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β-Crystallins insolubilized by calpain II in vitro contain cleavage sites similar to β-crystallins insolubilized during cataract

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Incubation of soluble proteins from rat lens with the protease calpain II caused the precipitation of β-crystallin polypeptides. Two-dimensional electrophoresis and sequence analysis identified β-crystallin polypeptides both before and after their precipitation by calpain II. β-crystallin polypeptides precipitated by calpain were cleaved at their NH_2-terminal extensions. These cleavage sites were similar to cleavage sites occurring in β-crystallin polypeptides precipitated during formation of experimental cataract induced by an overdose of selenite These data suggested that calpain II caused β-crystallin insolubilization during cataract formation, and indicated that the process can be mimicked in vitro.

Crystallin; Cataract, Calpain

1. INTRODUCTION

The majority of the dry weight of the lens of the eye is composed of proteins called crystallins. In the mammalian lens, these proteins are divided into three major classes: α-, β-, and γ-crystallins [1]. These divisions are based on the elution of soluble lens proteins during gel filtration. Native α-crystallin is an oligomer of approximately 800 kDa, and is composed of primarily two 20 kDa subunits named αA and αB. Native β-crystallins are also oligomers, which elute in two or more size classes during gel filtration, and range from 200–50 kDa. They contain 7 different types of subunits, named βB1, βB2, βB3, βA1, βA2, βA3, and βA4, ranging from 31–23 kDa. To date, genes coding for all 7 of the major β-crystallin subunits have been sequenced [2–5]. β-Crystallin polypeptides share sequence and structural homologies with γ-crystallins, reflecting their common evolutionary origin [1], however, unlike β-crystallins, γ-crystallins do not form oligomers, and contain no extensions at either their NH_2- or COOH-termini [1].

Crystallins have the remarkable ability of existing in high concentrations in the transparent lens without causing light scatter [6], however, this property is lost if lens crystallins become denatured. Crystallin denaturation is most readily observed during formation of experimental cataracts by the loss of their solubility in water [7].

Recent data in rodents suggests that removal of portions of the NH_2-terminal extensions of β-crystallins by the calcium-dependent neutral protease calpain II (EC 34.22.17) may contribute to crystallin denaturation and insolubilization during cataract. This hypothesis is supported by three observations.

(i) When 10-day-old rats are injected with a single overdose of selenite, their lenses undergo an elevation in calcium and form a dense opacity within 4 days [8]. The opaque insoluble protein from these lenses contained an abundance of partially degraded β-crystallin polypeptides missing from 5–49 amino acids from their NH_2- terminal extensions [9]. Calpain II was probably responsible for this partial proteolysis, since intact β-crystallin polypeptides were cleaved by purified calpain II at sites identical to cleavage sites appearing during cataract formation [9].

(ii) Cataracts produced in cultured rat lenses by addition of the cataractogenic agents selenite, calcium ionophore A23187, xylose, and diamide all underwent partial degradation of β-crystallin polypeptides and opacification. Addition of E64 or E64d, inhibitors of calpain and other cysteine proteases, decreased proteolysis induced by the various toxins, and temporally prevented the subsequent opacification [10,11].

(iii) Incubation of soluble lens crystallins with purified calpain II in vitro caused a rapid increase in turbidity, due to the insolubilization of partially degraded β-crystallin polypeptides. When examined by two-dimensional electrophoresis, the polypeptides insolubilized by calpain II migrated to positions that were identical to positions of partially degraded β-crystallin polypeptides appearing in the insoluble protein of cataractous lenses [12].

The finding that calpain II caused the insolubilization

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of crystallins in vitro was important, because it provided a model to study the mechanism of crystallin insolubilization during cataract. The purpose of the present study was to examine the validity of the in vitro insolubilization model by determining cleavage sites on β-crystallins following calpain-induced insolubilization. We found that β-crystallins insolubilized by calpain in vitro were cleaved at the same sites on their NH_2-terminal extensions as β-crystallins insolubilized during cataract. These data confirmed that in vitro insolubilization by calpain II is a useful model to study the mechanism of crystallin insolubilization during cataract formation.

2. MATERIALS AND METHODS

The lenses of 2-week-old Sprague–Dawley rats were dissected and homogenized at a ratio of 1 mg lens wet weight/2.5 μl buffer A containing 20 mM imidazole (pH 6.8), 0.1 mM EGTA, and 2.0 mM dithioerythritol (DTE). The soluble protein was isolated by centrifugation of the lens homogenates at $10,000 \times g$ for 15 min at 25°C. Calpain II was purified from porcine heart muscle and assayed as previously described [12]. Soluble lens protein at 30 mg/ml concentration was incubated for 60 min in buffer A containing 5 U calpain II/mg lens protein and 1.0 mM $CaCl_2$. The increase in turbidity during crystallin insolubilization was measured in the wells of a microtiter plate, and the extent of insolubilization was similar to previous experiments [12]. The resulting precipitated proteins were isolated by centrifugation at $10,000 \times g$ for 10 min at 25°C, washed once in buffer A containing no DTE, resuspended in water, a portion removed for protein assay using bovine serum albumin as a standard (bicinchoninic acid method; Pierce Chemical Company), and the remainder dried by vacuum centrifugation. Insoluble protein from the opaque lens nucleus of rats with selenite-induced cataracts was isolated as before [9].

Two-dimensional electrophoresis of proteins was carried out as before [12], followed by blotting onto polyvinylidene difluoride (PVDF) membranes (Immobilon P; Millipore, Bedford, MA) [9]. Blots were stained for 1 min in 0.1% Ponceau S, 1% acetic acid, and rinsed with MilliQ water (Millipore) to remove background stain. Individual polypeptides were then cut out, destained in pH 11 NaOH solution, rinsed with MilliQ water, then stored at −20°C. Six NH_2-terminal amino acid residues were then determined from combined spots from 2–4 gels (100 μg total protein/gel) using an Applied Biosystems model 470A protein-sequenator, and model 120 PTH analyzer.

Polypeptides with blocked NH_2-termini were identified by sequencing tryptic fragments produced by in situ digestion of blotted crystallin polypeptides on PVDF membranes. The polypeptides were separated by two-dimensional electrophoresis as above, except 125–250 μg total protein was applied, and spots from 3–16 gels were pooled. Membranes were then blocked by incubation for 30 min in 0.5% polyvinylpyrrolidone-40, 1% acetic acid, followed by ten MilliQ water rinses. The blotted polypeptides were then incubated for 24 h at 37°C in 50 μl 0.1 M NH_4HCO_3 (pH 8.0), 10% acetonitrile, and 4 μl of 1 mg/ml sequencing grade modified trypsin (Promega Corp., Madison, WI) dissolved in 0.1% trifluoroacetic acid (TFA). The tryptic fragments released from the membranes were then dried by vacuum centrifugation, dissolved in 0.1% TFA, and injected onto a 150×2.1 mm C_{18}

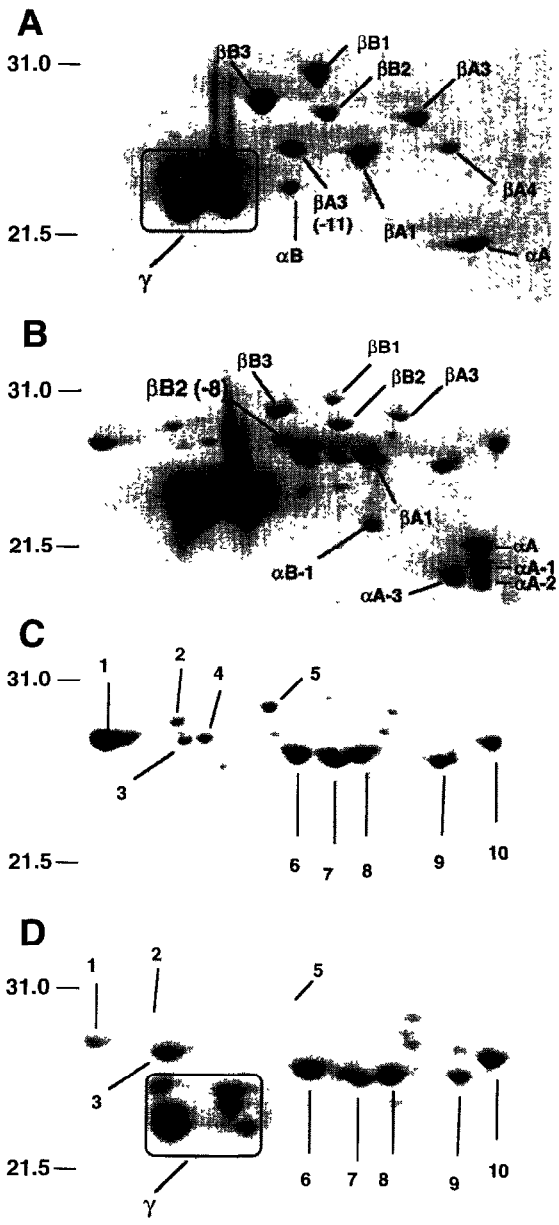

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Fig. 1. Two-dimensional electrophroresis of lens crystallins before and after partial degradation by calpain. (A) Soluble crystallin polypeptides from the lens of 2-week-old control rats. All α- and β-crystallin fragments appeared blocked, with the exception of βA3 (−11), which, based on its NH_2-terminal sequence, was βA3 missing 11 amino acids from its NH_2-terminal extension. The identification of all other major α- and β-crystallin polypeptides was based on the sequence of fragments produced by digestion of electroblotted polypeptides with trypsin (the tryptic maps for the β-crystallins are shown in Fig. 2). (B) Crystallin polypeptides from the lens of 2-week-old rats which remained soluble following incubation with calpain II. The positions of the remaining undegraded β-crystallin subunits are indicated, as well as the identity of partially degraded α- and β-crystallin polypeptides which, unlike the other proteolytic fragments which appeared, were not partially insolubilized during calpain incubation. (C) β-Crystallin polypeptides undergoing precipitation during incubation with calpain. The major insolubilized fragments were numbered and identified by Edman sequencing of their NH_2-terminus, and/or analysis of tryptic fragments (Table I, Fig. 3). (D) Insoluble protein from the opaque nuclear region of rat lens 4 days following selenite injection. Polypeptides with positions similar to polypeptides found in C were given the same numbers so that their identities could be compared (Table I). The boxed region shows γ-crystallin polypeptides were appeared in the insoluble fraction prior to cataract formation [9]

column (Vydac, Hesperia, CA). Chromatography was carried out at 50°C, using a 0.2 ml/min flow rate, and linear 75 min 0–55% acetonitrile gradient containing 0.1% TFA. Tryptic fragments were detected by absorbance at 216 nm, and 1 min fractions collected. Tryptic fragments were then identified by determining the sequence of 6–10 NH_2-terminal amino acid residues as above.

3. RESULTS

Soluble lens crystallins from normal 2-week-old rat lens were separated by two-dimensional electrophoresis, and 7 major β-crystallin polypeptides, and 2 major α-crystallin polypeptides were identified (Fig. 1A). The

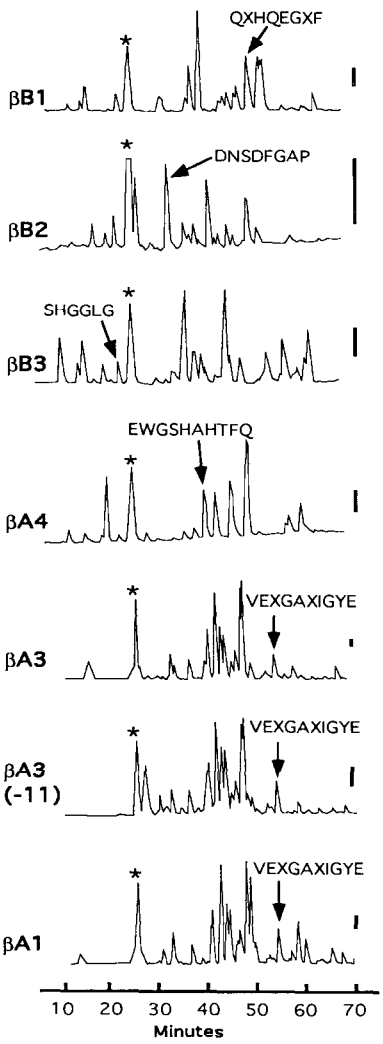

Fig 2. HPLC separation of tryptic fragments of control β-crystallin polypeptides shown in Fig 1A. Each polypeptide was identified by 6–10 cycles of Edman sequencing of the tryptic fragments indicated by arrows. The polypeptides were then identified by comparing the given sequence to published sequences of β-crystallins [2–5]. Due to their similar sequence, βA3 and βA1 were differentiated by their relative molecular weights (Fig. 1A), and βA3 (−11) was identified by its NH_2-terminal sequence. The asterisk indicates the elution position of trypsin. The bar on the right indicates 0.01 absorbance units at 216 nm. X indicates the position of cysteine or tryptophane residues which were not determined.

γ-crystallin polypeptides (Fig. 1A, boxed region) were not analyzed, because they were not calpain substrates. The α- and β-crystallin polypeptides from these normal lenses could not be identified by simple NH_2-terminal sequence analysis, because they were NH_2-terminally blocked. An exception was βA3 (−11). The sequence of the six NH_2-terminal amino acids of polypeptide βA3 (−11) were TLPTTK, confirming its identity as βA3 crystallin missing 11 amino acids from its NH_2-terminus [4,9]. The remaining β-polypeptides were identified by trypsinization, separation of fragments by HPLC, and determination of the sequence of 8–10 amino acids from individual fragments (Fig. 2). The tryptic map of each polypeptide was distinct, with the exception of βA3, βA3 (−11), and βA1, which had similar tryptic maps. The similarity of the tryptic maps and sequence of tryptic fragments confirmed that βA3, βA3 (−11), and βA1 were all products of the single βA3/A1 gene [4]. The lowest molecular weight of the three, βA1, is synthesized using an alternate initiation codon, resulting in a polypeptide 17 amino acids shorter than βA3 [4], while βA3 (−11) was probably produced by post-translational processing of βA3. The identities of αA and αB were similarly determined by fragmentation and sequencing of tryptic fragments (data not shown). Therefore, all α- and β-crystallin polypeptides were identified, with the exception of βA2 and αA (insert) [1], which were not major components in 2-week-old rat lens.

Soluble lens crystallins from 2-week-old rat lens were then incubated with calpain II for 60 min. As previously described, the turbidity of the incubation mixture increased due to insolubilization of approximately 4% of the total protein [12]. Following centrifugation of the incubation mixture, the resulting supernatant and pellet were separated by two-dimensional electrophoresis. Many new partially degraded polypeptides appeared in both the supernatant (Fig. 1B) and pellet (Fig. 1C). Changes in the relative concentrations of polypeptides during calpain incubation suggested that the majority of new polypeptides in both the supernatant and pellet were derived from partial degradation of βB1, βB3, βA3, βA4, αA, and αB. Furthermore, examination of the proteins insolubilized by calpain suggested that they were entirely composed of polypeptides, which, because of their apparent molecular weight, were all partially degraded β-crystallin polypeptides (Fig. 1C).

This hypothesis was confirmed by direct sequence analysis of the polypeptides precipitated by calpain. Polypeptides 2, 3, 4, 6, 9, and 10 (Fig. 1C) were identified as partially degraded βB1, βB3, βA3, and βA4 missing portions of their NH_2-terminal extensions (Table I). Insoluble polypeptides 1 and 7 (Fig. 1C) could not be identified by direct sequence analysis, due to indistinct NH_2-termini. Therefore, each was identified by sequencing tryptic fragments (Fig. 3). Insoluble polypeptide 1 was identified as βB3 missing a portion of its NH_2-terminus. This was based on finding the complete

sequence of the COOH-terminal tryptic fragment of βB3 (Fig. 3, Polypeptide 1). Furthermore, the appearance of alanine in the first two cycles during direct sequencing of untrypsinized polypeptide 1 suggested the calpain cleavage site was at or near residue 10 [3]. Similarly, polypeptide 7 was identified by the amino acid sequence of an internal tryptic fragment as a partially degraded βA3 or βA1 (Fig. 3, Polypeptide 7). Direct sequence analysis of the untrypsinized polypeptide then suggested that insoluble polypeptide 7 was derived by removal of a range of 17–21 amino acids from the NH_2-terminus of βA3. Insoluble polypeptides 5 and 8 appeared blocked at their NH_2-terminus, however, their positions suggested that they were intact βB3 and βA1, respectively (compare Fig. 1C with Fig. 1A). This was subsequently confirmed by the similarity of their tryptic maps (data not shown) to the tryptic maps of intact βB3 and βA1 (Fig. 2). The insolubilization of intact βB3 and βA1 during incubation with calpain illustrated that partial degradation was not always required for insolubilization to occur. Intact βA1 may have precipitated due to interaction with other β-crystallin subunits which were partially degraded.

Comparison of polypeptides insolubilized by calpain (Fig. 1C) with those remaining soluble folllowing calpain incubation (Fig. 1B) indicated that a significant proportion of partially degraded β-crystallin polypeptides identified in the pellet remained soluble. This comparison also indicated that some partially degraded polypeptides did not undergo significant insolubilization. Five of these partially degraded crystallins which remained soluble were identified. These included: βB2 missing 8 amino acids from its NH_2-terminus (Fig. 1B, βB2 −8), identified based on its NH_2-terminal sequence AGKPQP [5]; and polypeptides αA−1, αA−2, αA−3, and αB−1 (Fig. 1B), identified as partially degraded αA and αB polypeptides by similarity of their tryptic maps to the tryptic maps of intact αA and αB polypeptides (data not shown). NH_2-Terminal sequence analysis of these partially degraded a polypeptides also indicated that they remained NH_2-terminally blocked, suggesting that they were produced by cleavage at the COOH-terminus of both αA and αB.

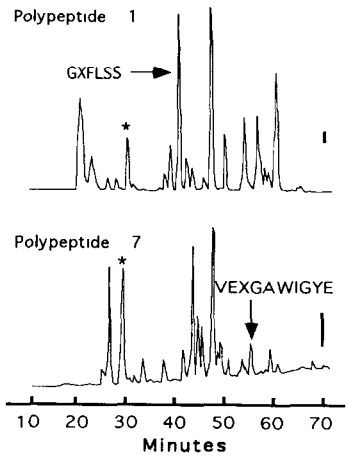

Fig 3 HPLC separation of tryptic fragments of β-crystallin polypeptides with indistinct NH_2-termini following insolubilization by calpain II. Polypeptide 1 (Fig. 1C) was identified as a partially degraded βB3, with an intact COOH-terminus, based on the sequence of the indicated COOH-terminal tryptic fragment Polypeptide 7 (Fig. 1C) was derived by partial degradation of either βA3 or βA1, based on the sequence of the indicated internal tryptic fragment. The bar on the right indicates 0.05 absorbance units at 216 nm X indicates the position of cysteine which was not determined.

The polypeptides insolubilized by calpain in vitro (Fig. 1C) were then compared to polypeptides insolubilized in vivo during formation of selenite induced cataract (Fig. 1D). Many of the polypeptides insolubilized by calpain II in vitro migrated to the same positions, and contained the same NH_2-terminal cleavage sites as polypeptides insolubilized during formation of cataract (Table I), however, there were minor differences between the insoluble protein formed in vitro (Fig. 1C) and the insoluble protein formed during cataract (Fig. 1D). The insolubilization during cataract was accompanied by more extensive degradation of βB3 than during insolubilization in vitro. For example, polypeptide 1 (βB3 missing approximately 10 residues) was the most abundant βB3 fragment in the protein insolubilized by calpain in vitro, while polypeptide 3 (βB3 −17) was the most abundant βB3 fragment in protein insolubilized during cataract formation. Another example of more extensive degradation during cataract formation was illustrated by the identities of polypeptides numbered 8 in both Fig. 1C and D. Polypeptide 8 insolubilized by calpain in vitro remained NH_2-terminally blocked, and was identified as intact βA1, while polypeptide 8 insolubilized during cataract was derived by removal of either 4 or 21 amino acids from βA1 or βA3, respectfully, and deamination of the resulting NH_2-terminal asparagine [9].

4. DISCUSSION

The major finding in the present study was that partially degraded β-crystallin polypeptides insolubilized by calpain II in vitro were cleaved at similar sites at their NH_2-terminal extensions as β-crystallin polypeptides insolubilized during cataract formation in vivo. This extends our earlier calpain cleavage site data for β-crystallins [9], and further supports the hypothesis that removal of NH_2-terminal extensions of β-crystallins by calpain is directly responsible for β-crystallin insolubilization during cataract formation. The data also suggested that in vitro insolubilization by calpain is a useful model to study crystallin insolubilization during experimental cataract.

The present study is also the first to demonstrate the utility of in situ tryptic digestion to identify crystallins following their separation by two-dimensional electrophoresis. The methodology was particularly useful for

Table I

The majority of β-polypeptides insolubilized by calpain II have the same cleavage sites at their NH_2-termini as insoluble β-crystallin polypeptides from cataractous lens

Polypeptide[a]	NH_2-termini[b]	Identity[c]	Found in cataract[d]
1	Indistinct[e]	βB3 (~ 10)	+
2	GAPEQA	βB3 (−5)	+
3	GGLGGS	βB3 (−17)	+
4	SHGGLG	βB3 (−15)	−
5	Blocked[f]	βB3	+
6	TLPTTK	βA3 (−11)	+
7	Indistinct[e]	βA3/A1 (−17 to −21)	+
8	Blocked[f]	βA1	−
9	XTKSAG[g]	βA4 (−18)	+
10	ELPPGS	βB1 (−49)	+

[a]The numbers correspond to the polypeptides marked in Fig. 1C and D.

[b]Polypeptides subjected to direct Edman sequencing from PVDF membranes.

[c]Polypeptides were identified by comparison of Edman sequencing results to published sequences of β-crystallin polypeptides [2–4]. In cases where NH_2-termini were blocked or indistinct, the identification was based on the sequence of tryptic fragments. Number in parentheses gives number of residues missing from the NH_2-terminus.

[d]Based on similarity of NH_2-terminal sequence and migration during two-dimensional electrophoresis (compare Fig 1C and D), these polypeptides were also found in the insoluble protein of rat lenses with selenite-induced cataract [9].

[e]These polypeptides contained several NH_2-termini, and were identified by sequencing tryptic fragments (Fig. 3), however, based on Edman sequence data, the approximate cleavage sites at the NH_2-termini are given.

[f]These polypeptides remained blocked, and were identified as intact βB3, and βA1, based on their position during two-dimensional electrophoresis, and similarity of their tryptic maps to the tryptic maps of intact βB3 and βA1 (Fig. 2)

[g]X indicates position of cysteine which was not determined.

study of lens proteins, because intact α- and β-crystallins are normally NH_2-terminally blocked, and we wished to identify the intact rat β-crystallins before their breakdown by calpain. While the positions to which many β-crystallins from other species migrate during electrophoresis are known, a direct comparison to identify rat crystallins was not possible. This is because orthologous crystallin subunits from various species can migrate to different positions during two-dimensional electrophoresis. For example, in bovine and human lens, βB1 is the most basic β-crystallin subunit [13,14], while in rat, βB3 is the most basic β-crystallin subunit. Also, without this technique, we could not have identified several partially degraded crystallins which contained indistinct termini following calpain cleavage. This technique will greatly facilitate the study of proteolysis and other post-translational changes in lens crystallins during aging and cataract formation.

The reason β-crystallins lose their solubility following removal of their NH_2-terminal extensions is unknown. It is also difficult to formulate a hypothesis for calpain induced insolubilization, because the function of the NH_2-terminal extensions of β-crystallin subunits remains unclear. It has been suggested that NH_2-terminal extensions in β-crytallins subunits could interact intermolecularly to stabilize oligomers [15]. While recent data on the structure of βB2 homodimers suggests that NH_2-terminal extensions are not used to stabilize βB2 dimers [16], it still remains plausible that NH_2-terminal extensions in other β-subunits could be responsible for dimer–dimer interactions [17]. If this is true, then insolubilization may result from decreased stability of the β-subunits following their dissociation when the NH_2-terminal extensions are removed. Alternatively, insolubilization following removal of NH_2-terminal extensions may occur because the extensions function as spacers to prevent unwanted interactions between β-crystallin oligomers. A third possibility is that the extensions may be required to maintain the proper secondary structure in individual β-crystallin subunits. Regardless of the mechanism of insolubilization, βB2 subunits appeared less susceptible to insolubilization, since they were more soluble than other β-crystallin subunits following their partial degradation.

Two recent unpublished findings are relevant to the present work. We recently found that physiological concentrations of KCl will delay the precipitation of partially degraded rat β-crystallin subunits by 48 h. This suggested that the initial interaction between partially degraded β-crystallin which causes insolubilization may be ionic in nature. Additionally, incubation of human lens soluble proteins with calpain did not cause significant precipitation of the resulting partially degraded β-crystallin subunits. This result could be due to the lower relative concentration of βB3, and higher concentration of βB2 in human lens, compared to rat lens.

Determining the mechanism of insolubilization is important, because it will not only provide important information regarding the mechanism of cataract formation, it will also assist in determining the function of NH_2-terminal extensions in various β-crystallin subunits. The relevance of partial degradation of β-crystallin subunits in human cataract remains unknown, but is currently under study.

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