The Sequence of Human βB1-Crystallin cDNA Allows Mass Spectrometric Detection of βB1 Protein Missing Portions of Its N-terminal Extension*

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Larry L. David,† Kirsten J. Lamp, Anders L. Lund, and Jean B. Smith

From the †Departments of Oral Molecular Biology and Ophthalmology, Oregon Health Sciences University, Portland, Oregon 97201 and the ‡Department of Chemistry, University of Nebraska, Lincoln, Nebraska 68588

The sequence of human βB1-crystallin cDNA encoded a protein of 251 amino acids in length. Mass spectrometric analysis of intact βB1 from young human lens confirmed the deduced amino acid sequence. Lenses of human donors newborn to 27 years of age also contained firmed the deduced amino acid sequence. Lenses of human residue 15 and 16 in human extensions. The similarity of the cleavage site between residues 15 and 16 in human βB1 to the cleavage occurring in bovine βB1 suggested that lenses of both species may contain a similar proteolytic activity. The remaining cleavage sites occurring in human βB1 did not closely match those occurring in other species, possibly due to the widely divergent amino acid sequence of the N-terminal extension of βB1 among species. Results from animal models suggest that cleavage of the N-terminal extension of βB1-crystallin could enhance protein insolubilization and cataract in lens. However, the presence of partially degraded βB1-crystallins in both water-soluble and water-insoluble fractions of lenses of young donors suggested that the rate that proteolyzed βB1-crystallins become water-insoluble is relatively slow in humans.

The lens is a unique organ in that 20–60% of its wet weight is composed of proteins called crystallins (1). Mammalian crystallins can be divided into two broad classes, the α-crystallins, which share homology to heat shock proteins and exhibit chaperone-like properties (2), and the γ-crystallin family, which like α-crystallins, also share a common ancestor but perform primarily a structural role. The individual proteins of γ-crystallin family contain four homologous motifs, each folded in a “Greek key” pattern (3). These motifs are organized into two equivalent domains, which are connected by approximately 4 amino acid residues, the entire structure thus forming the core of the protein. There are seven different β-crystallin proteins in bovine lens. These are named βA1, βB2, βB3, βA1, βA2, βA3, and βA4 (4). Each crystallin transcript is translated to yield a single protein species, except the mRNA coding for βA3, which also yields βA1, owing to an alternate downstream initiation codon (5). Orthologous β-crystallins are also found in other vertebrate species. Except for βA2, orthologs of all known β-crystallin proteins have been demonstrated in rat (6). While the presence of all protein products has not been established in the chicken, the cDNAs of all six β-crystallin transcripts have been sequenced (7). In human lens, the presence of βB1 (8), βB2 (9), βA3 (10), and βA4 (11) have been documented. However, to date, there is no evidence for the significant accumulation of βA1, βA2, or βB3 proteins in postnatal human lens.

All β-crystallins contain N-terminal extensions ranging from 10 to 58 amino acids in length as well C-terminal extensions of approximately 15 amino acids in the βB-crystallin subgroup (4). These N- and C-terminal extensions are missing in γ-crystallins. Since β-crystallins form complex mixtures of homodimers, heterodimers, and higher order structures, while γ-crystallins do not, it has been hypothesized that the extensions function to stabilize the intermolecular associations between β-crystallins (11). It is likely not a coincidence that βB1, which contains the longest N-terminal extension, is also selectively found in the largest β-crystallin aggregate (12). Thus, recent work has explored the possible function of β-crystallin N-terminal extensions in stabilizing the structure of β-crystallin oligomers (13, 14, 16, 17). We hypothesize that the presence of β-crystallin N-terminal extensions is important, because removal of from 2 to 49 residues from the N-terminal extensions of various β-crystallin subunits in lenses of young rodents was associated with protein insolubilization and cataract formation (6, 18). The mechanism of protein insolubilization following shortening of the N-terminal extensions is unknown. However, it presumably involves an alteration in the manner the β-crystallin subunits oligomerize. The specific cleavages in the N-terminal extensions may result from activation of the protease calpain II (18). Similar cleavage of β-crystallin N-terminal extensions and insolubilization of β-crystallins also occurs during maturation of normal rat lens (19). However, these older lenses may remain transparent due to the relatively slower rate that the process ensues during normal development.

A major focus of these laboratories is to determine if similar partial degradation and insolubilization of β-crystallins also occurs in human lenses. In human lenses, the amount of water-soluble β-crystallin decreases dramatically with aging, and partial degradation of βB2 has been reported (20). Similarly, a 29-kDa β-crystallin identified as βB1, was prominent in fetal human lenses, but it was nearly gone by age 5 (21). Thus, evidence exists for the partial degradation of β-crystallins during aging of human lenses. However, few detailed studies exist that examine cleavage sites in human β-crystallins. These studies are limited by the lack of sequence data for human

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†To whom correspondence should be addressed: 611 S.W. Campus Dr., Portland, OR 97201. Tel.: 503-494-8625; Fax: 503-494-5976; E-mail: david@ohsu.edu.

‡To whom correspondence should be addressed: 611 S.W. Campus Dr., Portland, OR 97201. Tel.: 503-494-8625; Fax: 503-494-8918; E-mail: david@apm.com.

†To whom correspondence should be addressed: 611 S.W. Campus Dr., Portland, OR 97201. Tel.: 503-494-8625; Fax: 503-494-8918; E-mail: david@apm.com.
Cloning and Sequencing of Human βB1 Crystallin—Total RNA was isolated from both freshly enucleated or –70 °C frozen lenses of human donors of 10 years of age or less (Lions Eye Bank, Portland, OR) (24). cDNA was synthesized by reverse transcription of total lens RNA using oligo(dT) containing adapter primer (3′-RACE2 system, Life Technologies, Inc.). Following degradation of the RNA template by RNase H, the total lens cDNA was purified by GlassMAX spin cartridges, and the cDNA was then homopolymer tailed using dCTP and terminal deoxynucleotidyl transferase (5′-RACE system, Life Technologies, Inc.). Cycling conditions for all PCR used 94 °C for 5 min, (94 °C for 45 s, 54 °C for 45 s, 72 °C for 60 s) × 35 cycles, ending with 72 °C for 10 min. Conditions recommended by the 5′-RACE system were used (Life Technologies, Inc.), except AmpliWax beads were employed (Perkin Elmer Corp.). The lens cDNA was subjected to 5′-RACE PCR using the 5′-RACE anchor primer, and the gene-specific antisense primer TTATCAGGCAGAC (primer 2) complementary to TGATGGGCCGGAAGGACATGAGCCGGTC (primer 3) corresponding to residues 293–320 of rat βB1 cDNA (25). The resulting sequence was used to choose two human βB1-specific primers, a sense primer CUACUAUCUAAGTCTGCAAATCTGGCAGAC (primer 1) complementary to GAAGTTGGCCCCTTCAAACAGGCAGA (primer 2) complementary to RACE anchor primer, and the gene-specific antisense primer TTATCAGCCGAGGCAGAC (primer 3) corresponding to residues 308–327 of human βB1 cDNA (26). The resulting sequence was directly sequenced using the AutoRead sequencing kit and automated laser fluorescence DNA analysis system (Pharmacia Biotech, Inc.). The sequencing reaction was primed using a 5′-fluorescein-labeled primer 2. The resulting sequence was used to choose two human βB1-specific primers, a sense primer CUACUAUCUAAGTCTGCAAATCTGGCAGAC (primer 1) complementary to GAAGTTGGCCCCTTCAAACAGGCAGA (primer 2) complementary to RACE anchor primer, and a nested gene-specific antisense primer TTATCAGGCAGAC (primer 3) corresponding to residues 308–327 of human βB1 cDNA. The human βB1 cDNA was amplified by 5′- and 3′-RACE PCR, cloned, and both strands were sequenced. The numbers to the right indicate the nucleotide numbers, while the numbers below indicate the amino acid number of the deduced protein sequence of 251 residues. The poly(A) addition signal is underlined.

B1 and its

The reported sequences represent the complete lengths of the deduced protein sequence of 251 residues. The poly(A) addition signal is underlined.

β-crystallins. Only the complete sequences of human βB2 and βA3 are known (22, 23). This study reports the sequence of human βB1 cDNA and confirms the deduced amino acid sequence by mass spectrometry. With the knowledge of the sequence, we then found that partially degraded forms of βB1 missing various portions of its N-terminal extension were present in both water-soluble and water-insoluble fractions of human lens.

**Materials and Methods**

**Cloning and Sequencing of Human βB1 Crystallin—** Total RNA was isolated from both freshly enucleated or –70 °C frozen lenses of human donors of 10 years of age or less (Lions Eye Bank, Portland, OR) (24). cDNA was synthesized by reverse transcription of total lens RNA using oligo(dT) containing adapter primer (3′-RACE2 system, Life Technologies, Inc.). Following degradation of the RNA template by RNase H, the total lens cDNA was purified by GlassMAX spin cartridges, and the cDNA was then homopolymer tailed using dCTP and terminal deoxynucleotidyl transferase (5′-RACE system, Life Technologies, Inc.). Cycling conditions for all PCR used 94 °C for 5 min, (94 °C for 45 s, 54 °C for 45 s, 72 °C for 60 s) × 35 cycles, ending with 72 °C for 10 min. Conditions recommended by the 5′-RACE system were used (Life Technologies, Inc.), except AmpliWax beads were employed (Perkin Elmer Corp.). The lens cDNA was subjected to 5′-RACE PCR using the 5′-RACE anchor primer, and a nested gene-specific antisense primer TTATCAGGCAGAC (primer 2) complementary to TGATGGGCCGGAAGGACATGAGCCGGTC (primer 3) corresponding to residues 293–320 of rat βB1 cDNA (25). The resulting sequence was used to choose two human βB1-specific primers, a sense primer CUACUAUCUAAGTCTGCAAATCTGGCAGAC (primer 1) complementary to GAAGTTGGCCCCTTCAAACAGGCAGA (primer 2) complementary to RACE anchor primer, and the gene-specific antisense primer TTATCAGGCAGAC (primer 3) corresponding to residues 308–327 of human βB1 cDNA (26). The human βB1 cDNA was amplified by 5′- and 3′-RACE PCR, cloned, and both strands were sequenced. The numbers to the right indicate the nucleotide numbers, while the numbers below indicate the amino acid number of the deduced protein sequence of 251 residues. The poly(A) addition signal is underlined.

**Confirmation of the Deduced Protein Sequence of βB1—** The tryptic digests of the proteins on the two-dimensional gel corresponding to βB1 and its degradation product from newborn human lens were each analyzed by a laser fluorescence DNA analysis system (Pharmacia Biotech, Inc.). The resulting sequence was used to choose two human βB1-specific primers, a sense primer CUACUAUCUAAGTCTGCAAATCTGGCAGAC (primer 1) complementary to GAAGTTGGCCCCTTCAAACAGGCAGA (primer 2) complementary to RACE anchor primer, and the gene-specific antisense primer TTATCAGGCAGAC (primer 3) corresponding to residues 308–327 of human βB1 cDNA. The human βB1 cDNA was amplified by 5′- and 3′-RACE PCR, cloned, and both strands were sequenced. The numbers to the right indicate the nucleotide numbers, while the numbers below indicate the amino acid number of the deduced protein sequence of 251 residues. The poly(A) addition signal is underlined.
Bovine sequence identity with bovine, rat, and chicken \( \beta \)-crystallin species are indicated. The parent \( \beta \)-B1 protein, and its partial degradation product, \( \beta \)-B1 \((-15)\), were cut from these blots and trypsinized, and the masses of the resulting fragments were measured as shown in Fig. 4.

**RESULTS**

The Nucleotide and Deduced Amino Acid Sequence of Human \( \beta \)-B1 cDNA and Protein—The cDNA of human \( \beta \)-B1-crystallin was amplified by 5'- and 3'-RACE PCR, ligated into plasmid vector pAMPP10, and both strands of each insert were sequenced. Excluding the dc homopolymer region introduced for 5'-RACE PCR and the poly(A) tail on the 3'-end, the overlapping sequences of the 5'- and 3'-RACE PCR products resulted in the 923-base pair sequence shown in Fig. 1 (GenBank accession number U35340). The initiation codon began at nucleotide 73, resulted in an open reading frame of 756 nucleotides, and ended with a stop codon at nucleotides 829-831. The 3'-end of the cDNA contained a 92-base pair noncoding region, and a poly(A) addition site at nucleotides 905-910. The 5'-end of the cDNA contained a 72-base pair noncoding region, suggesting that the 5'-end of the cDNA may be complete. Excluding the N-terminal methionine, the human \( \beta \)-B1 cDNA coded for a protein 251 amino acids in length.

There are evolutionary constraints upon the sequence of the water-soluble portion of the 27-year-old lens was digested with trypsin (50:1, substrate/enzyme) at pH 8.2 for 4 h. The resulting peptides were separated by reversed-phase HPLC using a Vydac C-18 column and a linear gradient of 1-40% CH\(_3\)CN over 60 min and then to 65% CH\(_3\)CN at 70 min. The fractions were collected, and the molecular masses of the peptides present in each fraction were determined by fast atom bombardment mass spectrometry (FABMS) (Kratos MS-50, Kratos Analytical, Manchester, United Kingdom) or by ESIMS. The water-insoluble guanidine-soluble portions of all three young adult lenses were examined for the absence of intact and partially degraded \( \beta \)-B1. The monomeric portion isolated by gel filtration was fractionated by reverse-phase HPLC, resulting in five poorly resolved peaks (28). Each peak, except for the first for which there was insufficient material, was digested with trypsin as described previously for the water-soluble portion, and the molecular masses of the resulting peptides were determined by fast atom bombardment mass spectrometry or ESIMS. Accuracy of the molecular mass determinations was within 0.3 Da. Identification of peptides was facilitated by computer programs written in our laboratory (29). When a peptide identification based on molecular mass was ambiguous, the portion (90%) of the sample that had been diverted before the ESIMS analysis was further analyzed 1) by peptide sequencing (Purdue Laboratory for Macromolecular Structure), 2) by further digestion with carboxypeptidase or pepsin followed by mass spectrometric analysis of the products, or 3) by analysis of the MS/MS fragmentation pattern of the peptide. MS/MS analyses were performed on a Fisons VG-Autospec Mass Spectrometer equipped with an orthogonal acceleration time-of-flight analyzer.

ESIMS Analysis of \( \beta \)-Crystallins without Tryptic Digestion—Undigested \( \beta \)-crystallins, isolated from a 20-year-old lens by gel filtration, were analyzed by reverse-phase HPLC/ESIMS to obtain the molecular masses of the proteins eluting at 43-45% CH\(_3\)CN where \( \beta \)-B1 was expected to elute. Because this sample was a mixture, the molecular masses of only the major components are reported. Mass accuracy for the ESIMS determinations was within 0.025%.

Confirmation of the Deduced \( \beta \)-B1 Amino Acid Sequence by Mass Analysis of \( \beta \)-B1 from Newborn Human Lenses—Water-soluble protein from newborn human lenses was separated by two-dimensional electrophoresis, and individual protein species were identified by either direct Edman sequence analysis, or Edman sequence analysis of tryptic fragments followed by comparison to deduced amino acid sequences from reported cDNA sequences. The identities of the major protein species are indicated in Fig. 3. The partially degraded \( \beta \)-B1-crystallin
labeled β1 (1–15) was identified based on the N-terminal sequence PGPDTH, which indicated it was missing 15 residues from its N terminus (Fig. 2).

The regions containing intact β1 and βB1 (1–15) were digested with trypsin, and the masses of the resulting fragments were analyzed. The peptides from these digests gave masses corresponding to all expected tryptic peptides in the mass range analyzed, confirming the deduced amino acid sequence (Fig. 4). The mass of 545 Da for the N-terminal peptide indicated that the N-terminal methionine was removed, and that the N-terminal Ser was acetylated. The mass of the peptide containing Cys-79 appeared 71 Da higher than expected due to the formation of an acrylamide adduct during the two-dimensional gel separation (33). The deduced protein sequence in Fig. 1 has a calculated molecular mass of 27,935 Da, agreeing very well with the ESIMS-determined molecular mass of 27,933 Da reported by He et al. (10). Mass spectral analysis of β1 (1–15) also gave all the expected tryptic peptides, except those corresponding to residues 1–5 and 6–21. The presence of a peptide with a molecular mass corresponding to peptide 16–21 (613 Da) confirmed the Edman sequencing data, indicating that the protein had been cleaved between Asn-15 and Pro-16 (Fig. 4). A peptide with a mass corresponding to the C-terminal tryptic fragment indicated the presence of an intact C terminus in the young lens.

Detection of Additional Truncated Forms of β1 in the Water-soluble Crystallins of Lenses of Increasing Age—The HPLC/ESIMS analysis of the first eluting β-crystallin peak from gel filtration (also called β1H) from the 20-year-old lens indicated the presence of many components. The masses of the major components suggested that several proteins were degraded with a molecular mass of 27,935 Da for the intact protein, and the other masses, 26,538, 24,833, 24,394, and 24,296 Da were similar to the calculated molecular masses for residues 16–251 (26,535 Da), 35–251 (24,834 Da), 40–251 (24,391 Da), and residues 41–251 (24,294 Da), respectively. An ESIMS spectrum showing three of the identified degradation products is shown in Fig. 5. Analysis by ESIMS of the β1H-crystallins from a 14-month-old human donor also gave molecular masses indicating the presence of intact β1, βB1 (16–251), and βB1 (41–251), suggesting that further cleavage of the N-terminal extension of βB1 begins quite early in life.

Mass spectral analysis of the tryptic peptides produced from the water-soluble lens crystallins of young adult (age 27) suggested that additional forms of partially degraded βB1-crystallin were present. Even though this digest was a mixture of peptides from several proteins, peptides encompassing over 75% of βB1-crystallin could be identified from comparisons of their molecular masses and HPLC elution times with those found in the digest of βB1 from the newborn lens. Expected peptides derived from the N terminus of intact βB1 and βB1 (16–251) were not evident, probably because the N-terminal peptides from these species were present at very low levels, and the mass spectral response was masked by the presence of other more responsive peptides. However, the ESIMS data summarized above indicated that cleavage at Lys-49 by trypsin should produce peptides corresponding to peptides 35–49, 40–49, and 41–49. Ions with molecular masses corresponding to these three peptides were found. In addition, the digest contained ions with molecular masses consistent with the presence of peptides 34–49, 36–49, 37–49, and 42–49, indicating that there are numerous other N-terminal degradation sites of βB1-crystallin evident by age 27.

Detection of β1 in the Water-insoluble Crystallins—The water-insoluble portions of all three young adult lenses were examined for the presence of β1-crystallin. The results were very similar to the water-soluble β-crystallin fraction of adult lenses. Ions with masses corresponding to the majority of peptides from β1 were identified. However, the data suggested...
that most of the N-terminal extension of insoluble βB1 was missing. Neither peptide 1–5, peptide 6–21, nor peptide 16–21 were detected, and the response for peptide 24–49 was very weak. Also, the presence of peptides corresponding to residues 41–49 (916 Da) and 42–49 (815 Da) indicated N-terminal cleavage at these sites.

Because the tryptic digests of water-insoluble proteins were a complex mixture, the identities of many of the peptides, particularly those indicating the presence of N-terminal degradation, were confirmed. One of three methods was used for confirmation: 1) further digestion with another enzyme, such as pepsin or carboxypeptidase, followed by a second mass spectral analysis of these products; 2) Edman sequencing, if the peptide was pure; or 3) MS/MS analysis of the collision-induced fragmentation pattern of the peptide. Analysis of the MS/MS fragment patterns of the peptides at 916 Da and 815 Da, isolated from the 16-year-old lens, showed several fragments that confirmed the identities of the peptides as residues 41–49 and 42–49, respectively. The MS/MS spectrum for peptide 41–49 is shown in Fig. 6.

**DISCUSSION**

The major finding of this study was that βB1-crystallin of human lens undergoes extensive cleavage at its N-terminal extension during lens maturation. The first cleavage product of human βB1, βB1 (16–251), was already abundantly evident in lenses of a newborn donor. This suggested that βB1 may be the most protease susceptible crystallin in the human lens.

Alcala et al. (21) showed that from 8 months of gestation to 5 years of age, the percentage of intact βB1 in the water-soluble protein of human lens dropped from 10 to 0.5%. During the same interval, the percentage of a 27-kDa protein, corresponding to βB1 (16–251) of the present study, increased from 3.5 to 7%. Thereafter, the 27-kDa protein decreased, so that by 87 years of age it composed only 1.2% of the water-soluble lens protein. The loss of intact βB1 and transient accumulation of the 27-kDa protein also correlated with the age of the lens fiber. The conversion from intact βB1 to 27-kDa protein and the subsequent loss of 27-kDa protein was more pronounced in the deeper lens cortical and nuclear fibers than in the superficial cortex. These results are consistent with the present findings. βB1 and βB1 (16–251) were most abundant in newborn lenses. However, by early adulthood, tryptic fragments corresponding to the N terminus of intact βB1 and βB1 (16–251) were not detected, while tryptic fragments from forms of βB1 with more extensively degraded N termini were abundant.

Bovine βB1 also undergoes a similar cleavage as that producing human βB1 (16–251). Bovine βB1 is cleaved between Asn-14 and Pro-15 (34). The 6 amino acid residues surrounding this cleavage site are identical between bovine and human βB1 (Fig. 2). This suggested that both species contain a similar protease capable of cleaving at this site. Rat βB1 does not undergo cleavage at this site, possible due to the loss of the Pro residue found at positions 18 and 17 in human and bovine βB1, respectively (Fig. 2). Bovine βB1 also contained a cleavage site between Ala-11 and Ala-12 (34). Neither mass spectrometric analysis or Edman sequencing in the present study detected a similar cleavage in human βB1, possibly due to the lack of sequence identity in this region between bovine and human βB1.

Further cleavage of human βB1 occurred between residues 33 and 41. ESIMS analysis of βH-crystallins from a 2-year-old bovine lens did not yield molecular masses indicative of similar N-terminal degradation in this region. Instead, the bovine βB1 from a 2-year-old lens appears to have undergone even more extensive degradation, since the molecular masses of the proteins in βH-crystallin were all less than 25,000 Da (35). Bovine lens βB1 may not have undergone cleavage in regions similar to human βB1, because residues 35–44 in bovine βB1 contained Pro-Ala repeats not found in human βB1. Pro-Ala repeats in bovine βB1 and Pro-X repeats in rat βB1 may be more resistant to proteolytic attack than the corresponding region in human βB1.

Cleavage at five sites within the N-terminal extension of βB1 has also been reported during maturation of rat lens (Fig. 2). At least two of these sites in rat βB1 are confirmed calpain II cleavage sites (19). However, none of the cleavage sites within the N-terminal extension of rat βB1 corresponded in relative position to cleavage sites found in human βB1 (Fig. 2). The difference in the manner rat βB1 and human βB1 are degraded could be due to a lack of sequence identity at the respective...
cleavage sites. Sequences within the N-terminal extensions of βB1 from various species may have evolved to exhibit specific susceptibilities to proteolytic attack. Alternatively, the lenses of each species may contain proteolytic activities with different specificities. The predominate proteolytic activity responsible for partial degradation of β-crystallin N-terminal extensions in rat lens may be calpain II (19). However, the proteases responsible for degradation of the N-terminal extension of bovine and human βB1 remain unknown.

The extensive cleavage of the N-terminal extension of βB1 occurs quite early in life. This suggested that the cleavage plays an important role in the maturation process. In rat, the partially degraded forms of βB1 were found in only the water-insoluble fraction of the lens (19). Therefore, the cleavage of the rat βB1 N-terminal extension, as well as the N-terminal extensions of other β-crystallins, may rapidly induce protein insolubilization (6). Such insolubilization may be important in initiating dehydration and hardening of the rat lens (36). However, the results in the present study indicated that human β-cristallins respond quite differently following partial proteolysis. Partially degraded βB1 was found in both water-soluble and water-insoluble fractions of young human lens. Also, the majority of human βB1-crystallin is partially degraded before adulthood, but most crystallin insolubilization occurs in human lenses after the third decade of life (15). Future studies will determine if the water-insoluble fraction of aged lenses contains a greater proportion of partially degraded β-crystallins than does the water-soluble fraction. Also, the relationship between the extent of proteolysis and lens opacification requires closer examination.

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B1 Protein Missing Portions of Its N-terminal Extension

Larry L. David, Kirsten J. Lampi, Anders L. Lund and Jean B. Smith

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