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Alteration in the ubiquitin structure and function in the human lens: a possible mechanism of senile cataractogenesis

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Abstract High-performance liquid chromatography purification followed by mass spectrometry analyses highlighted that human senile cataractous lens includes a 8182 Da species which is absent in the normal lens, whereas a 8566/8583 Da species is present in both lenses. Western blot analysis identified both species as ubiquitin. The species at lower molecular weight is a shorter form due to the cleavage of the C-terminal residues 73–76. As it is the last amino acid of ubiquitin which is involved in the protein degradation mechanism, we suggest that this structure modification compromises the function of ubiquitin and consequently the physiologically occurring degradation of the lens proteins.

Key words: Senile cataract; Ubiquitin; Protein degradation

1. Introduction

The mammalian lens mainly consists of proteins, which account for over 30% of its weight [1]. Their highly ordered structure is responsible for the transparency of the lens [2]. Numerous changes in crystallin structure occur with age, mainly due to post translational non-enzymatic reactions [3]: protein synthesis and turnover are present mainly in the cortical region, and decrease with age in the nucleus. One important point to be considered is that lens denatured proteins are not removed by devoted cellular mechanisms; the numerous proteolytic enzymes which are present in the lens require structural markers to support their action.

Ubiquitin, a normal component of the lens, conjugate to proteins as a signal to initiate degradation. In the low molecular weight (LMW) fraction of human crystallins from cataractous lenses, we noted the presence of two forms of ubiquitin, one of which was not active, unlike the single form present in the clear human lenses of age-matched controls. Due to the age-related increase in sodium and calcium in the human lens [4], calcium-activated calpain II [5] (EC 34.22.17) may therefore be responsible for the alteration of the structure and function of ubiquitin, which is no longer capable of inducing degradation in damaged crystallins.

2. Materials and methods

2.1. Preparation of LMW fraction

The water-soluble fractions were processed by means of high-performance liquid chromatography (HPLC) apparatus (Waters Instruments Model 660) by using two sequential gel filtration BIO-SIL SEC 400 and 250 columns (Bio-Rad), equipped with a diode array photodetector. The columns were equilibrated with a 50 mM phosphate buffer, 0.1 M NaCl, pH 6.8 (flow rate 1 ml/min).

The suitable fractions were pooled, selectively filtered with a 10 kDa and 3 kDa Centricon filter and concentrated by lyophilization; the fractions were rechromatographed by reverse-phase (RP)-HPLC by using an analytical Vydac C_{18} column (4.6×150 mm) (Separation Group, Hesperia, CA, USA). The column was equilibrated with 0.01% trifluoroacetic acid TFA), elution was performed at room temperature with a linear gradient of acetonitrile from 0 to 60% in 40 min (flow rate of 1 ml/min). Fractions of 0.2 ml were collected, and absorbance peaks (260 nm) were pooled and evaporated to dryness. The dry samples were dissolved in distilled water and subjected to MALDI-TOF (matrix-assisted laser desorption/ionization-time-of-flight) and Western blot analyses.

2.2. MALDI-TOF

α-Cyano-4-hydroxy cinnamic acid (Fluka, Buchs, Switzerland) was used as matrix; the protein or peptide samples (1 μl from a solution 1 g/l in water) were loaded on the target and dried. Afterwards, 1 μl of a solution 10 mg/ml of matrix in a mixture of 0.1% TFA in H_2O and acetonitrile. The samples were analyzed with a Voyager DE-Pro (PerSeptive Biosystem, Framingham, MA, USA) mass spectrometer operating either in linear or in reflector mode.

2.3. Western blot analyses

The purified LMW fractions were separated by SDS–PAGE on a 20% gel and transferred to nitrocellulose membrane by a Bio-Rad transfer system. Blotting was performed for 1 h at 100 mA. After blotting, the membrane was treated with blocking buffer (0.2% casein, 1×PBS, 0.1% Tween-20) for 1 h. Thereafter the blot was treated with the primary antibody (rabbit anti-ubiquitin polyclonal antibody diluted 1:1000) for 2 h. The blot was then washed three times with PBS–Tween for 10 min. Immunoreactivity was detected using conjugated antibody anti-rabbit IgG alkaline phosphatase (diluted 1:5000) by a Western-Light™ immunodetection system (Tropix Company).

3. Results

Non-pathological clear human lenses (68–75 years old), obtained from an Eye Bank, were divided into cortex and nucleus by a sham surgical extracapsular approach. Nuclei from

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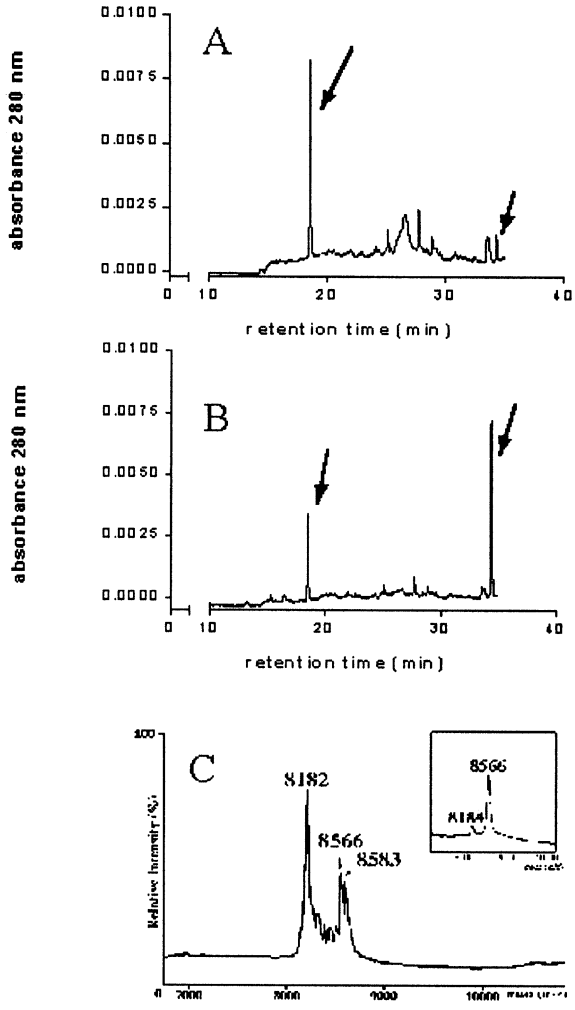

Fig. 1. RP-HPLC elution pattern by C_{18} column of LMW fraction purified from clear (panel A) and from cataractous lens (panel B). Elution was carried out at a flow rate of 1 ml/min, with a gradient of acetonitrile from 0 to 60% in 40 min. Panel C reports the MALDI-TOF spectrum of the fraction with rt 34.6 min obtained by cataractous lens and by clear lens (inset).

total cataractous lenses (age 70–75 years old) were obtained by surgery. Nuclei were homogenized in a 50 mM phosphate buffer, 0.1 M NaCl, pH 6.8, and centrifuged at 15 000 rpm for 15 min to obtain the water soluble fraction. Analytical HPLC of this fraction from clear and cataractous lens highlighted the presence of a very small protein fraction in the region of the LMW proteins.

This fraction (retention time, rt 23 min) was filtered and lyophilized. The normalized samples obtained from the nuclei of both clear and cataractous lenses were rechromatographed by RP-HPLC by using a Vydac C_{18} column. Fig. 1A shows the elution patterns of both samples. There is a clear difference between the areas of the two peaks, eluted at rt 18.6 min and 34.3 min, respectively, which were collected for further investigation. The absorption spectra of these fractions (not shown) highlighted different features: the first peak (rt 18.6 min) of both clear and cataractous lenses showed maximum absorbance centered at 260 nm, with a shoulder at 276 nm, which may identify a protein complex with a nucleotide cofactor. The second peak (rt 34.3 min) showed maximum absorbance at 276 nm in clear lenses, while the maximum shifted to 274 nm in cataractous lenses. The maximum absorbance at 276 nm is typical of proteins containing tyrosines, but not tryptophans. The shift at 274 nm, observed in the cataractous lenses, may have been due to the structural modification, or denaturation, of the same protein. Upon denaturation (3 M urea) of this purified fraction only one peak, with rt 38 min, was eluted from both samples (data not shown), with a maximum absorbance at 274 nm. This change in elution pattern may indicate that both peaks obtained in native conditions may contain the same protein, with or without a cofactor. We believe that under denaturant conditions, the weak bonds are broken and the cofactor is released. The shift to a higher retention time may be a consequence of protein denaturation, which increases the exposure of hydrophobic side chains.

Each peak obtained from RP-HPLC was analyzed by MALDI-TOF mass spectrometry (MS) in order to determine the molecular mass of the actual species. Fig. 1C shows the mass spectra of the 34.3 rt peaks from the cataractous and the transparent (inset) human lenses. In the cataractous lens, an intense peak at 8182 *m/z* was present, together with two less intense peaks at 8566 and 8583 *m/z*; in the transparent nuclei fraction, only the 8566 *m/z* peak was clearly present together with a barely detectable peak at 8184 *m/z*. Search in sequence databases suggests that the value 8566 *m/z*, which is present in both spectra, is coincident with the molecular weight of human ubiquitin (8565 Da), followed by its oxidized form at 8583 ± 1 Da. The identity of the 34.3 rt peak was confirmed by peptide mapping (see Fig. 2). The peak from transparent lens was digested using endoproteinase Asp-N, and the resulting peptide mixture analyzed by MALDI MS (see Fig. 2a). Peptides present in the spectrum were sequenced by MALDI PSD MS and the set of peptides obtained was mapped onto the known amino acid sequence of ubiquitin (see Fig. 2b), allowing verification of the entire sequence of ubiquitin with the exception of the region 18–31. This unambiguously demonstrated that the HPLC peak under investigation was indeed ubiquitin, and it also confirmed our spectral data, in good agreement with the absence of tryptophan in human ubiquitin and the presence of one tyrosine. Spectral features of the 18.6 min rt peak might be due the presence of native ubiquitin and ATP, which is required for ubiquitin function, or another nucleotide. The LMW protein (8182 *m/z*) which is present in high amounts in cataractous lenses may be an anomalous product generated by the action of protease. The peptide map obtained by Endoproteinase Asp-N digestion of the peak from cataractous lens (see Fig. 2c) was comparable to that of the control, but contained a further peptide at 1831.3 Da, corresponding to the sequence 58–72, in mixture with the expected C-terminal peptide 58–76 at 2214.3 Da, both identified by PSD experiments. The same component is observed as a small trace (8184 *m/z*) in transparent lenses (68–75 years old), while it is absent in young transparent lenses (20–23 years old, data not shown). The ratio of three forms observed in the cataractous lens, i.e. truncated form (8181 *m/z*), native form (8566 *m/z*), and oxidized form (8583 *m/z*), is approximately 3:1:1.

As a further support to the identification of the proteins under investigation, we performed a Western Blot analysis using a rabbit polyclonal anti-ubiquitin antibody (Fig. 3). Only one band proved evident in all the fractions tested from transparent lens nuclei, thus agreeing with the mass spectra, which indicated the presence of only one molecular

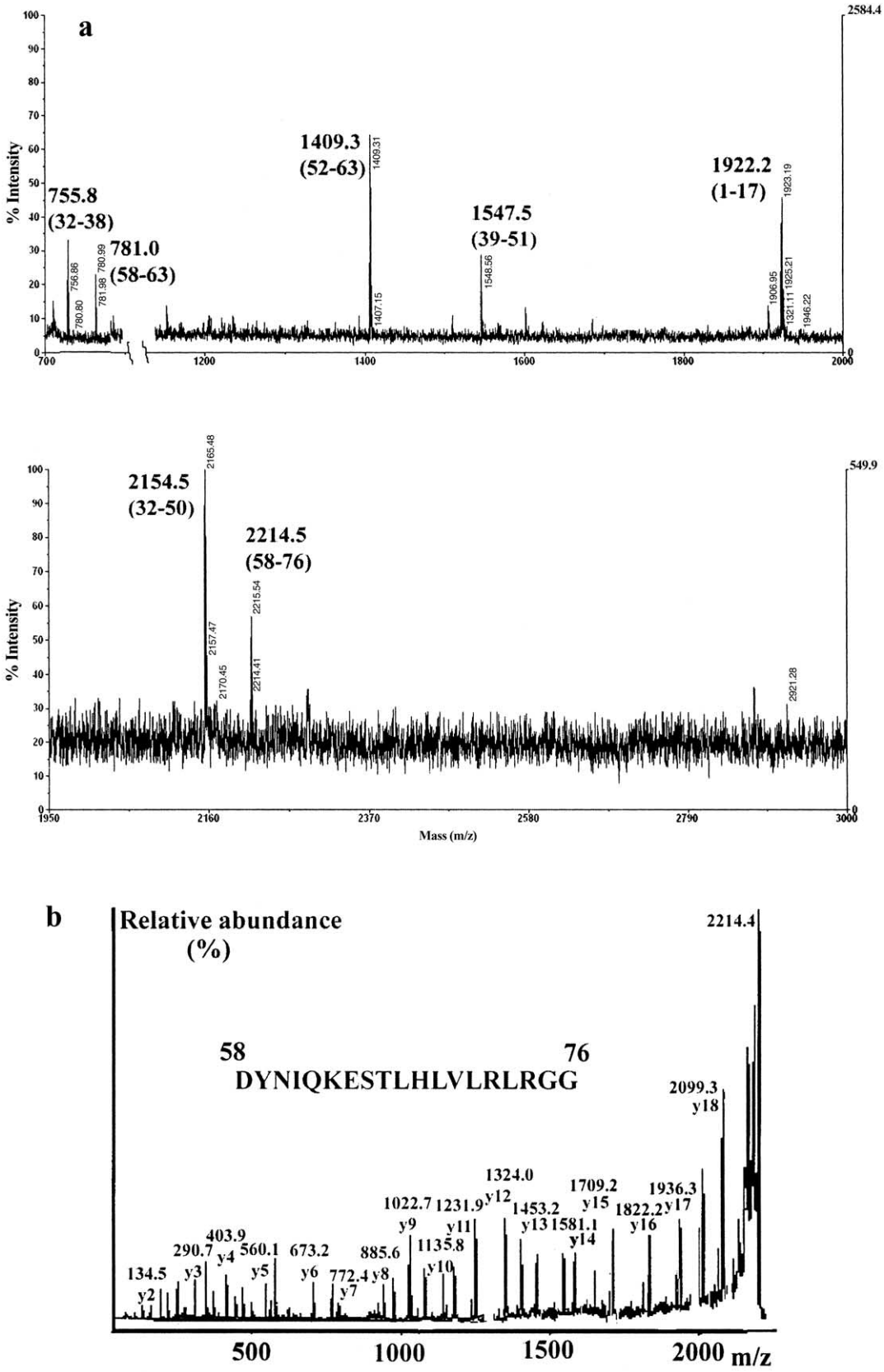

Fig. 2. Peptide mapping and sequencing by MS. a: MALDI-TOF mass spectrum of the endoproteinase Asp-N digest of the peak at 34.3 rt from transparent lens in the HPLC separation of Fig. 1A. The peptide fraction was collected from the HPLC separation of Fig. 1, under the conditions described in Section 2, concentrated to 10 μl, and then analyzed by MALDI MS. b: MALDI-TOF mass spectrum in post source decay mode of the peptide at 2214.5 *m/z* in the spectrum of (a) corresponding to the peptide 58–76 from the Endoproteinase Asp-N digest of ubiquitin (peak at 34.3 rt in the HPLC separation of Fig. 1A) from a normal subject. Signals diagnostics of peptide structure are shown in the figure. c: MALDI-TOF mass spectrum of the endoproteinase Asp-N digest of the peak at 34.3 rt from cataractous lens.

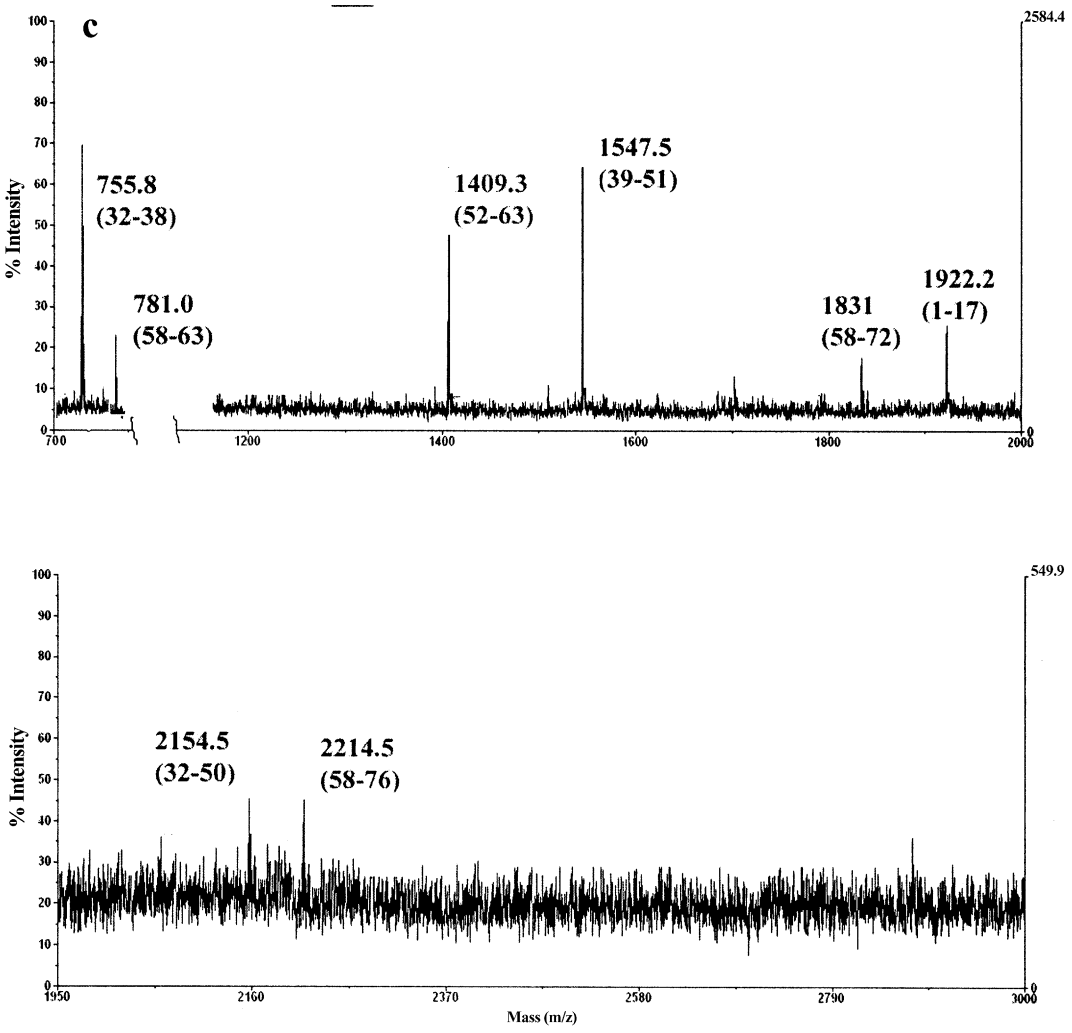

Fig. 2 (*Continued*).

form. The fraction from cataractous lens nuclei showed two forms binding anti-ubiquitin antibody, and this was similar to the mass results which highlighted two peaks (8182 and 8566/8583 *m/z*). This heterogeneous content of the peak may explain the subtle difference of migration, in comparison to the transparent lens samples, concerning the native ubiquitin band.

All these results demonstrate that the LMW fraction purified from both clear and cataractous lenses is ubiquitin, in native and truncated forms.

4. Discussion

Ubiquitin is a signal for protein protection from oxidative stress [6] or for protein degradation [7]. The proteolytic removal of aged or damaged proteins plays a considerable role in maintaining lens transparency [8]. Human cataractous lenses accumulate damaged proteins, so the mechanism of cataractogenesis may be related to the inactivation of the protein turnover systems [3].

We purified two forms of ubiquitin: in the transparent lenses we found an ubiquitin of 8566 Da, whereas in the cataractous lenses, we found the presence of an 8566/8583 Da component as well as a truncated form of 8182 Da in higher amount. It may be hypothesized that ubiquitin is inactivated by the cleavage of a short segment, either at the N-terminus or C-terminus. The mass of 8192 Da is in good agreement with the cleavage of the C-terminus segment 73–76 (Leu-Arg-Gly-Gly) which generates a truncated ubiquitin with theoretical mass of 8181.44 Da. The cleavage of segment 1–3 (Met-Gln-Ile) should generate a truncated form with theoretical mass of 8192.40 Da. We confirmed that the truncated ubiquitin corresponds to the cleavage of the segment 73–76 by carboxypeptidase assay, according to the experimental procedure previously described [9]. To study the molecular mechanism of this cleavage in detail, we used molecular graphics techniques to analyze the three-dimensional structure of human ubiquitin, solved by X-ray crystallography [10–11] (Fig. 4). According to the CATH classification [12], ubiquitin is an α-β roll structure, consisting of a mixed β-sheet (five strands) and two helices. The last four amino acids (segment 73–76) do not assume a defined secondary structure (Fig. 4), whereas the N-terminus segment is involved in an internal strand of the β-sheet. The disordered region at the C-terminus is well suitable for proteolytic cleavage, as demonstrated by different authors [13–15]. In these articles, it was demonstrated that beta

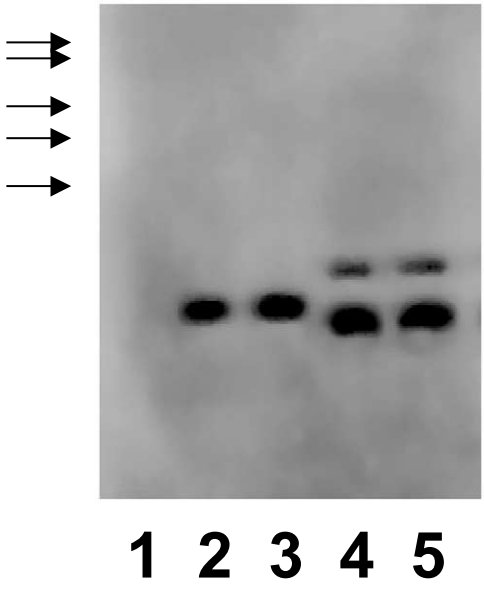

Fig. 3. Western Blot of the fractions with rt 34.3 min from clear lens (lane 2–3) and cataractous lens (lane 4–5) obtained by RP-HPLC. Lane 1 has been loaded with molecular weight markers. The arrows indicate the position of bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), lactalbumin (14.4 kDa).

strands are not hydrolyzed by proteases (even if this opportunity could not be excluded for the external strands, which may leave the β-sheet, whereas the internal strands are very strongly constrained). Well exposed segments, without secondary structure constraints, are the most suitable for proteolytic attack. Furthermore, we found a possible protease to specifically cleave the segment 73–76. The calcium-activated neutral proteinase (calpain II, EC 34.22.17) is a component of lens [5] which preferentially hydrolyzes the peptide bond between Tyr, Met or Arg residues followed by Leu or Val. In our case, Arg72 is followed by Leu73 and therefore it constitutes a possible proteolytic site for this proteinase. Abnormal activation of calpain is known to induce cataract [16–17], and this phenomenon has been interpreted as the effect of calpain activity on lens proteins, with the consequent insolubilization of the proteolytic products. It has to be stressed that, under physiological conditions, the ubiquitin pathway should degrade such products. However, if the ubiquitin is still inactivated by calpain, its function can result strongly reduced. It must be remembered that, by the mass spectrum (Fig. 1C), the native ubiquitin appears to be about 1/5 of the total protein observed, being 1/5 inactivated by oxidation, and 3/5 inactivated by proteolysis.

Modifications occurring at the C-terminal amino acid of ubiquitin may be responsible for alterations in the protein degradation mechanism, determining post-translationally modified protein accumulation in the lens. In fact, ubiquitin joins the substrate proteins via an isopeptide linkage between its carboxyl terminus and the epsilon amino group of an internal lysine of the target protein. This event is followed by the formation of a polyubiquitin chain, which once again requires the linking of the carboxyl terminal group of ubiquitin to the epsilon amino group of Lys48 belonging to another ubiquitin molecule. For this reason, we suggest that one of the cataractogenesis mechanisms might be induced, or at

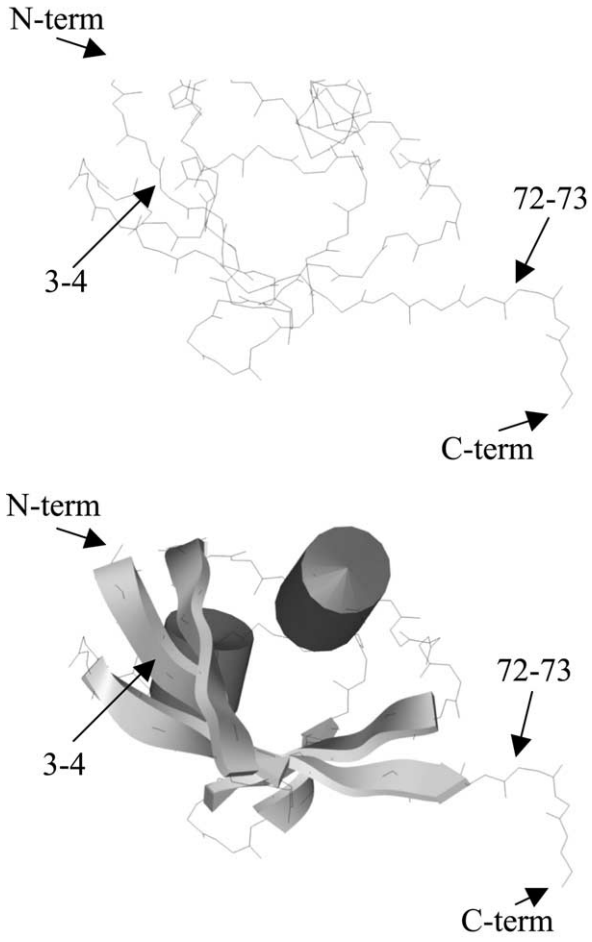

Fig. 4. Schematic view of ubiquitin, in wireframe representation (top image) and secondary structure cartoon (bottom image) with helix as cylinders and β-strands as arrows. The position of N-terminus, C-terminus, and both 3–4 and 72–73 peptide bonds are highlighted with arrows. The picture has been created with the Insight II program (Accelrys, San Diego, CA, USA), using the PDB entry 1UBI.

least facilitated, by a complex mechanism involving the increase of calcium in lenses, due to aging. A consequent increase in calpain activity, which truncates ubiquitin molecules, determines the inactivation of ubiquitin-mediated protein degradation mechanisms, bringing about a final accumulation of damaged proteins.

In conclusion, HPLC purification and MS analyses evidenced that human senile cataractous lens includes a 8182 Da species which is absent in the normal lens, whereas a 8566/8583 Da species is present in both lenses. Western blot analysis and MS identified both species as ubiquitin. The 8182 Da species is due to the cleavage of residues 73–76 at the C-terminus of the chain. The last amino acid of ubiquitin is involved in the protein degradation mechanism, thus, we suggest that this structure modification compromises the function of ubiquitin and consequently the physiologically occurring degradation of the lens proteins.

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