined by 2-DE. The masses of truncated crystallins were determined after elution from 2-DE gels.

Results. The ovine lens contained the normal complement of crystallins and, similar to other mammalian lenses, underwent partial proteolysis of βB1-, βA3-, and βB3-crystallin during maturation. Cataract development was associated with enhanced truncation of α- and β-crystallins. C-terminal truncations of αA- and αB-crystallin and N-terminal truncation of βB2-crystallin were observed as well as a loss of γ-crystallin.

Conclusions. These data provide the first 2-DE gel maps for ovine lens crystallins and indicated that ovine lens crystallins are truncated during lens maturation. The differences in proteolysis appearing in normal and cataractous lenses suggested that calpain isoforms may be differentially activated during lens maturation and cataract. The ovine hereditary cataract is a useful nonrodent model to study the role of calpain proteolysis in cataract formation. (*Invest Ophthalmol Vis Sci.* 2008;49: 1016–1022) DOI:10.1167/iovs.07-0792

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Development of human cataracts may be caused by posttranslational modifications of lens crystallin proteins.[1] Major modifications detected in human lenses include deamidation, oxidation of methionine and tryptophan, backbone cleavage, disulfide bonding, methylation of cysteines, and phosphorylation.[2–4] Such posttranslational changes in crystallins may lead to protein unfolding and exposure of hydrophobic regions, allowing crystallins to aggregate and form opacities.[1,2] For example, deamidation of βB1 decreased the stability of the protein in urea and altered its associative properties.[5]

Animal models are frequently used to understand the effects of specific posttranslational modifications on cataract formation. For example, the selenite cataract in rats demonstrated that rapid partial proteolysis of crystallins by the calcium-activated calpains, Lp82 and calpain II, leads to precipitation of truncated crystallins.[6–8] However, no evidence of increased activation of calpains in nonrodent models of cataract has been published. Calpains have been purified from rat, human, and bovine lenses, and their ability to degrade crystallins partially in vitro is well documented.[9–13] Since an in vivo model of bovine cataractogenesis is not available, the hereditary ovine cataract is an alternative model to test the hypothesis that calpains are activated in nonrodent species during cataract formation. Recent data have shown that ovine lenses contains calpain I, calpain II, and Lp82.[14]

The hereditary ovine cataract occurs spontaneously in Romney sheep and is inherited in dominant fashion, affecting approximately 40% of the offspring.[14] The early stages of the cataract are localized in the cortical region, but eventually affect the entire lens in the mature stage, which usually occurs before 10 months of age.[14] Although the genetic defect causing this hereditary cataract remains unknown, studies have implicated calcium-induced proteolysis as a possible contributor to opacification. Calcium levels were markedly elevated in lenses undergoing opacification, and significant proteolysis was observed in α- and β-crystallins, as well as the cytoskeletal proteins spectrin and vimentin, known substrates for calpains.[14]

Since the earlier studies of ovine hereditary cataract examined only proteolytic changes by using single dimension SDS-PAGE, a more detailed study with two-dimensional electrophoresis was performed to define which crystallins are being degraded and which possible proteases are activated. The present study compares two-dimensional gel electrophoresis maps for ovine crystallins of both normal lenses and lenses with hereditary cataracts.

Materials and Methods

Ovine Lens Isolation and Preparation

Normal and cataractous eyes from Coopworth lambs at Lincoln University, New Zealand (Lincoln University ethics approved protocol LU 15/01, which complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research) were examined by slit lamp biomicroscopy. Lenses were selected from 12 26-week-old animals: 6 with mature hereditary cataracts and 6 age-matched control

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subjects. The lenses were obtained immediately after death by excision from a posterior approach, and the lens weight was recorded. Each whole lens was then homogenized in 2.0 mL buffer containing 20 mM sodium phosphate (pH 7), 1 mM EGTA, and one tablet of freshly dissolved protease inhibitor cocktail (Minicomplete; Roche, Mannheim, Germany) per 10 mL buffer. For construction of a reference crystallin, two-dimensional electrophoresis (2-DE) map, lenses were also obtained from normal 10 month-old lambs (Pel-Freez Biologicals, Rogers, AR). The lenses were dissected into cortex and nuclear fractions of approximately equal size and each fraction homogenized as just described. Lens homogenates were centrifuged for 1 hour at 48,500*g* at 4°C, and the water soluble lens proteins in the supernatants were collected. The water insoluble pellets were washed twice in dissection buffer with no protease inhibitors to remove residual soluble proteins, and then were resuspended in 500 μL of the homogenizing buffer by brief sonication (20 seconds burst) on ice. Protein concentrations were measured by protein assay (BCA; Pierce, Rockford, IL) using BSA as a standard. Samples were then stored at −20°C until use for electrophoresis.

Two-Dimensional Electrophoresis

Proteins from each experimental group were separated by 2-DE with immobilized pH gradient (IPG) gel strips (18 cm, pH 5–9) in the first dimension, by application of 400 μg protein in 400 μL rehydration buffer containing 8 M urea, 2% CHAPS, 50 mM DTT, 2% IPG buffer (pH 6–11; GE Healthcare, Piscataway, NJ), 2% glycerol, and 0.001% bromophenol blue per strip.[15] Focusing was then performed (with a Multiphor II horizontal electrophoresis apparatus with an Immobiline Drystrip Kit; GE Healthcare, or in a Protean IEF cell; Bio-Rad, Hercules, CA). Focusing began with a 500- to 3500-volt linear gradient over 1.5 hours and continued for 20 hours at 3500 volts. Focused IPG strips were frozen at −70°C before second-dimension separation.

Second-dimension, molecular weight separation of the proteins on the IPG strips was completed by using 23 × 20 cm 12% SDS PAGE gels in an electrophoresis apparatus (IsoDalt; GE Healthcare) with a monomer (Duracryl; Proteomics Research Services, Inc., Ann Arbor, MI). IPG strips containing lens proteins were reduced and alkylated as previously described[15] and then fixed to SDS PAGE gel slabs with 25 mM Tris base, 192 mM glycine, 1% SDS, and 1% low-melt agarose. The second-dimension gels were run for 2 hours at 25 volts, then overnight at 100 volts, for a total of 1750 volt × hours. The gels were stained with Coomassie G250[16] and imaged at a resolution of 100 pixels per inch with a flat-bed scanner with transparency lid (Expression 1600; Epson, Long Beach, CA) and saved as a 12-bit TIFF file.

Identification of Lens Proteins Isolated from 2-DE Gels of Ovine Lens Cortex

Coomassie-stained proteins were excised from 2-DE gels and in-gel digested with trypsin, as previously described.[17] Tryptic peptides extracted from the gel pieces were then identified by tandem mass spectrometry using an ion trap mass spectrometer (LCQ Classic; Thermo Scientific, San Jose, CA). Peptides were either separated by reversed-phase chromatography and ionized by electrospray as previously described[17] or were spotted onto metal plates and ionized by atmospheric pressure matrix-assisted laser desorption (MALDI), as previously described,[18] except samples were solid-phase extracted in preparation for MALDI (ZipTip μ-C18 pipette tips; Millipore, Bedford, MA). Automated analysis of tandem MS spectra was performed with software (Sequest; ver. 2.7 rev. 12; Thermo Scientific) that correlates experimental tandem MS spectra with theoretical tandem MS spectra calculated from protein sequences in databases.[19] Since the sequences of all ovine crystallins are unknown, except for the sequences of αA, αB, and βB3 reported in the following description, ovine β- and γ-crystallins were identified based on sequence homology to the known sequences of bovine crystallins. The searches used a subset database of bovine and ovine proteins created from the Sprot and TrEMBL databases (Uniprot release 10.1, download date, March 27, 2007)[20] containing 13,773 entries, supplemented with the ovine α-crystallin and βB3 sequences reported later (the corresponding three bovine sequences were removed). A sequence-reversed database was created and appended to the normal sequences to allow estimation of protein false-positive identifications. The LC/MS protein identification criteria were a minimum of two fully tryptic peptides from each in-gel digest with Xcorr values greater than 1.8, 2.5, and 3.5 for singly, doubly, and triply charged peptides, respectively. For MALDI data for which only 10 MS/MS spectra were collected, two singly charged peptides with Xcorr values greater than 1.0 were required. Peptides identified in each digest are summarized in Supplementary Table S1, online at http://www.iovs.org/cgi/content/full/49/3/1016/DC1. Protein identifications were used to label the spots on the 2-DE gels for Figures 1 and 2 in the body of this article.

Determination of Ovine αA-, αB-, and βB3-Crystallin cDNA Sequences

Given that ovine α-crystallins were subject to proteolysis in cataract, cDNAs for both proteins were sequenced. cDNA for βB3 was also sequenced to confirm the identity of a major truncated species seen in both normal and cataractous lenses. Total RNA was isolated from lens epithelia of two normal lambs by using an animal tissue protocol (RNeasy kit; Qiagen, Valencia, CA) and the mortar and pestle with needle and syringe homogenization method. Based on the cDNA for bovine αA-, αB-, and βB3-crystallin (GenBank accession numbers NM_174289, NM_174290, and AF013259; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD), the PCR primers used to amplify full-length ovine αA and αB-crystallin cDNA were: αA upstream ^{1}ccg ggt gcc cac aga gcc^{18}, αA downstream ^{702}gct act ctc tca aac cct ca^{683}; αB upstream (human NM_001885) ^{9}aca ctc acc tag cca cca tg^{27}, αB downstream ^{547}tgg ttt aga gaa agg gca tct a^{526}; and βB3 upstream ^{25}cct ctc tcc tca cgg cga c^{43}, βB3 downstream ^{765}gcc tcc tct tct tgc ctt tgc^{745}. One microliter of total RNA was reverse transcribed with oligo $(dT)_{12\text{-}18}$ primer (Invitrogen, Carlsbad, CA). Reverse transcription (RT) was performed for 50 minutes at 42°C, and the mixture was then heated to 70°C for 15 minutes to inactivate the RT enzyme (SuperScript II; Invitrogen). One microliter of RT reaction mixture was subsequently transferred to a PCR tube containing 0.2 mM dNTP mix, 0.04 U/μL *Taq* DNA polymerase, 1× PCR buffer, and 0.2 μM of each upstream and downstream primer in a 50-μL reaction. The PCR cycling profile was as follows: 94°C for 2 minutes initial denaturation, then 30 cycles of denaturation at 94°C for 30 seconds; annealing at 66°C (αA), 54°C (αB), and 64°C (βB3) for 30 seconds; and extension at 72°C for 1 minute. A final extension of 72°C for 10 minutes was performed immediately after the 30-cycle profile. Ten microliters of PCR product was electrophoresed on a 1.2% agarose gel containing ethidium bromide for band staining.

PCR products with expected sizes of 701 (αA), 561 (αB), and 741 (βB3) bp were ligated directly into a vector (PGEM-T Easy Vector System I; Promega, Madison, WI) overnight at 4°C. JM109 competent cells were transformed and colonies positive for the insert were selected, and the DNA was purified (Qiaprep spin miniprep kit; Qiagen) and sequenced. Ovine αA- and αB-crystallin cDNAs (GenBank accession numbers AY819022 and AY819023, respectively) encoded proteins 173 and 175 amino acid residues in length, respectively, whereas ovine βB3-crystallin (GenBank accession AY995197) was a 210-amino-acid protein.

Analysis of Crystallin Masses from Normal and Cataractous Lenses

The masses of intact and partially truncated crystallins were determined from proteins eluted by passive diffusion from duplicate zinc-stained 2-DE gels and protein masses determined by ESIMS (electrospray ionization mass spectrometry), as previously described.[8] The number of amino acid residues missing from the C or N terminus of

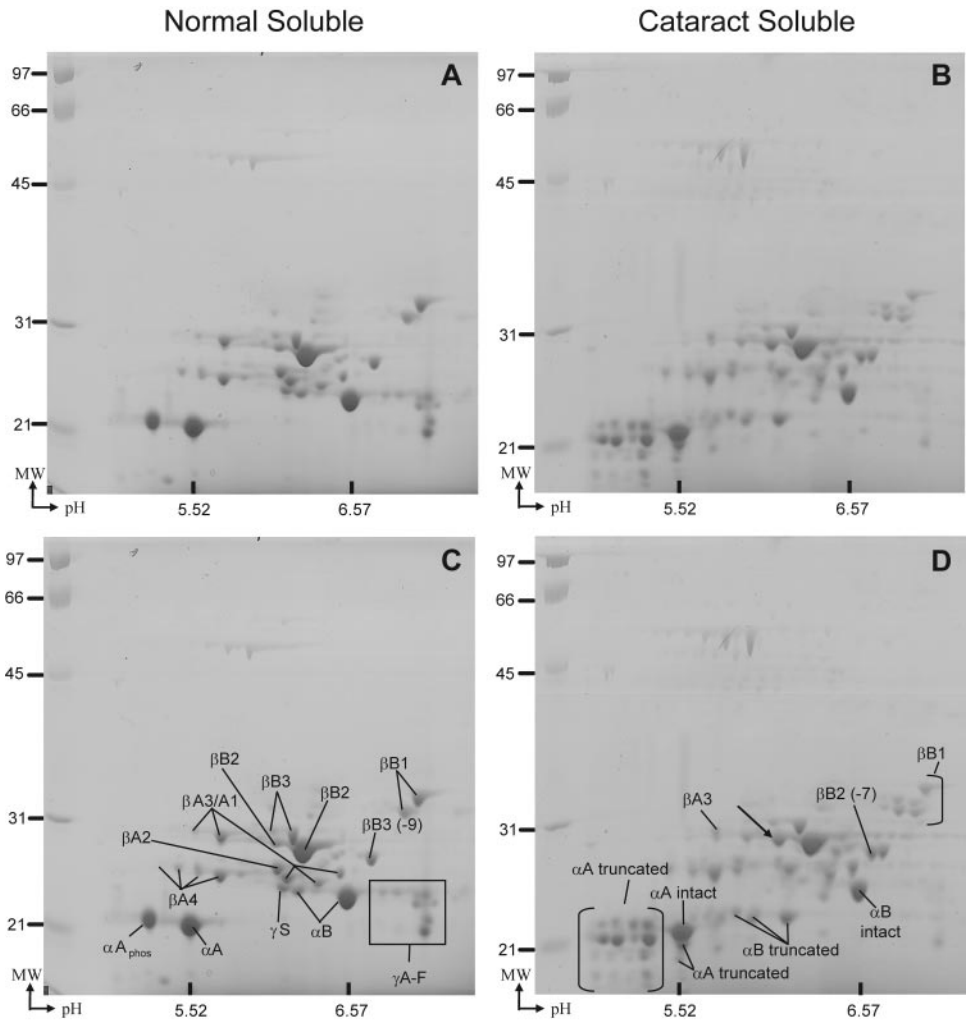

FIGURE 1. (**A**) 2-DE of soluble proteins from the lens of a normal 26-week-old lamb. Protein identification of each gel spot was determined by tandem mass spectrometry (data shown in Supplementary Fig. S1, http://www.iovs.org/cgi/content/full/49/3/1016/DC1). Upper and lower panels are duplicated so the images are not obscured by the labels. (**B**) 2-DE of soluble proteins from the lens of a 26-week-old lamb with cataract. (**C**) Spots identified by MS are marked in the normal soluble fraction. The spot marked βB1(−9) was a truncated form of βB3-crystallin missing 9 amino acids from its N-terminus. (**D**) The positions of intact αA- and αB-crystallin are labeled in the soluble cataract fraction. The positions of the truncated form of βB2-crystallin missing seven residues from its N-terminus, and truncated αA-, αB-, and βB1-crystallin are also marked. *Arrow*: position of an acidic form of βB2-crystallin increased in cataractous lenses. The pH range of the gels are indicated using the calculated pIs of αA- and αB-crystallins.

partially degraded crystallins was inferred by comparison of the measured mass to the mass calculated from protein sequences (PAWS program; Genomic Solutions, Ann Arbor, MI).

RESULTS

Cataract Formation and Ovine Lens Properties

On dissection, the normal ovine lens on average weighed 0.79 ± 0.13 g (SD; $n = 6$) and was perfectly transparent with a biconvex circular shape. In contrast, the 26-week-old cataract lens weighed an average of only 0.31 ± 0.11 g ($n = 6$; $P < 0.01$), had a fragile opaque lens capsule, and often contained a solid nuclear opaque mass and liquefied cortex. The fragile nature of the lens capsule made dissection difficult, and pipetting of lens remnants was necessary to collect all cataractous tissue. Lens-insoluble protein content also rose significantly with cataract formation. Normal 26-week-old cataract lenses contained an average 12.2 ± 1.9 mg (SD) insoluble protein/g lens wet weight ($n = 6$), and this content increased in cataractous lens to 40.3 ± 6.4 mg/g wet weight ($n = 6$; $P < 0.01$).

2-DE Map of Sheep Lens Proteins

A 2-DE reference map of the water-soluble and -insoluble cortex fractions of a 10-month normal ovine lens cortex illustrated the normal complement of crystallins found in other mammals (Supplementary Table S1, http://www.iovs.org/cgi/content/full/49/3/1016/DC1). In 26-week-old normal whole lenses α- and β-crystallins were present in high abundance (Figs. 1C, 2C). Spots yielded peptides matching unique sequences found in γS, whereas it was difficult to identify γA-F crystallins due to either low abundance or the lack of peptides matching unique sequences in bovine γ-crystallins. Ovine lenses contained a high abundance of phosphorylated αA crystallin, comprising nearly 50% of the total αA. Similar to bovine lens,[21] phosphorylation of ovine αA-crystallin was observed at serine residue 122 during analysis of the digest of this phosphorylated αA spot by tandem MS (data not shown). Two acidic αB spots were also observed that were presumedly due to phosphorylation of αB; however, phosphorylated peptides were not recovered from the digests to confirm this assignment. Similar to bovine lens,[22] ovine lens βB1, βB3, and βA3 underwent partial truncation and were found in positions similar to those of the N-terminally truncated forms of these proteins in bovine lens (David L, unpublished results, 2008). The partial truncation of these crystallins was also more pronounced in the water-insoluble fraction, in which the lower-molecular-weight form of βB3-crystallin was a major component, and a second more acidic and lower-molecular-weight form of βB1-crystallin was in nearly equal abundance to intact βB1-crystallin. In addition to these differences, the water-insoluble fraction contained a markedly lower abundance of βB2-crystallin, as well as a higher abundance of the cytoskeletal and intermediate filament proteins actin, vimentin, filensin, and CP49.

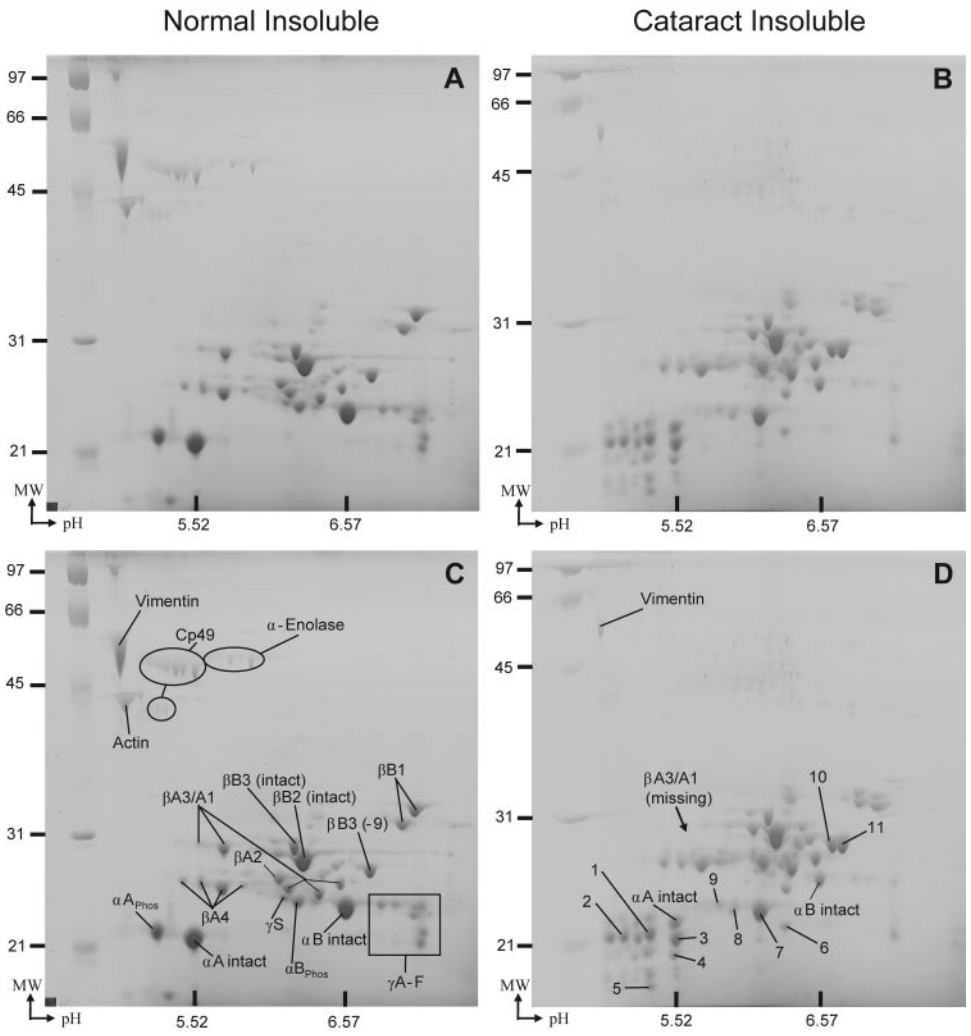

FIGURE 2. (**A**) 2-DE of insoluble proteins from the lens of a normal 26-week-old lamb. (**B**) 2-DE of insoluble proteins from the lens of a 26-week-old lamb with cataract. (**C**) 2-DE gel spots identified by mass spectrometry in the normal insoluble lens fraction. (**D**) Crystallin degradation specific to the insoluble cataract fraction is depicted by numbered gel spots. The identity of these spots and their specific modification are located in Table 1. The pH range of the gel is indicated using the calculated pI of αA- and αB-crystallin.

Changes in Sheep Lens Proteins during Cataract Formation

Cataracts were produced by mating cataract-affected heterozygous rams with normal-eyed ewes to produce offspring that developed mature cataracts by 26 weeks of age.[14] Comparison of the 2-DE gels of both soluble and insoluble proteins from the whole lenses of normal sheep and those with mature cataracts showed pronounced differences in crystallin migration (Figs. 1, 2): (1) The cataractous lenses showed several distinct truncated α-crystallin spots in contrast to the predominant intact and phosphorylated spots in the normal lenses; (2) βB2-crystallin underwent truncation and migrated next to a truncated form of βB3 that was also present in the normal lens; (3) an acidic form of βB2 became more abundant (Fig. 1D, arrow); (4) several lower-molecular-weight spots for βB1 appeared, suggesting more extensive proteolysis; (5) βA3-crystallin was decreased; and (6) cataractous lenses were void of most γ-crystallins compared with the age-matched control lenses.

Many of the changes just mentioned were also more pronounced in the water-insoluble fraction (compare Figs. 1, 2). The truncation of αA- and αB-crystallins was more extensive in the insoluble fraction of cataractous lenses than in the soluble fraction, since the spots corresponding to intact αA- and αB-crystallins became minor components (Fig. 2D). The intact forms of βB1- and βA3-crystallins were also absent from the insoluble fraction of cataractous lenses. In addition to the proteolysis of crystallins in the water-insoluble fraction, the cytoskeletal proteins abundant in the normal lens were all but lost in the mature cataract (Figs. 2C, 2D).

Masses of Truncated Forms of Crystallins in Cataractous Lenses

The whole mass measurements (detailed below) indicated that initial methionines were retained on α-crystallins, the initial methionine was absent in βB3, and all three proteins were N-terminally acetylated. The proteins from several intact and truncated αA-, αB-, βB2-, and βB3-crystallin spots (Figs. 2C, 2D) were eluted from the gels and whole protein masses measured using electrospray mass spectrometry. The masses of intact αA- and αB-crystallins (Table 1; Figs. 3A, 3B) were nearly identical with the masses predicted from the cDNA sequences (see the Methods section). Furthermore, the mass of phosphorylated αA was 80 mass units higher than unmodified αA, confirming that the full-length protein was phosphorylated. The whole-mass measurements of the truncated α-crystallin spots from cataractous lenses observed in Figure 3D were in agreement with the loss of various numbers of C-terminal amino acids (Table 1). αA-crystallin was truncated into two major products, one missing 5 residues and the other missing 11. The 2-DE protein spot for the $\alpha A_{1\text{-}168}$ species was more abundant than the $\alpha A_{1\text{-}162}$ species (spots 3 and 4, respectively; Fig. 2D). Additional identically truncated species of αA were also observed (Fig. 3D, spots 1 and 2), but were the result of trunca-

TABLE 1. Whole-Mass Measurements Showing αA- and αB-Crystallin Modifications in the Normal and Cataractous Insoluble Lens Fractions

Spot (Figs. 3C, 3D)	Crystallin	Mass	Predicted Mass with Modification*	Modification
αA†	$\alpha A_{(1-173)}$	19,876.9	19,875.1	Intact
αA_{phos}†	αA_{phos}	19,955.3	19,955.1	Intact+phos
1	$\alpha A_{(1-168)}$	19,447.0	19,445.7	−5 aa
2‡	$\alpha A_{(1-168)}$	19,448.6	19,445.7	−5 aa
	$\alpha A_{phos(1-168)}$	19,527.5	19,525.7	−5 aa+phos
3	$\alpha A_{(1-168)}$	19,445.9	19,445.7	−5 aa
4	$\alpha A_{(1-162)}$	18,719.9	18,718.9	−11 aa
5	$\alpha A_{(1-151)}$	17,613.5	17,613.7	−22 aa
αB†	$\alpha B_{(1-175)}$	20,109.5	20,108.8	Intact
6	$\alpha B_{(1-163)}$	18,858.9	18,858.3	−12 aa
7	$\alpha B_{(1-170)}$	19,614.0	19,613.1	−5 aa
8	$\alpha B_{(1-170)}$	19,613.0	19,613.1	−5 aa
9	$\alpha B_{phos(1-170)}$	19,693.0	19,693.1	−5 aa+phos
βB2§	$\beta B2_{(1-204)}$	23,321.8	23,322.7‖	Intact
10	$\beta B2_{(8-204)}$	22,513.8	22,512.9‖	−7 aa
βB3§	$\beta B3_{(2-210)}$	24,322.7	24,324.0	Intact
11§	$\beta B3_{(10-210)}$	23,302.7	23,302.9	−9 aa

aa, amino acid; phos, phosphorylated form of protein.

* Predicted masses according to the ovine crystallin sequence, except where indicated.

† Whole masses reported were from spots cut from normal insoluble gels.

‡ Gel spot contained two protein species.

§ Whole mass measured from 2-DE gels of purified β-crystallin from ovine lens.

‖ Predicted masses from bovine βB2 sequence.

tion of phosphorylated and deamidated species. Phosphorylation was indicated by an 80-mass-unit increase in the truncated species, and deamidation was the only modification that could increase the acidity of the truncated species, but not appreciably alter their observed masses (Table 1). Truncated species of αB-crystallin were also observed in cataractous lenses that were missing either 5 or 12 residues from their C terminus (Table 1), with the form missing 5 residues being the predominant species (Fig. 2D, spot 7). Similar to αA-crystallin, additional species of αB-crystallin that were either deamidated or phosphorylated were also observed (Table 1).

Masses for intact and truncated forms of βB2 and βB3 were similarly determined. The measured mass of intact ovine βB3-crystallin (Table 1) was similar to the mass predicted from the c-DNA sequence (the Methods section). While its c-DNA sequence is still unknown, ovine βB2-crystallin had a mass that was less then 1 mass unit from the calculated mass of bovine βB2-crystallin, suggesting that the sequences of βB2-crystallins are identical in the two species. The masses of the truncated βB3-crystallin species found in both normal and cataractous lenses suggested that they were missing 9 residues from their N terminus, whereas the truncated species of βB2-crystallin unique to cataractous lenses had a mass consistent with a species missing 7 residues from its N terminus (Table 1, Figs. 3C, 3D).

DISCUSSION

This is the first 2-DE study to identify crystallin species and their modifications in the ovine lens. The ovine lens contained a similar array of crystallins found in other mammalian lenses.[15,22,23] αA-, αB-, and βB3-crystallin sequences for the ovine lens were determined and used to identify several posttranslational modifications associated with cataract formation and maturation.

Modifications of the crystallins may disrupt their normal structure and function in the lens and lead to improper interactions and light scatter. Ovine lens crystallins underwent numerous maturationally related truncations as seen in cows,[22] rats,[24] mice,[25] and humans.[26] Most notably, truncated species of βB1, βA3, and βB3 were observed in normal ovine lenses. Additional posttranslational modifications observed in normal ovine lenses included phosphorylation of αA- and αB-crystallin. The phosphorylation in ovine αA-crystallin was also localized to serine residue 122, similar to αA-crystallin from other species.[21,27,28]

Formation of the hereditary cataract increased the extent of proteolysis in ovine lens. Cataract specific forms of αA-, αB-, and βB2-crystallin were observed to lack from 5 to 22 residues in their termini. Truncated forms of αA- and αB-crystallin were also more abundant in the water-insoluble fraction, suggesting that their truncation may contribute to the formation of insoluble protein in cataractous lenses. Loss of 11 or more C-terminal residues in αA-crystallin dramatically decreases its chaperone activity[29] and may lead to the observed precipitation of truncated α-crystallins in cataractous ovine lens. The preferential loss of the phosphorylated form of αA-crystallin in the soluble fraction of the cataractous lenses compared with the unmodified form of αA also suggested that phosphorylation may render the protein more susceptible to proteolysis.

The specificity of the observed cleavages in α- and β-crystallins and the previously measured elevation in lenticular calcium in cataractous ovine lenses[14] suggests that the calcium-dependent proteases calpains were activated during cataract formation. Rat and bovine αA-crystallins were both preferentially cleaved by Lp82 to produce αA_{1-168}, whereas calpain II preferentially produced αA_{1-162}. This suggested that Lp82 may be responsible for the observed proteolysis of crystallins and cytoskeletal proteins in cataractous ovine lenses. Although the cleavage site specificity of ovine Lp82 for βB2 and βB3 have not been determined, rat βB2 and βB3 are cleaved by rat calpain II to produce species missing 7 and 9 residues from their N termini, respectively,[12] and ovine Lp82 could produce the same species. However, the appearance of $\beta B3_{10-210}$ during maturation, and the appearance of $\beta B2_{8-204}$ and truncated α-crystallins in only cataractous lenses suggest that different proteolytic activities are induced during ovine lens maturation and cataract formation. By zymography, calpain II is clearly the

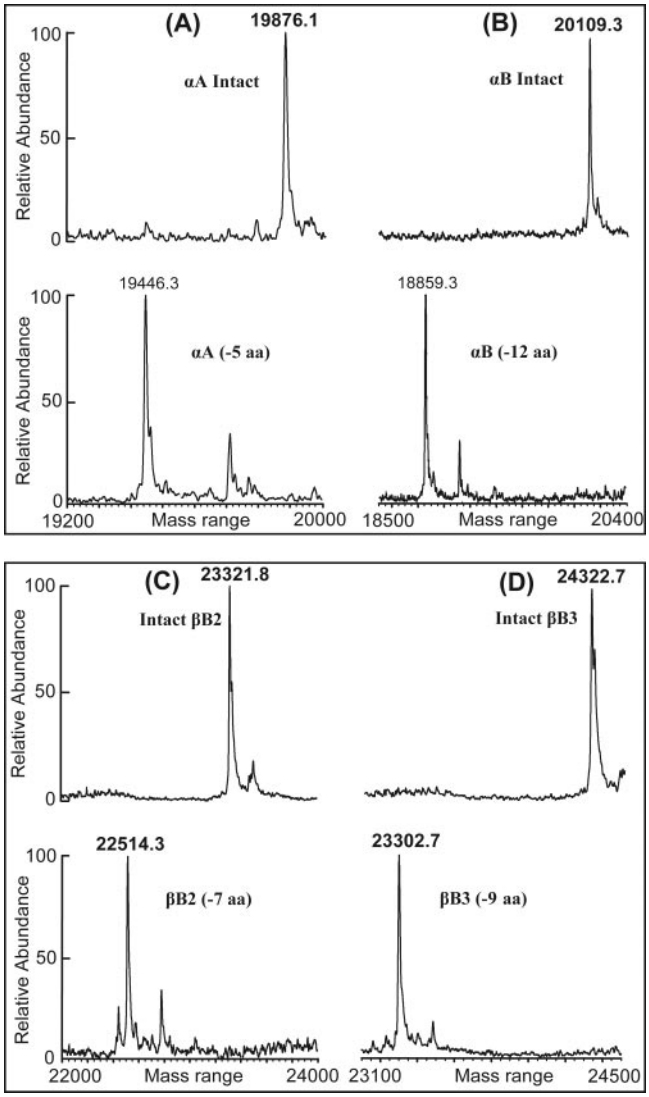

FIGURE 3. (**A**) Deconvoluted whole-mass measurements of intact αA crystallin (Fig. 2D) and αA-crystallin missing five amino acid residues from the C terminus (Fig. 2D, spot 1). (**B**) Deconvoluted whole-mass measurements of intact αB crystallin (Fig. 2D) and αB-crystallin missing 12 amino acid residues from the C terminus (Fig. 2D, spot 6). (**C**) Deconvoluted whole-mass measurements of intact βB2 crystallin (Fig. 2C) and βB2-crystallin missing seven amino acid residues from the N terminus (Fig. 2D, spot 10). (**D**) Deconvoluted whole-mass measurements of intact βB3 crystallin (Fig. 2C) and βB3-crystallin missing nine amino acid residues from the N terminus (Fig. 2D, spot 11).

most active isoform in the ovine lens.[14] However, Lp82 may still cause most of the proteolysis observed during cataract formation, because unlike calpain II, Lp82 in the bovine lens was not inhibited by the endogenous inhibitor calpastatin, and it was less susceptible to autolytic inactivation than was calpain II.[9] Additional experiments should test the substrate and site specificity of calpain isoforms in ovine lenses after their purification, to determine which are differentially activated during maturation and cataract.

The loss of γ-crystallins from ovine lenses during cataract formation could have been due to either proteolysis or leakage from lenses. Recent data from knockout mice missing lens Lp82 suggest that γ-crystallins can act as Lp82 substrates.[30] The reported low-molecular-weight fragments would have been lost from the 12% polyacrylamide gels used in the present study. Alternatively, the γ-crystallins may be leached from the lens during cataract formation because the membrane and cytoskeletal proteins are degraded and the ovine lens essentially liquefies with mature cataract. Also, the γ-crystallins are monomers and are relatively small compared with the α- and β-crystallins. During cortical cataracts in human subjects the concentration of γ-crystallin in the aqueous humor increases significantly compared with that in subjects with normal lenses.[31] Protein analysis of aqueous humor and possibly vitreous humor could test this hypothesis.

The loss of the cytoskeletal proteins in the cataract insoluble fraction of the lens could be due to a combination of increased precipitation of crystallins and increased proteolysis of cytoskeletal proteins during cataract formation. Cataractous lenses contained larger quantities of insoluble crystallins, and these may have decreased the relative abundance of cytoskeletal proteins on 2-DE gels. However, we have also observed proteolysis of the cytoskeletal protein vimentin in the insoluble fraction of ovine lens epithelium during cataract formation in Western blot analysis.[14] Thus, the loss of cytoskeletal proteins in the insoluble fraction during cataractogenesis could be a due to a combination of increased crystallin precipitation and protein breakdown.

In conclusion, our data provide the first 2-DE maps of ovine lens proteins and support the role of calpain activation in sheep cataracts. These data are important, because they provide the first evidence that calpains are activated during experimental cataract formation in a nonrodent species. Elucidation of the remaining ovine crystallin cDNA sequences is needed to confirm truncation sites in βB1-, βB2-, and βA3-crystallins. Further work is also needed to determine which calpain isoforms are activated in ovine lens during maturation and cataract and to determine which gene defect initiates the loss of calcium homeostasis in this cataract model.

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