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Cleavage of β-Crystallins During Maturation of Bovine Lens

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Purpose: (1) Identify major crystallin proteins in fetal and adult bovine lens, (2) examine the N-termini of β-crystallins for truncation, and (3) determine if the protease m-calpain (EC 3.4.22.17) is responsible for the cleavage of bovine β-crystallins during maturation.

Methods: Crystallins from fetal and adult bovine lenses were analyzed by one and two-dimensional electrophoresis and Edman sequencing of separated proteins and their tryptic fragments. Identical techniques were used to analyze crystallins following their incubation with purified m-calpain.

Results: The identities of the major crystallins and several additional crystallin species missing portions of their N-terminal extensions were identified in the fetal bovine lens. Besides the previously identified form of βB1 missing 15 residues from its N-terminus, forms of βA3 missing 11 and 22 residues were identified. With aging, the βA3 (-22) species became a major protein in the adult bovine lens, and minor forms of βB2 and βB3 missing 8 and 22 residues from their N-termini, respectively, appeared. Purified m-calpain cleaved within the N-terminal extensions of bovine β-crystallins and removed: 12 or 15 residues from βB1; 8 residues from βB2; 5 or 10 residues from βB3; and 11 or 17 residues from βA3.

Conclusions: Based on the cleavage sites in vitro, m-calpain may be partially responsible for cleavage of bovine βB1, βB2, and βA3 during lens maturation. However, the preference of m-calpain to remove 12 residues from βB1, and 11 and 17 residues from βA3, suggested that the βB1 (-15) and βA3 (-22) species found in vivo were produced by a different protease. This identified protease may have a preference for the asparagine-proline-X-proline sequence found in the N-terminal extensions of βB1 and βA3.

Bovine crystallins were some of the very first lens proteins whose sequences were determined [1]. As a result of these early studies, the modern nomenclature of the α, β, and γ-crystallin subunits were partially defined based on the isoelectric points of these proteins. Each β–crystallin subunit was named according to the order that individual β–crystallin subunits emerged during ion exchange chromatography and their relative positions following isoelectric focusing [1]. Therefore, unlike the orthologous β–crystallins found in other species, there was never a need to identify the major bovine β–crystallin subunits on two-dimensional electrophoresis (2-DE) gels. Their identities were explicitly defined by their positions. However, many of the less abundant crystallin species observed on 2-DE gels of bovine lens crystallins remained unidentified due to post-translational modifications which altered both their relative molecular weights and isoelectric points [1]. The recent analysis of 2-DE separated crystallins from both human [2] and rat [3] lenses suggested that many of the post-translationally modified proteins in bovine lens could be β–crystallins missing portions of their N-terminal extensions.

The loss of β–crystallin N-terminal extensions is important because it may significantly alter crystallin interactions following lens maturation [3]. When the loss of N-terminal extensions is accelerated, increased light scatter occurs in experimental rodent models of cataracts [4]. The protease(s) responsible for the degradation of N-terminal extensions on β–crystallin may differ between species. Results suggest the loss of β–crystallin N-terminal extensions in rodent lenses is caused by the calcium-dependent protease m-calpain (also termed calpain II, EC 3.4.22.17) [3,5]. In contrast, the loss of β–crystallin N-terminal extensions in human lenses may result from activation of an as yet unidentified protease(s) with a cleavage site specificity unlike that of m-calpain. These unidentified human lens protease activities may specifically recognize and cut between asparagine and proline within the asparagine-proline-X-proline (NPXP) sequence found in both human βB1 and βA3/A1 N-terminal extensions [2].

The purpose of this study was to first establish if β–crystallin N-terminal extensions in bovine lens also undergo truncation during maturation, and then to determine if the cleavage sites in these crystallins were consistent with the activation of m-calpain, as found in rat lens, or activation of the putative NPXP recognizing protease, as observed in human lens. We found that βB1, βB2, βB3, and βA3/A1 all undergo partial cleavage in bovine lens, and that the observed cleavage sites in these proteins were consistent with the activation of both proteolytic activities. These data also demonstrate that the proteases responsible for loss of N-terminal extensions on β–crystallins during lens maturation may differ between species. Maturationally related proteolysis in bovine lenses shares similarities to both rodent and human lenses.

METHODS

Lenses were obtained by a posterior approach from fetal and adult bovine eyes (Ferry Brothers, Portland, OR) and homogenized in 20 mM imidazole (pH 6.8), 50 μM EGTA, 2

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mM dithioerythritol, and 0.02% NaN_3. Soluble and insoluble proteins were obtained by centrifugation at 16,000 x g for 5 min and assayed for protein using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) with bovine serum albumin as a standard. Lens proteins were separated by 2-DE using non-equilibrium pH gradient electrophoresis in the first dimension with pH 3.5-10 ampholytes, electroblotted onto polyvinylidene difluoride (PVDF) membranes, and subjected to direct N-terminal Edman sequence analysis as previously described [4]. Proteins containing blocked N-termini were identified by digestion of the 2-DE separated proteins on the surface of PVDF membranes with trypsin, separation of the resulting peptides by HPLC, and Edman sequencing of individual tryptic fragments [2,6]. It was not possible to calculate the pIs of the separated proteins, because they do not reach their isoelectric points in this non-equilibrium procedure. Equilibrium isoelectric focusing of crystallins using free carrier ampholytes is not advised, since the most basic crystallins are lost from the gel. The approximate molecular weights and pIs of the displayed regions of the gels containing the crystallins were determined by calculating the theoretical molecular weights and pIs of βB1 (the highest molecular weight crystallin subunit with one of the most basic pIs) and αA-crystallin (the lowest molecular weight crystallin subunit with one of the most acidic pIs). Molecular weights and pIs of the N-acetylated forms of these proteins were calculated using the programs PAWS (Protein Analysis Worksheet version 8.1.1, by Dr. Ronald Beavis), and GeneWorks 2.5 (Oxford Molecular Group, Inc., Beaverton, OR), respectively.

The m-calpain cleavage sites within the N-terminal extensions of bovine β–crystallins were determined by incubating soluble protein from fetal lenses with purified porcine heart m-calpain [7]. The use of porcine heart m-calpain was justified, since previous studies indicated that m-calpain isolated from different mammalian species and tissues exhibited similar cleavage site specificity [4,6]. The relative susceptibility of the various crystallin subunits to m-calpain digestion were estimated by incubating crystallins for a constant amount of time with increasingly higher concentrations of m-calpain. This resulted in more linear loss of substrate than did incubation with a constant amount of m-calpain for increasing lengths of time. This observation was likely due to autolytic inactivation of m-calpain in the presence of calcium [8]. The 0.05 ml m-calpain digestion mixture contained 250 µg of soluble protein from fetal bovine lens, 20 mM Tris (pH 7.5), 1.0 mM dithioerythritol, 1.25 mM $CaCl_2$, and a range of 0-1.25 units of m-calpain. One unit of m-calpain activity was defined as the amount of m-calpain producing 1 µg acid soluble fluorescein isothiocyanate labeled casein fragments per min at 30 °C [9]. Following incubation for 10 min at 30 °C, the reaction was stopped by addition of an excess of EGTA over $CaCl_2$. The N-terminal cleavage sites in the resulting partially degraded crystallins were determined by separating the proteins by 2-DE followed by direct N-terminal Edman sequence analysis as above.

One-dimensional and 2-dimensional electrophoretic gels prepared for image analysis or publication were stained with colloidal Coomassie Brilliant Blue G-250. Images were digitized using a Gel Doc 1000 camera (Bio-Rad, Hercules, CA), and analyzed with NIH Image (version 1.61, from the Research Services Branch, National Institutes of Health). The second dimension of the 2-DE gels was run on 1.5 mm thick, 16 x 14 cm, 12% polyacrylamide gels as previously described [4]. All 2-DE gels used in this study were loaded with 100-

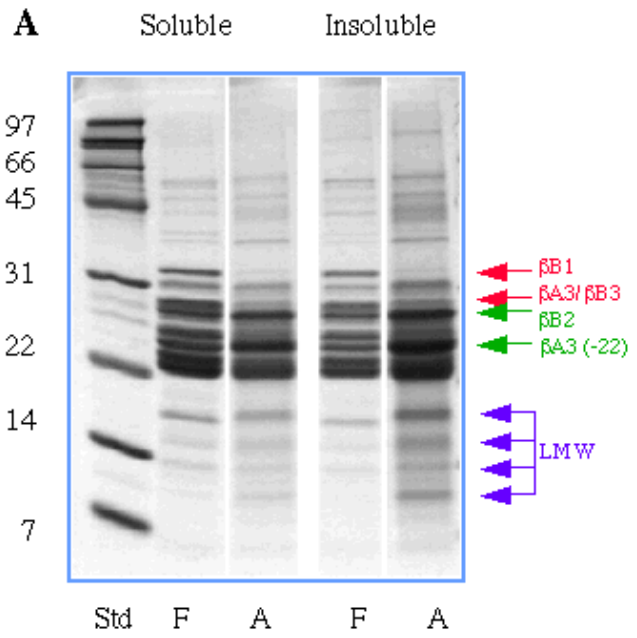

Figure 1A. One-dimensional electrophoretic analysis of fetal and adult bovine lens proteins. SDS-PAGE of water-soluble and water-insoluble bovine lens proteins. F= fetal, A = adult.

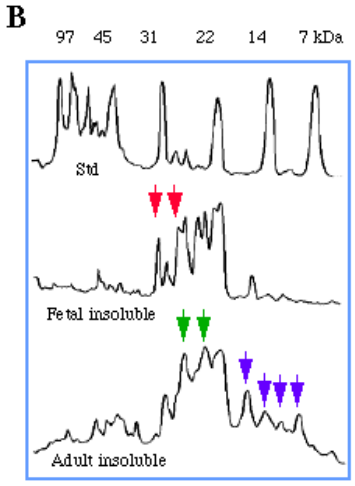

Figure 1B. Densitometric scan of lanes in (A) containing molecular weight standards, water-insoluble protein from fetal lenses, and water-insoluble protein from adult lenses. Red arrows indicate the positions of intact βB1 and comigrating βA3/βB3 crystallins which decrease with age. Green arrows indicate intact βB2 crystallin and partially degraded βA3 crystallin missing 22 residues from its N-terminus which increase with age. Purple arrows show positions of extensively degraded crystallins found in highest concentration in the insoluble protein of adult lenses. The identity of crystallins in this figure were inferred based on their apparent molecular weights and comparison to proteins identified on two-dimensional electrophoretic gels shown in Figure 2.

250 µg of protein. Single dimension SDS-PAGE gels were run on 1.5 mm thick, 8 x 8 cm, 18% polyacrylamide gels (Novex, San Diego, CA, catalog number EC6508) containing 5-6 µg of protein per lane.

RESULTS

One dimensional electrophoretic analysis of fetal and adult bovine lens proteins— An overview of the pattern of proteins in fetal and adult bovine lenses by SDS-PAGE revealed that lens contained at least eight prominent bands ranging in molecular weight from approximately 22 to 32 kDa (Figure 1A). Adult lenses showed a less complicated pattern dominated by three to four bands at lower molecular weights ranging from 22 to 27 kDa. Analysis of 2-DE gels in experiments described below allowed identification of several of the changes observed in protein composition by one-dimensional electrophoresis shown in Figure 1. The protein bands corresponding to intact βB1 and co-migrating βA3/βB3 decreased (marked in red), and two prominent protein bands corresponding to intact βB2 and partially truncated βA3 increased (marked in green) during aging. The protein pattern was similar between the soluble and insoluble fractions in both age groups, except that the adult insoluble fraction accumulated more low molecular weight components below 17 kDa (marked in purple) than the soluble fraction. These low molecular weight components may be similar to extensively degraded forms of crystallins described in human lenses [10-12]. This study focused instead on examining the larger, partially degraded crystallins in the range of 17-31 kDa. Since these partially degraded crystallins are best resolved using 2-DE, the proteins from both fetal and adult lenses were examined below using this technique.

Table I. Identification of crystallins in fetal bovine lens shown in Figure 2A.

Crystallin	Sequence Determined	Residues	Accession Number
βB1	AELPPGSYK	53-61	P07318
βB1 (-15)	PGPDGKGKAGPPP	16-28	P07318
βB3	GEQYVLEK	76-83	2338456
βB2	DSGDFGAPQP	173-182	P02522
βB3*	MEIVDDDVPSL	129-139	2338456
βA3 (-11)	SLPTTKMAQT	12-21	P11843
βA3 (-22)	PMPGSVGPWKIXIY	23-36	P11843
βA1	EWGSHAQTSQ	180-189	P11843
βA2	NYSEFGTQAH	176-185	P26444
βA3	GYQYILEXDH	178-187	P11843
βA4	IVVWDEEGFQGR	14-25	P11842
αB1	PFFPFHSPS	13-21	P02510
αB2	HFSPEELK	83-90	P02510
αA1	VQEDFVEIHG	89-98	P02470
αA2	TVLDSGISEV	55-64	P02470
γS	PVDWGAASP	160-168	P06504
γD/E	GKITFYEDRGFQGRH	2-16	P08209/P23005
γD/E	GKITFYEDRGFQGRH	2-16	P08209/P23005

The identities of the crystallins labeled in Figure 2A were determined by partial Edman sequence analysis of blotted proteins from 2-DE gels. The identities of β-crystallins in red were determined by direct Edman sequencing of the truncated and resulting unblocked N-termini. Numbers in parenthesis indicate the number of residues missing from the N-terminus, based on reported sequences in Swiss-Prot and Genbank databases. Numbering begins with the N-terminal methionine as residue 1, even though this residue may be absent in the parent protein. Intact γ-crystallins were also directly sequenced because they are normally unblocked. The remaining N-terminally blocked α- and β-crystallins were identified by sequencing of internal tryptic fragments. The βB3 marked with an asterisk was of lower molecular weight than unmodified βB3, but unlike the other shortened β-crystallins contained a blocked N-termini. Regions marked γD/E in Figure 2A may contain either γD or γE, since the N-terminal sequences determined matched the reported sequences of both proteins. An "X" in the "Sequence Determined" indicates where an amino acid identity could not be determined. The partial sequences determined by Edman degradation matched the given residue numbers of the previously reported sequences of bovine crystallins appearing in the given Swiss-Prot or Genbank entry. The numbering of residues again included the N-terminal methionine as residue 1. However, this residue may be missing in the actual protein. Note that the given partial sequence for βA4 does not match the numbers of the sequence of βA4 appearing in P11842, since recent sequences of chicken βA4 (P49152) and human βA4 (P53673), and the measured mass of bovine βA4 [24] indicated that the actual sequence is 14 residues shorter at the N-terminus than reported in P11842.

Identification of crystallin subunits of fetal bovine lenses by 2-DE analysis and Edman sequencing— The major proteins of the water-soluble fraction of the fetal bovine lens were separated by 2-DE (Figure 2A) and individual species identified by partial Edman sequencing of either free N-termini, or internal tryptic fragments (Table I). Intact crystallin subunits in Figure 2 are labeled in black, while partially degraded crystallin subunits are labeled in red. The identities of the intact crystallin subunits labeled in Figure 2A and identified in Table I agreed with the previously published identities of soluble crystallins from calf lens cortex [1]. However, the present study

Table II. Identification of crystallins in adult bovine lens shown in Figure 2B.

Crystallin	Sequence Determined	Residues	Accession Number
βB2 (-8)	AGAPQPLNPK	9-18	P02522
βB3 (-22)	SYKVIVYEME	23-32	2338456
βA3 (-22) (basic)	PMPGSVGPWK	23-32	P11843
βA3 (-22) (acidic)	PMPGSVGPWK	23-32	P11843
γS	VLEGAWIFYE	132-141	P06504

The identities of the crystallins labeled in Figure 2B were determined by partial Edman sequence analysis of blotted proteins from 2-DE gels. The identities of βB2 (-8), βB3 (-22), and both acidic and basic forms of βA3 (-22) were determined by direct Edman sequencing of the truncated and resulting unblocked N-termini (red). The identity of γS was determined by Edman sequencing of an internal tryptic fragment (black). Numbers in parenthesis give the number of residues missing from the N-terminus of the truncated proteins based on the previously reported sequences of each crystallin in the given Swiss-Prot or Genbank entry. All partial sequences matched the reported sequences in Swiss-Prot or Genbank entries, except residue 11 of βB2 which yielded alanine (in green) instead of the reported lysine at this position in P02522. This alanine was likely an artifact, since residue 11 of a m-calpain cleaved bovine βB2 was confirmed as lysine in Table III. The identities of other labeled crystallin subunits in Figure 2B were determined by comparison of their migration positions to crystallins identified in fetal lenses shown in Figure 2Aand Table I.

identified several previously uncharacterized components produced by in vivo partial cleavage of β–crystallin subunits. A previously observed component called βB1b [1] was confirmed to contain a form of βB1 missing 15 residues from its N-terminal extension [13]. However, unlike the previous study, an additional form of βB1 missing 12 residues was not detected. Two partially degraded forms of βA3 were found, missing either 11 or 22 residues from their N-terminal extensions. Since both βA3 and βA1 contain the same sequence past residue 17 of βA3 due to the use of alternate in-frame initiation codons within a single βA3/A1 m-RNA [14], it was unknown if the βA3 (-22) species was produced by cleavage of either βA3, βA1, or both proteins. A lower molecular weight form of βB3 was also observed in bovine lens (Figure 2A, asterisk). This partially degraded β–crystallin was unusual, since it contained a blocked N-terminus, suggesting possible cleavage at its C-terminus. Both acidic and basic forms of αA and αB were identified in Figure 2A. The basic forms (αA2 and αB2) were likely the unmodified proteins, while the acidic forms (αA1 and αB1) were probably phosphorylated or deamidated forms [15]. Two regions containing γ-crystallins were identified in Figure 2A. N-terminal sequence analysis of these unblocked proteins yielded identical sequences matching the reported residues 2-16 of both γD and γE. Therefore, it was unknown which of the two proteins were found in regions marked γD/E in Figure 2A. The crystallins in the water-insoluble fraction of fetal bovine lenses were also analyzed by 2-DE. This gel is not shown because it yielded a pattern which was identical to the 2-DE gel of soluble protein shown in Figure 2A.

Changes in crystallins during aging of bovine lens— During aging, bovine lens proteins undergo extensive alterations (Figure 2B). This resulted in a 2-DE pattern dominated by a few proteins and accumulation of degradation products (shown in red). The identities for βB1, βB1 (-15), βB2, βB3, βA3, βA4, and all α- and γ-crystallins on the 2-DE gel of adult lens protein (Figure 2B) were inferred based on the positions of these proteins identified from fetal lenses (Figure 2A). The remaining labeled protein species in Figure 2B were identified by Edman sequencing. N-terminal sequence analysis of proteins below βB2 identified four β–crystallins missing portions of their N-terminal extensions. Two minor species of βB2 and βB3 missing 8 and 22 residues from their N-terminal extensions, respectively; and two major species of βA3, both missing 22 residues from their N-terminal extensions, were identified (Figure 2B, Table II). The more

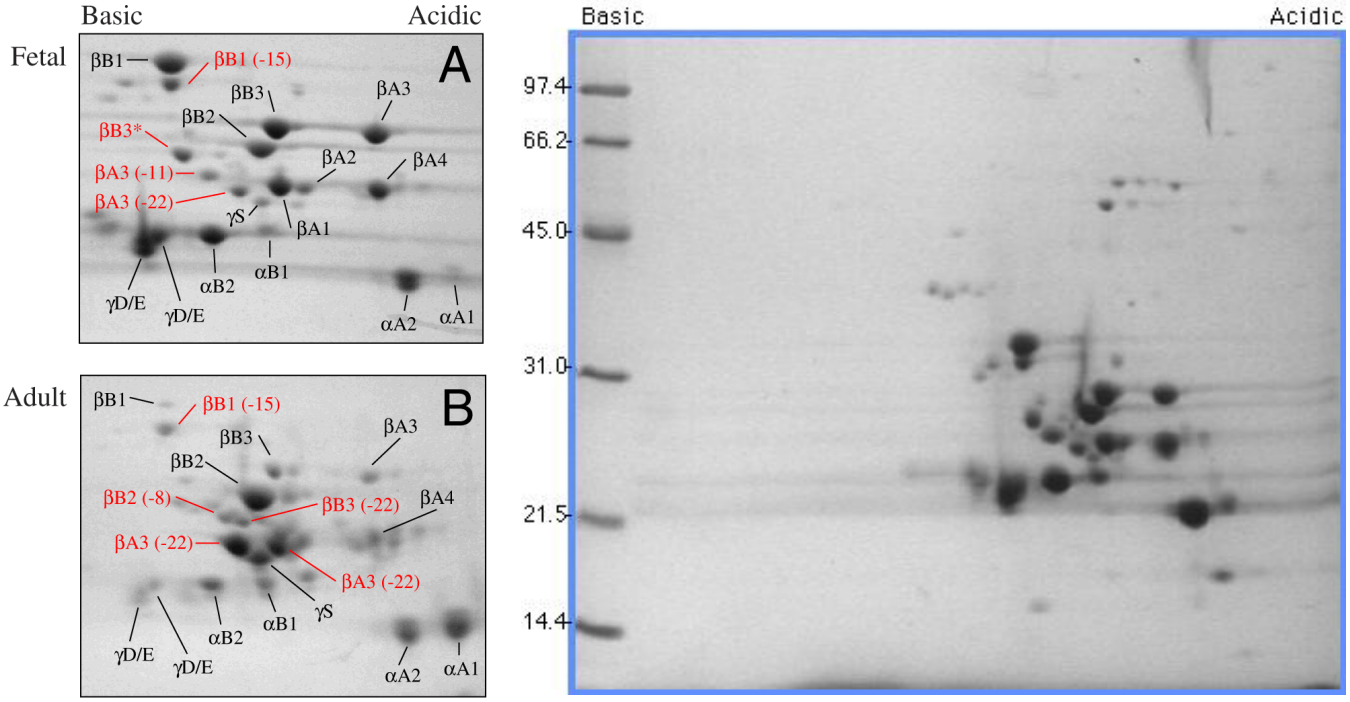

Figure 2. Two-dimensional electrophoresis of water-soluble proteins from fetal and adult bovine lenses. Identities of crystallins in fetal lens (A) were established by Edman sequencing shown in Table I. Undegraded crystallin subunits are labeled in black, and partially degraded crystallins are in red. The number of amino acids missing from the N-termini of the partially truncated β-crystallins are shown in parentheses. The major truncated β-crystallins in fetal lenses were βB1 (-15), βA3 (-11), and βA3 (-22). The identities of βB1, βB1 (-15), βB2, βB3, βA3, βA4, γD/E, αB2, αB1, αA2, and αA1 in adult lenses (B) were assigned by comparison to the position of the corresponding proteins in fetal lenses. The identities of γS, βB2 (-8), βB3 (-22), and acidic and basic forms of βA3 (-22) in adult lenses were determined by Edman sequence analysis as summarized in Table II. Intact βB1, βB3, βA3, βA4, γD/E, and αB decreased with age, while βB2, and the two forms of βA3 both lacking 22 amino acids from their N-terminus became major protein species. For reference, the calculated molecular weight and pI of acetylated bovine βB1 are 28,054 and 7.13, respectively, and the calculated molecular weight and pI of acetylated bovine αA are 19,832 and 5.52, respectively. These images only show the region of the gels containing crystallins; the entire gel for Figure 2a is shown below. For orientation, the uncropped two-dimensional gel of water-soluble protein from fetal bovine lens (Figure 2a) is also shown.

basic βA3 (-22) species on the left matched the position of the βA3 (-22) species observed in fetal lenses (Figure 2A). The more acidic species on the right was likely due to deamidation of βA3 (-22). The identity of the diffuse species directly above the acidic form of βA3 (-22) and the species directly to its right remain unknown. As before, these partially degraded βA3 crystallins could also have been produced by removal of 5 residues from the N-terminus of βA1. The major protein species observed directly below the two βA3 (-22) species was blocked at its N-terminus. However, this protein, which migrated to the same position as γS from fetal lens, was confirmed to be γS by the sequence of an internal tryptic peptide (Table II).

While losses of βB1, βB3, βA3, and βA4 occurred in adult bovine lenses, the concentration of intact βB2, and partially truncated βA3 (-22) increased dramatically (compare Figure 2A and Figure 2B). These changes are likely the result of both selective expression of βB2 and βA3/A1 crystallin genes, and selective degradation of βA3/A1 crystallin protein. Similar 2-DE analysis of the water-insoluble fraction of adult bovine lens detected no difference in the protein composition between the water-soluble fraction shown in Figure 2B and water-insoluble fraction (gel not shown). This was unlike rat lens, where partially truncated β–crystallin selectively entered the water-insoluble fraction [3].

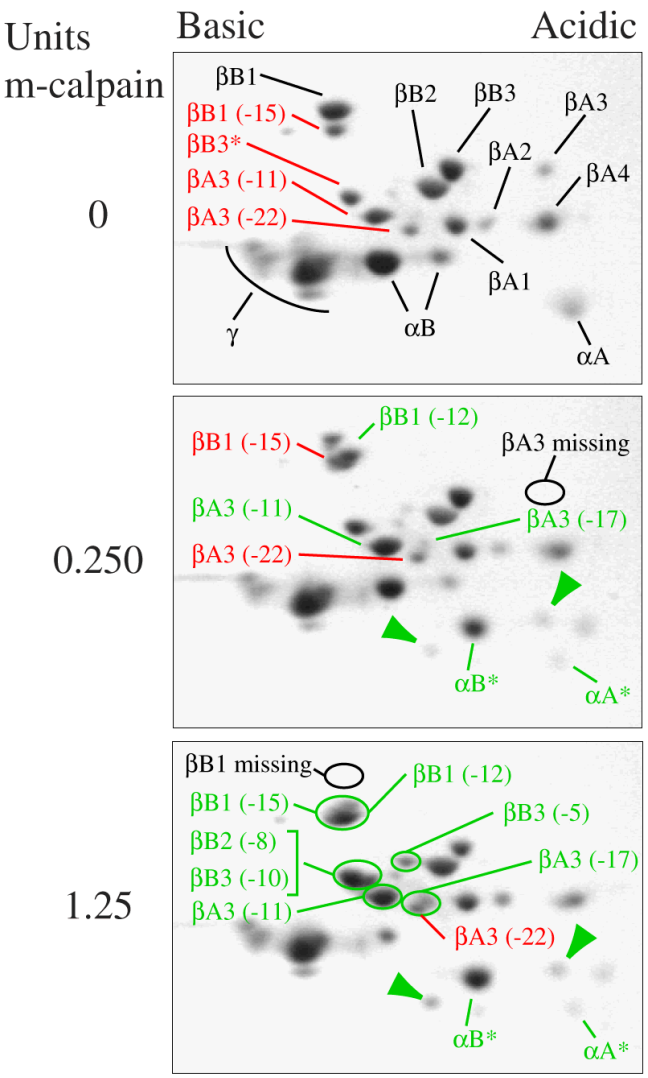

Figure 3. Digestion of water-soluble proteins from fetal bovine lenses with purified m-calpain. The digestion was performed for 10 min with either 0 units (top), 0.25 units (middle), or 1.25 units (bottom) of m-calpain. Following incubation, the appearance of truncated crystallins was observed by 2-DE. The identities of proteins in the sample incubated without m-calpain (top) were based on results of Edman sequencing as summarized in Table I. Partially truncated crystallins found in fetal lenses before m-calpain incubation are labeled in red, while intact crystallins are labeled in black. Partially degraded crystallins produced by m-calpain incubation (middle and bottom panels) are labeled in green. Regions where intact βA3 and βB1 were lost during incubation are indicated in the middle and bottom panels. The identities of truncated β-crystallins produced by m-calpain digestion and the number of amino acids removed from their N-termini (given in parentheses) were determined by Edman sequencing summarized in Table III. The identification of C-terminally degraded αA- and αB-crystallins (αA* and αB*) was based on comparison to m-calpain digested rat α-crystallins [17]. The identity of the m-calpain degradation products marked with green arrows was not determined.

Table III. Partially truncated β-crystallins observed following incubation of fetal bovine lens soluble protein with m-calpain.

Crystallin	Sequence determined	Residues	Accession Number
βB1 (-12)	AVNPGPDGKG	13-22	P07318
βB1 (-15)	PGPDGKGKAG	16-25	P07318
βA3 (-11)	SLPTTKMAQTNPMPG	12-26	P11843
βB2 (-8)	AGKPQPLNPKIIIFE	9-23	P02522
βB3 (-10)	AAAGKSHGGLGGSYK	11-25	2338456
βB3 (-5)	STPEQAAA	6-13	2338456
βA3 (-17)	MAQTNPMP	18-25	P11843
βA3 (-22)	PMPGSVGP	23-30	P11843

Soluble protein from fetal bovine lenses was incubated for 10 min at a ratio of 5 units m-calpain/mg lens protein, the mixture separated by 2-DE, and five distinct regions containing partially truncated β-crystallins with free N-termini either produced by m-calpain or found prior to m-calpain incubation subjected to Edman sequence analysis. The analyzed regions correspond to areas circled in green on the 2-DE gel shown in the third panel of Figure 3. The partially truncated β-crystallin(s) identified in each region which either increased in concentration or first appeared following m-calpain incubation are shown in green. Underlined β-crystallins produced by m-calpain in vitro were also observed in vivo in either fetal or adult bovine lenses (see Table I and Table II). Unlike the other β-crystallins listed, βA3 (-22) shown in red did not increase following incubation, but is shown here since it co-migrated with the m-calpain digestion product βA3 (-17). Numbers in parenthesis give the number of residues missing from the N-terminus of the truncated proteins based on the previously reported sequences of each crystallin in the given Swiss-Prot or Genbank entries. Three of the five regions contained mixtures of two proteins which did not fully resolve during 2-DE. However, the presence of only two amino acids per cycle allowed the identification of the two truncated β-crystallins present. The experimentally determined sequences in the second column matched the listed residue numbers of the previously known sequences of bovine β-crystallins in the given Swiss-Prot or Genbank entries.

Role of m-calpain in proteolytic changes in bovine lens— Since m-calpain activity has been demonstrated in bovine lens [16], and this calcium-dependent protease was previously implicated in the removal of N-terminal extensions in rat lens β–crystallins [3], the susceptibility of bovine β–crystallin to m-calpain digestion was determined. Water-soluble protein from fetal bovine lenses was incubated with either 0.25 or 1.25 units of purified m-calpain to determine the relative susceptibility of crystallins to the protease, and to detect possible intermediate products of degradation (Figure 3). The most abundant degradation product produced by m-calpain was in a region matching the position of a C-terminally degraded rat αB-crystallin produced by m-calpain (Figure 3, αB*) [17]. This was accompanied by the production of a species migrating to a similar position as C-terminally degraded rat αA-crystallin produced by m-calpain (Figure 3, αA*) [17]. The susceptibility of bovine α-crystallin C-termini to m-calpain digestion was previously reported [18].

Several β–crystallins exhibited nearly equal susceptibility to m-calpain digestion as the α-crystallins. The β–crystallins most susceptible to m-calpain digestion were βA3 and βB1. Incubation with 0.25 units and 1.25 units m-calpain caused the total loss of intact βA3 and βB1 spots (Figure 3, middle and bottom panels). Concurrent with these losses were the accumulation of degradation products of βB1, βB2, βB3, and βA3 missing various portions of their N-terminal extensions. These truncated β–crystallins were identified by Edman sequence analysis which is summarized in Table III. The m-calpain degraded βB1 by removing either 12 or 15 residues from the N-terminal extension. Removal of 12 residues from βB1 by m-calpain was the preferred cleavage site, since only βB1 (-12) appeared following incubation with 0.25 units of m-calpain (Figure 3, middle panel). In contrast, incubation with 1.25 units of m-calpain produced both βB1 (-15) and βB1 (-12) species which did not fully resolve from one another (Figure 3, bottom panel, and Table III). The production of additional βB1 (-15) over that found before incubation can be most easily observed by comparing the top and bottom panels of Figure 3. Incubation with m-calpain also produced two cleavage products of βA3 missing either 11 or 17 residues from their N-termini. The production of βA3 (-11) was also difficult to observe following incubation with m-calpain, because like βB1 (-15), this protein was already present before incubation. However, the mean density of the region containing βA3 (-11) in the sample incubated with 0.25 units m-calpain increased by 27% over the mean density of βA3 (-11) found in lens protein before m-calpain incubation. In contrast, the density of the βA3 (-11) region in the sample incubated with 1.25 units m-calpain (Figure 3, bottom panel) was not significantly increased above the beginning density of the βA3 (-11) region (Figure 3, top panel), because βA3 (-11) underwent further cleavage to produce significant amounts of βA3 (-17) in this sample. βA3 (-17) was detected very near the βA3 (-22) species observed before m-calpain incubation (Figure 3, bottom panel). For this reason, Edman sequence analysis of the region containing βA3 (-17) also detected some βA3 (-22) (Table III). However, unlike βA3 (-17), there was little evidence that significant quantities of βA3 (-22) were produced by m-calpain. Incubation with m-calpain also produced two forms of βB3 missing either 5 or 10 residues from their N-terminus. While these two species were well resolved from one another, the βB3 (-10) species migrated very near a cleavage product of βB2 missing 8 residues from its N-terminus. This resulted in the simultaneous detection of both proteins during the Edman sequence analysis summarized in Table III. Note that the βB3 (-10) cleavage product migrated to a position identical to the partially truncated form of βB3 found before incubation which contained a blocked N-termini (Figure 3, top panel, βB3*). It was unlikely that the βB3 (-10) cleavage product was derived from this unusual form of βB3, since the parent βB3 diminished greatly during incubation with m-calpain. Furthermore, cleavage of βB3* by m-calpain, even if it involved only removal of a N-terminal blocking group, would have likely altered the position of βB3* on the 2-DE gel. Of interest was the finding that m-calpain cleaved rat βB2, βB3, and βA3 at precisely the same positions within the N-terminal extensions as bovine βB2 (-8), βB3 (-10), and βA3 (-11) when the N-terminal extensions of the three proteins from the two species were aligned [3].

DISCUSSION

The present study confirmed the identities of the major crystallins of the fetal bovine lens, and detected several partially truncated forms of β–crystallins not previously identified [1]. This additional mapping of modified proteins on 2-DE gels of adult bovine lens protein will also facilitate the future analysis of the other post-translational modifications of crystallins in bovine lens. The truncated form of βA3/A1 crystallin found in fetal lens, βA3 (-22), accumulated with age and became a major protein in the adult bovine lens. The abundance of the truncated βA3 (-22) species on the 2-DE gels of adult bovine lenses was striking. The presence of both βA3 (-22) and βA3 (-11) in bovine lens was also recently reported by Werten et al. [19]. While the protease m-calpain could reproduce at least three of the cleavages in bovine β–crystallins found in vivo, its apparent inability to reproduce the βA3 (-22) cleavage suggested that at least one other proteolytic activity was also responsible for truncation of crystallins in adult bovine lens.

These results and those of earlier studies examining the maturational changes of crystallins in both rat and human lenses suggested that the loss of β–crystallin N-terminal extensions occurs in many species. We hypothesize that this important event decreases the dispersive forces between crystallins and facilitates increases in protein concentration in maturing fiber cells. The higher protein concentrations and refractive index of the lens fiber cytosol may minimize the effect of protein aggregation. However, it remains largely speculative that this proteolysis is programmed and performs a specific function. Alternatively, it may just be a normal degradative process without function, amplified by the lack of protein turnover.

Determining which proteases remove the N-terminal extensions of β–crystallins is important not only because it could provide information concerning normal lens

development and function, but it will test the hypothesis that a loss of protease regulation during maturation could contribute to cataract. An accelerated loss of β–crystallin N-terminal extensions in young rat lens during induction of cataracts by selenite administration was associated with rapid protein insolubilization and lens opacity [3]. Examination of truncated β–crystallins in both normal and cataractous rat lens suggested that the protease m-calpain was responsible for the majority of β–crystallin degradation in this species. In contrast, no evidence for degradation of human β–crystallins by m-calpain in vivo was found. This conclusion is based on cleavage sites found in β–crystallins from young human lenses [2], and m-calpain induced cleavage sites in human β–crystallins (unpublished data). This suggested that an as yet unidentified proteolytic activity was responsible for β–crystallin truncation in human lens. The present study suggested that the proteolysis of β–crystallins in bovine lenses is performed by both m-calpain and additional unidentified protease(s) similar to that found in human lens.

The results suggesting that m-calpain was partially responsible for β–crystallin truncation in bovine lenses is as follows. (1) Analysis of partially truncated β–crystallins in both fetal and adult bovine lenses identified at least two truncated β–crystallins containing known m-calpain cleavage sites. These were βA3 (-11) found in fetal lenses, and βB2 (-8) found in adult lenses. An earlier study also reported the presence of a partially truncated βB1 in the cortex of calf lens missing 12 residues from its N-terminus [13]. While this species was not detected in the present study in vivo, m-calpain readily produced this cleavage in vitro (Table III). (2) The production of βB1 (-15), the major cleavage product of βB1 in bovine lens, may have also been caused by m-calpain. While βB1 (-12) was the preferred m-calpain cleavage product of βB1, incubation with a high concentration of m-calpain was capable of also producing βB1 (-15). (3) While the other known m-calpain products βB3 (-5), βB3 (-10), and βA3 (-17) were not detected in bovine lens, they may have been obscured in the 2-DE gels of adult lens protein by other modified crystallins, or they may have been further cleaved by other lens proteases. For example, the proposed m-calpain product βA3 (-11) found in fetal bovine lens was absent in adult bovine lens. The m-calpain product βA3 (-11) was likely cleaved by another protease to βA3 (-22) during lens maturation. In a similar manner, other m-calpain products may have been further cleaved so they were no longer observed in mature lens.

The results suggesting that an additional as yet unidentified protease is also active in bovine lens is: (1) βB1 (-15) and βA3 (-22) found in bovine lens were also observed in human lens [2], a species where no evidence for proteolysis of crystallins by m-calpain has yet been found. (2) The cleavage sites within the N-terminal extensions of both βB1 and βA3 were within the sequence NPXP between asparagine and proline residues. The presence of proline residues at the new N-termini of both proteins could be interpreted as exopeptidase cleavage which paused at the prolyl residues. However, the presence of other prolyl residues, nearer the N-terminus of the intact crystallins than the prolyl residues at the cleavage site, and resistance of the other β–crystallins to cleavage suggest a specific endopeptidase. This endopeptidase may recognize the NPXP site within the relatively long N-terminal extensions of βB1 and βA3. (3) The hypothetical protease cleaving within the NPXP sequence may have already been described in the lens, but its specific role in crystallin cleavage may not be recognized. Besides m-calpain, a partial list of endopeptidases demonstrated in lens include: several new proteases with activities against synthetic substrates [20], proteasome [21], serine protease [22], and membrane bound protease [23].

The present study illustrates the importance of interspecies comparisons while studying the mechanisms altering the structure of lens crystallins. Lenses from the longer lived cow may be a more appropriate model to understand the mechanism of crystallin modification in human lens than is the short lived rat. Maturation of crystallins in rat lenses may be predominately performed by m-calpain, while crystallin maturation in cow and human lens is more complex due to the involvement of other proteases and possible non-enzymatic hydrolysis. The bovine lens may be an appropriate tissue to purify and identify the proteolytic activities responsible for crystallin modification in human lens.

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