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Biochemical and Biophysical Research Communications 301 (2003) 44–49
BBRC
www.elsevier.com/locate/ybbrc

βB2-crystallin undergoes extensive truncation during aging in human lenses

O.P. Srivastava* and K. Srivastava

Department of Physiological Optics, School of Optometry, University of Alabama at Birmingham, Worrell Building, 924 South 18th Street, Birmingham, AL 35294-4390, USA

Received 9 December 2002

Abstract

Based on the present literature, it is unclear whether βB2-crystallin undergoes age-related truncation in human lenses. To answer this question, the purpose of this study was to determine in vivo truncation of βB2-crystallin in human lenses during aging by examining its fragments in the β_H-crystallin fraction. The WS-protein fraction was isolated from lenses of desired ages and separated by a size-exclusion Agarose A 1.5 m column to recover α-, β_H-, β_L-, and γ-crystallin fractions. The β_H-crystallin fractions, isolated from lenses of 24- and 70-year-old donors, were utilized for two-dimensional (2D) gel electrophoresis (isoelectric focusing in the first dimension followed by SDS–PAGE in the second dimension). The partial N-terminal sequences of the desired fragments (Molecular weights $[M_r] < 18$–19 kDa) from a 2D-gel of WS-proteins from lenses of a 70-year-old donor were determined. More than 37 crystallin fragments with M_r between 4 and 19 kDa were observed on a 2D-gel. Nine fragments in β_H-crystallin fraction were from βB2-crystallin but additional single fragments of αA-, γs-, βA4, and of either γB-, γC- or γD-crystallins were also observed. Seven cleavage sites in the βB2-crystallin were identified, which included two sites at Q_7–A_8 and A_8–G_9 bonds in the N-terminal extension, two sites at E_{46}–K_{47} and G_{49}–S_{50} bonds in the motif 1, one site at S_{94} –S_{95} in the motif 2, and two sites at N_{115}–F_{116} and Q_{135}–Y_{136} in motif 3. No fragments with cleavage in the motif 4 and C-terminal extension of βB2-crystallin were seen. Apparently, three βB2-crystallin fragments with only N-terminal cleavage and five with both N- and C-terminal cleavages were observed. Additional fragments with cleavage sites at Q_{54}–Y_{55} in αA-crystallin, at E_{112}–N_{113} in βA4-crystallin, at G_4–T_5 in γs-crystallin, at M_{69}–G_{70} in either γB-, γC- or γD-crystallins (three have identical sequences at the cleaved bond), and at G_1–K_2 in γB or γC (both have identical sequences at the cleavage site) were observed.

Conclusions. The results showed that βB2-crystallin undergoes age-related truncation producing fragments with M_r between 4 and 19 kDa that existed in the β_H-crystallin oligomer. The β_H-crystallin fraction also contained single fragments of α-, βA4-, γs-, and other γ-crystallins.

Mammalian lens contains three major structural proteins that are known as α-, β-, and γ-crystallins. Among these, α- and β-crystallins exist as oligomers whereas γ-crystallin exists as a monomer. These structural proteins, by virtue of their special structural interactions and high concentrations, contribute to the transparency of the lens and provide refractive index for focus of light onto the retina. With aging, crystallins undergo several modifications that lead to their aggregation and cross-linking, water insolubilization, and finally to cataract development. Based on recent studies of water insoluble proteins from human lenses [1,2], the major in vivo modifications of human water insoluble lens crystallins are disulfide bonding, deamidation, methionine oxidation, and backbone cleavage.

The truncation of α- [1,3,4], β- [5–9], and γ- [10,11] crystallins has been well established. An understanding of these cleavage sites in crystallin is important because after truncations, the crystallin function to maintain lens transparency may be affected. Furthermore, the crystallin fragments per se may undergo post-translational modifications.

* Corresponding author. Fax: 1-205-934-5725.
E-mail address: Srivastava@icare.opt.uab.edu (O.P. Srivastava).

0006-291X/02/$ - see front matter © 2002 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0006-291X(02)02975-3

Based on the present literature, it is unclear whether βB2 crystallin undergoes age-related truncation in human lenses. A recent report suggested that human βB2-crystallin resists age-related in vivo modifications including truncation during aging [12]. It is proposed that by virtue of the property of being least modified than any other crystallin, the βB2-crystallin might keep other β-crystallins soluble [12]. This report contrasts with the earlier reports showing age-related truncation of βB2-crystallin at the N-terminus [13,14]. The authors of the recent report [12] suggested that a truncated 23-kDa βB2-crystallin species, detected in the earlier report [14], could be due to false immunoreactivty of the antibody to a truncated βA3 species. This might have happened because the antibodies, prepared against a synthetic peptide corresponding to residue nos. 1–12 and 195–204, were based on erroneous bovine βB2-crystallin sequences [15] and not on a later published human βB2-crystallin sequence [16]. Furthermore, the peptide used for antibody production had five residues with four contiguous that were identical to N-terminus of βA3 [12]. Additionally, the βA3-crystallin on aging showed a truncated species of about 23 kDa [7] with M_r identical to the previously immunologically detected truncated βB2 species [13]. However, a major problem in both previous investigations was that none of the species lower than 23 kDa were detected because of use of 12% polyacrylamide gels. Therefore, the analysis of truncated βB2-crystallin species with $M_r < 23$ kDa in human lenses is warranted and this is the focus of our study.

In the human lens, the earliest post-translational modification was reported to be the truncation of βB1- and βA3/A1-crystallins [17], and both crystallins were further degraded with age [7]. Generally in the mammalian lenses the truncations of βA3/A1-crystallin [7,9] and βB2 [5] mainly occur at the N-terminal region. The C-terminal region of βB2-crystallin is also proteolyzed, suggesting its exposure on the outside of the molecule in its native state [18]. A possible consequence of the C-terminal truncation of β-crystallin may be a change in its interaction with other β-crystallins for oligomerization, because the NMR studies showed that the C-terminal of βB2 crystallin is involved in interaction with other crystallins [19]. Furthermore, the crystallin fragments could also undergo extensive modifications [20]. A 10-kDa crystallin fragment has been shown to be ten times more glycated compared to non-truncated (native) crystallins in the human cataractous lenses [21]. This suggested that the crystallin fragments might be relatively more prone to glycation in vivo compared to full-length crystallins.

The purpose of this study was to determine in vivo cleavage of βB2-crystallin by examining its fragments in the β_H-crystallin fraction of human lenses. As stated above, because previous studies have identified β-crystallin fragments with $M_r > 18$–19 kDa (e.g., Lampi et al. [7] identified cleavage sites in human β-crystallins with M_r's ranging between 19,951 and 27,935), the focus of this study was to identify the truncated βB2-crystallin species with $M_r < 18$–19 kDa in the β_H-crystallin fraction of human lenses. The results presented show extensive truncation of βB2-crystallin during aging with certain specific cleavage sites in the crystallin.

Materials and methods

Materials. Normal human lenses with no apparent opacity were obtained from Dr. Robert Church of the Emory University. The lenses were retrieved within 48-h post-mortem and stored in medium-199 without phenol red at −20 °C until used. The prestained and unstained molecular weight markers were from Life Sciences and Amersham Biosciences, respectively. Unless indicated otherwise, all the other chemicals used in this study were purchased from Sigma or Fisher companies.

Two-dimensional gel electrophoretic analysis of crystallin fragments present in β_H-crystallin fraction. The WS protein fraction was isolated from lenses of desired ages by a procedure as previously described [10]. The WS-protein fraction was further fractionated by a size-exclusion Agarose A 5 m column to recover α-, β_H-, β_L-, and γ-crystallin fractions. Because the column fractions analyzed M_r of proteins by SDS–PAGE prior to pooling of different fractions, the fractions truly represented individual α-, β_H-, β_L-, and γ-crystallin peaks. Each preparation was concentrated by lyophilization and dialyzed at 5 °C for 24 h prior to its use. The 2-D gel electrophoretic analysis of β_H-crystallin fractions isolated from WS-protein fractions of lenses of 27- and 70-year-old donors was performed as described previously [11]. During the protein separation in the first dimension, Immobilized Dry Strips (Amersham Biosciences) with a pH range of 3–10 were used, whereas SDS–PAGE with 15% polyacrylamide gels was used in the second dimension.

Miscellaneous methods. The partial N-terminal sequencing of the desired fragments was performed at the core facilities of University of Missouri at Columbia or University of Alabama at Birmingham. For this purpose, the crystallin fragments were transferred from a 2D-gel to a PVDF membrane [22], briefly stained with Coomassie blue, and individual spots were excised and used for sequencing. The sequences of desired fragments were matched with those of published sequences of human lens crystallins. For a sequence search, the on-line search (via SEQSRCH) of database of Protein Research Foundation (Japan) was used. Protein concentration was determined by using BCA Protein Assay Reagent (Pierce) with bovine serum albumin as a standard. SDS–PAGE was performed by the method of Laemmli [23].

Results

The WS protein fractions from lenses of 27-year-old and 70-year-old donors were separated by an agarose A 5 m (BioRad) column to recover α-, β_H-, β_L-, and γ-crystallin fractions as shown in Fig. 1. As stated above, the M_r's of proteins in the column fractions under each peak were carefully examined by SDS–PAGE to avoid cross-contamination during the recovery of the above four fractions. The protein elution profile of individual crystallin peaks from lenses of 27-year-old donors (not shown) was similar to the protein elution profile from lenses of 70-year-old donors and the fraction nos. 35–44 constituting the β_H-crystallin peak were collected (Fig. 1).

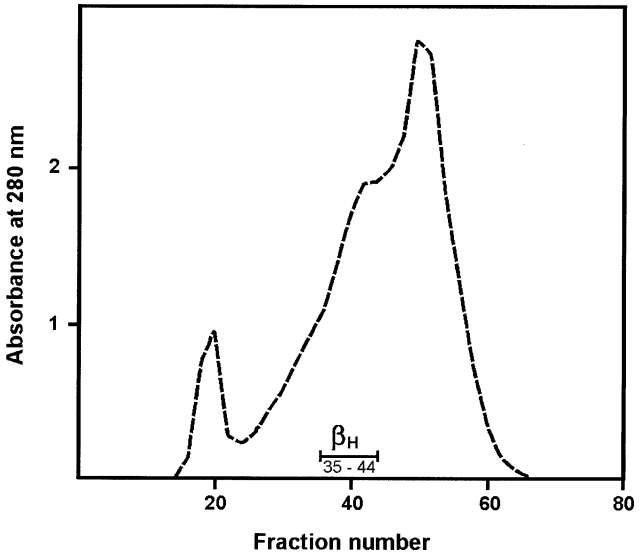

Fig. 1. Agarose A 5 m size-exclusion chromatography of WS proteins isolated from lenses of 70-year-old donors. The WS protein fraction (30 mg) was fractionated by an Agarose A 5 m column (2.5 × 75 cm) and the protein peaks were visualized at 280 nm. The α-, β_H-, β_L-, and γ-crystallins were identified by their elution peaks and from M_r of the protein species present in the column fractions after SDS–PAGE analysis. The β_H-crystallin fractions represented by fraction nos. 35–44 were pooled, concentrated by lyophilization, dialyzed, and analyzed by 2D-gel electrophoresis. The elution profile at 280 nm of the WS protein fraction from lenses of 27-year-old donors was similar (not shown) and after SDS–PAGE analysis, the β_H-crystallins constituting fraction nos. 35–44 were pooled, treated as above, and used for 2D-gel electrophoretic analysis.

On comparison of 2D-gel electrophoretic profiles of β_H-crystallin fractions from lenses of 70-year-old (Fig. 2) and 27-year-old (Fig. 3) donors, several common crystallin fragments (M_r < 18–19 kDa, identified by numbers) were seen. A careful comparison was carried out by identifying 37 crystallin fragments (referred to as

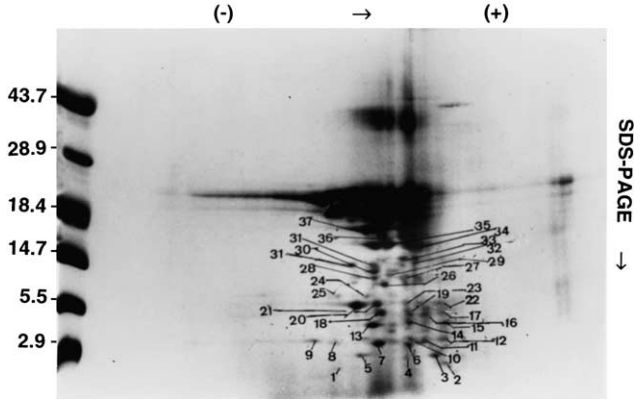

Fig. 2. Two-dimensional gel electrophoretic separation of protein species present in β_H-crystallin fraction of lenses from 70-year-old donors. Approximately 2.5 mg protein was used for the 2D-gel analysis. The spot identified by numbers in the figure is comparable to the spot numbers of Fig. 3.

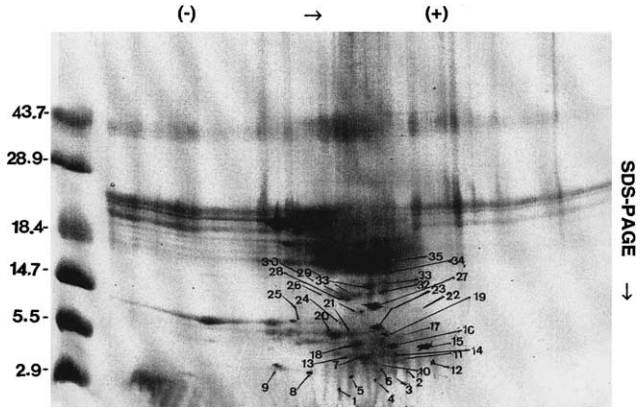

Fig. 3. Two-dimensional gel electrophoretic separation of protein species present in β_H-crystallin fraction of lenses from 27-year-old donors. Approximately 3.0 mg protein was used for the 2D-gel analysis.

spots) with M_r between 3 and 19 kDa in the β_H-crystallin fraction of 70-year-old lenses (Fig. 2). Each spot was assigned a number, beginning with the one with the lowest molecular weight of 3 kDa to the highest of 19 kDa. The spots, previously identified as βA3/A1-crystallin fragments [9], were not assigned a number. Because of the overlapping spots in β_H-crystallin fractions of both types of lenses, their common presence was noted. However, certain spots that existed in the 27-year-old lenses were absent in the 70-year-old lenses or vice versa.

Because of our focus on age-related degradation of βB2-crystallin, the spots in the 2D-gel of 70-year-old lenses were used for further sequence analysis. The partial N-terminal sequences of the desired fragments from 70-year-old lenses were matched with the human lens crystallin sequences using an on-line search (via SEQSRCH) of database of Protein Research Foundation (Japan). The spot numbers, their amino acid sequences, matching with the crystallin sequences, and their cleavage sites are shown in Table 1. The majority of fragments were from βB2-crystallin, but additional single fragments of αA-, βA4-, γs-crystallins, and of either γB-, γC- or γD- (all three showed identical sequences at the cleavage site) were also observed. Seven cleavage sites in the βB2-crystallin were identified which included, Q_7–A_8, A_8–G_9, E_{46}–K_{47}, G_{49}–S_{50}, S_{94}–S_{95}, N_{115}–F_{116}, and Q_{135}–Y_{136}. A cleavage site at E_{112}–N_{113} in βA4- crystallin, at G_4–T_5 in γs-crystallin, and at M_{69}–G_{70} in either γB-, γC- or γD-crystallins (the three have identical sequences at the cleaved bond) and G_1–K_2 in γB or γC (both have identical sequences, and the cleavage site), and at Q_{54}–Y_{55} in αA-crystallin were observed.

The cleavage sites in different regions of βB2-crystallin are identified in its sequence in Fig. 4. Two cleavage sites at Q_7–A_8 and A_8–G_9 bonds were observed in the N-terminal extension of the crystallin. Similarly,

Table 1
Identification of cleavage sites in crystallins to generate fragments present in β_H-crystallin fraction of lenses from a 70-year-old donor

Spot number (in Fig. 1)	Approximate mol. wt. (kDa)	Sequence	Cleavage site in crystallin	
1	4	S–L–R–P–I	βB2	S_{94}–S_{95}
3	4	N–F–L–G–K–K	βA4	E_{112}–N_{113}
4	4.5	F–T–G–K–K	βB2	N_{115}–F_{116}
5	4.5	A–G–K–P–Q–S	βB2	Q_7–A_8
6	5	S–L–R–P–I	βB2	S_{94}–S_{95}
8	5	S–V–L/R–L/V–Q	βB2?	G_{49}–S_{50}
9	5.5	Y–Q–E–K–V	βB2	G_{135}–Y_{136}
13	5.5	S–L–R–P–I	βB2	S_{94}–S_{95}
19	9	T–K–I–T–F	γS	G_4–T_5
27	12	G–L–S–D–S	γB/γC/γD	M_{69}–G_{70}
29	13	G–K–I–T–F	γB/γC	G_1–K_2
32	14	Y–F–L–R–R	αA	Q_{54}–Y_{55}
33	14.5	K–A–G–S–V–L	βB2	E_{46}–K_{47}
37	19	G–K–P–Q–S	βB2	A_8–G_9

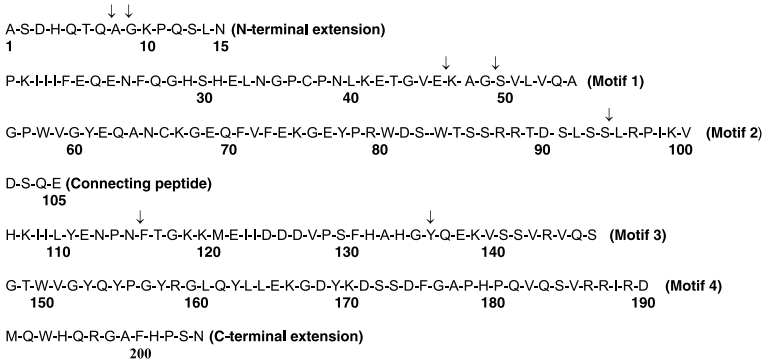

Fig. 4. Cleavage sites in different regions of human βB2-crystallin based on the partial N-terminal sequences of βB2-crystallin fragments as listed in Table 1.

two cleavage sites at E_{46}–K_{47} and G_{49}–S_{50} bonds in the motif 1, one cleavage site at S_{94}–S_{95} in the motif 2, and two cleavage sites at N_{115}–F_{116} and Q_{135}–Y_{136} in the motif 3 were observed. No fragment with cleavage sites in the motif 4 and in the C-terminal extension of βB2-crystallin were seen in our analysis.

Discussion

The purpose of this study was to identify in vivo produced fragments of βB2-crystallin in the β_H-crystallin fraction of human lenses and also to identify the bonds cleaved during βB2 truncation. Our focus was to identify the fragments with M_r lower than 18–19 kDa because the fragments with M_r's > 18 kDa have been previously identified [7].

A major finding of the study was that the β_H-crystallin fraction contained nine βB2-crystallin fragments with M_r between 3 and 18 kDa, which were generated due to cleavages at seven different sites in the crystallin. These cleavages were observed in the N-terminal extension and in the motifs 1, 2, and 3, but not in the motif 4 and the C-terminal extension. Although this finding was in contrast to a recent report [12], which suggested a greater age-related stability of the βB2-crystallin relative to other β-crystallins, yet it confirmed two previous reports [13,14] of age-related truncation of βB2-crystallin. The M_r of the fragments of βB2-crystallin ranged between 4 and 19 kDa (Fig. 3), which included three βB2-crystallin fragments of 5.5 (spot no. 9), 14.5 (spot no. 33), and 19 kDa (spot no 37) with cleavage sites at G_{135}–Y_{136}, E_{64}–K_{65}, and A_8–G_9 bonds, respectively. Because these three fragments contained the expected number of amino acids that matched with their approximate molecular weights, this suggested that the three species were apparently cleaved only at their N-termini but not at the C-termini. A similar comparison between approximate M_r' and cleavage sites in the remaining six βB2-crystallin fragments suggested that these were cleaved at both N- and C-terminal regions. For instance, the spot number 1 (Fig. 2 and Table 1) with M_r of about 4-kDa with only the N-terminal cleavage sites at S_{94}–S_{95} would produce a fragment with 100 amino acid residues (approximate M_r of 10,000) because human βB2 has a total of 204 residues. Instead, the M_r of only 4-kDa of the fragment

suggests a further truncation at the C-terminal region. A similar argument could be made regarding cleavage at both N- and C-termini for other the βB2-crystallin fragments, i.e., two 4.5-kDa fragments (spot nos. 4 and 5), two 5-kDa fragments (spot nos. 6 and 8), and a 5.5-kDa fragment (spot no. 9).

Additional single fragments of αA-, γs-, βA4-, and β-crystallins were also seen in the β_H-crystallin fraction. The αA-crystallin fragment of 14-kDa (spot no. 32) with a cleavage site at Q_{54}–Y_{55} might be produced because of the cleavage at both N- and C-terminal regions in the crystallin. Similarly, the fragments of γs- and other γ-crystallin (spot nos. 19 and 27, respectively) were apparently derived due to cleavages at both N- and C-terminal regions in the respective crystallins. However, the cleavage site and M_r of a 13-kDa γ-crystallin fragment (spot no. 29) suggested that it might be produced due to cleavage only at the N-terminal region in the crystallin.

The association of an αA-crystallin fragment, following cleavage at Q_{54}–Y_{55}, with β_H-crystallin oligomer was surprising, it shows in vivo oligomer formation between an αA-fragment and native β-crystallins. The same arguments can be made regarding an association of fragments of γs- and of other γ-crystallins with the β-crystallin oligomer. Because the results were from lenses from a 70-year-old donor, the oligomerization among αA- and γs- and other γ-crystallin fragments with β-crystallins in the β_H-crystallin fraction could be an age-related phenomenon. However, because this was contrary to previous finding that even after truncation, the crystallin fragments remained associated with their parent crystallins [24], a more careful analysis is warranted. Nevertheless, the cross-contamination of peaks could be precluded because the α-crystallin fraction (isolated by Agarose A 5 m column) from lenses of 70-year-old donors contained over 20 truncated αA and αB-species (Srivastava; unpublished results), but only one αA-crystallin fragment (with cleavage at Q_{54}–Y_{55}) was found to be associated with the β_H-crystallin fraction.

Among crystallins, β- and γ-crystallins have a common tertiary structure containing two homologous domains. Each domain consists of two symmetrical "Greek key" motifs which are formed by four antiparallel β-sheets [25]. In mammalian lens, a family of seven homologous polypeptides of 22–32 kDa of β-crystallin are coded by a disperse gene family [26]. The β-crystallin aggregates of 50–200 kDa (designated as β_H- and β_L-crystallins) contain individual species, which are either acidic (βA1, βA2, βA3, and βA4), with only N-terminal extensions, or basic (βB1, βB2, and βB3), with both N- and C-terminal extensions. The β_H-crystallin fraction (M_r, 200,000) from bovine lenses contains aggregates of most of the β-crystallin subunits [27].

When bovine β_H-crystallin was dissociated into two fractions, the fraction without βB2-crystallin showed relatively less water solubility than the one with the crystallins [27]. The fact that β_H-crystallin can be concentrated to about 150 mg/ml and still remains soluble suggests that βB2 has a role in controlling oligomer formation and its stability [27]. This is further supported by a study in Philly mouse (a strain with inherited cataract with frame shift mutation in βB2-crystallin gene), which showed that an intact βB2-crystallin structure was essential for the proper folding into its tertiary structure [28]. Therefore, the truncation in βB2-crystallin at different sites as reported here may also lead to destabilized β_H-oligomer and may contribute to light scattering.

The N- and C-terminal extensions of β-crystallins are believed to play a role in oligomer formation via molecular interactions between different β-crystallins [29–31]. However, this role of the N- and C-terminal extensions is presently under debate [32]. The removal of 30-residues N-terminal extension from mouse recombinant βA3/A1-crystallin suggested that the extension was needed for the dimer formation [30]. A ^{1}H NMR spectroscopic study showed that the first 22 amino acid residues of βA3-crystallin could readily be detected, suggesting that this part of the molecule is not involved in the oligomerization [33]. Further, the N-terminal arm of βB2 retained its flexibility in complexes such as β_H- and β_L-crystallins fraction; whereas βA1- and βA3-crystallins showed an absence of resonance from the NMR spectra, suggesting that their N-terminal extensions are constrained [33]. The fact that these extensions are cleaved very early in life in rat [34], bovine [35], and during aging in human as reported here suggests their exposure to endogenous proteinases.

In a recent report, cleavages in the N-terminal extension of βB2-crystallin were predicted adjacent to helical domain [36]. A series of hydrophilic amino acid residues from Q9 to Q11 in βB2-crystallin were predicted to be with high propensity of helical conformation, and the cleavages by cysteine proteinases such as lens calpain were shown to be outside of helical region in the AKG triplet region. Our results also show (Fig. 2 and Table 1) that the triplet was cleaved in vivo in the N-terminal arm of human βB2-crystalin and therefore calpain may be involved. This is consistent with the fact that in the rat lens, a calpain-cleaved βB2-crystallin fragment with cleavage site at Q_6–A_7 was observed [34].

Six in vivo cleavage sites in βB2-crystallin as described in this report may be located in the non-helical regions of the molecule. Presently, we do not know the role of the endogenous proteinases in this process and neither the functional consequences of these truncations. As shown in Table 1, the in vivo cleavage sites in different crystallins suggest that the C-terminal regions of S, G, E, and Q are the preferred sites by lens endogenous proteinases. We are presently examining proteolysis of human βB2-crystallin by endogenous proteinases to determine whether indeed the above are the preferred sites.

Acknowledgments

The authors express their sincere appreciation to Ms. Martha Robbins for help during the preparation of the manuscript. This work is supported by Grants from the National Eye Institute (EY 06400 and EY O339), Retirement Research Foundation, and Faculty Development Award from the University of Alabama at Birmingham.

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