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αA and αB peptides from human cataractous lenses show antichaperone activity and enhance aggregation of lens proteins

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Purpose: To identify and characterize properties of αA- and αB-crystallins' low molecular weight peptides (molecular weight [Mr] < 5 kDa) that were present in a 62-year-old human nuclear cataract, but not in normal 62-year-old human lenses.

Methods: Low molecular weight peptides (< 5 kDa) were isolated with a trichloroacetic acid (TCA) solubilization method from water-soluble (WS) and water-insoluble (WI) proteins of nuclear cataractous lenses of a 62-year-old donor and normal human lenses from an age-matched donor. Five commercially synthesized peptides (found only in cataractous lenses and not in normal lenses) were used to determine their chaperone and antichaperone activity and aggregation properties.

Results: Mass spectrometric analysis showed 28 peptides of αA-crystallin and 38 peptides of αB-crystallin were present in the cataractous lenses but not in the normal lenses. Two αA peptides (named αAP1 and αAP2; both derived from the αA N-terminal domain (NTD) region) and three αB peptides (named αBP3, αBP4, and αBP5, derived from the αB NTD-, core domain (CD), and C-terminal extension (CTE) regions, respectively) were commercially synthesized. αAP1 inhibited the chaperone activity of αA- and αB-crystallins, but the other four peptides (αAP2, αBP3, αBP4, and αBP5) exhibited mixed effects on chaperone activity. Upon incubation with human WS proteins and peptides in vitro, the αBP4 peptide showed higher aggregation properties relative to the αAP1 peptide. During in vivo experiments, the cell-penetrating polyarginine-labeled αAP1 and αBP4 peptides showed 57% and 85% aggregates, respectively, around the nuclei of cultured human lens epithelial cells compared to only 35% by a scrambled peptide.

Conclusions: The antichaperone activity of the αAP1 peptide and the aggregation property of the αBP4 peptide with lens proteins could play a potential role during the development of lens opacity.

Crystallins (α-, β-, and γ-crystallins) constitute > 90% of the total vertebrate lens proteins. Alpha crystallin (composed of two subunits of αA- and αB-crystallins) exhibits chaperone function [1] to keep other crystallins soluble in their native states, and therefore, it plays a major role in maintaining lens transparency. β- and γ-crystallins are mainly structural proteins and constitute a superfamily due to their origin via gene duplication. In addition, interactions among crystallins play a major role in maintaining lens transparency [2]. However, during aging and cataract development, the native structures of crystallins and their interactions are altered. These factors, along with mutations in crystallins [3-6] and a variety of post-translational modifications (PTM), such as truncation, deamidation, oxidation, and glycation that cause aggregation of crystallins [7-17], are considered causative factors in cataract development.

Despite the low turnover of crystallins during aging [18], α-, β-, and γ-crystallins show truncations during aging and cataract development, resulting in low molecular weight (LMW) polypeptides [7-12]. These LMW crystallin polypeptides exist in water-soluble (WS) and water-insoluble (WI) protein fractions [7-12], and they are part of in vivo existing covalent complexes of human lenses [19,20]. Mass spectrometry studies of aged human lenses show that the majority of polypeptides are derived from αA-, αB-, and βA3/A1-crystallins. They are mostly present in the cortical region of the lens. The truncation process of crystallins begins in the outer cortex and continues to the nucleus [21]. Together, these reports suggest a potential role of crystallin fragments in the aggregation of crystallins and cataract development. However, presently, the mechanism of such a role of crystallin fragments in the aggregation process is not well understood.

Truncation of crystallins in human lenses starts in early middle age. In older human lenses, peptides with molecular weight (Mr) greater than 1,778 Da are mostly present in the WI-protein fractions, but to a lesser extent in the WS-protein fractions [22]. These LMW polypeptides are believed to compromise the structure and interaction among crystallins, facilitate protein aggregation, and reduce the chaperone activity of α-crystallin [23-26]. For example, the αA(66–80), αB(1–18), βA3/A1(59–74), and βA3/A1(102–117) peptides increased light scattering during in vitro experiments and inhibited the chaperone functions of α-crystallin. Furthermore, when two of the hydrophobic residues of the βA3/

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A1(102–117) peptide were replaced with hydrophilic residues, the substituted peptide (SDADHGERLMSFRPIC) did not show the antichaperone property [25].

Our studies and those of others [12,19,20,23-33] have indicated the involvement of crystallin fragments in aggregation and cross-linking processes in aging and cataractous human lenses. However, because of the lack of in vivo studies, the potential in vivo mechanism of lens crystallins' induced aggregation mechanism remains elusive.

Cell-penetrating peptides (CPPs), also known as protein transduction domains (PTDs), are well established for internalization with many types of cargo, such as proteins, nucleotides, siRNA nanoparticles, and peptides [34,35]. They are highly cationic and usually rich in arginine and lysine amino acids [34,36], and are able to internalize themselves into the cells by crossing the plasma membrane. These are noncovalently or covalently conjugated with the cargo to carry them inside the cells, and therefore, are an interesting tool for drug delivery [36]. Antenapedia, penentratin, HIV-tat, tranportan, pVEC, MAP, and polyarginine are well established CPPs [37]. In the present study, we used polyarginine with six arginine residues that were covalently conjugated at the N-terminal region of the desired polypeptides for cell penetration experiments.

The focus in the present study was peptides that are present in cataractous lenses but not in age-matched normal lenses. Among these peptides, five peptides of αA- and αB-crystallins were selected to determine their in vitro effects on the chaperone activity of αA- and αB-crystallins, and on aggregation of crystallins. In addition, the effects of selected fluorescently labeled, cell-penetrating peptides were examined on protein aggregation under ex vivo conditions in cultured human lens epithelial cells.

METHODS

Materials: Normal human lenses with no apparent opacity were obtained from Dr. Robert Church (Emory University, Atlanta, GA), and the lenses were stored at −20 °C until used. Two normal lenses (from a 62-year-old donor and stored at −20 °C in medium 199 without phenol red) and two age-matched lenses with nuclear cataracts (recovered from a local surgeon and stored at −20 °C in medium 199 without phenol red) were separately pooled and processed as described below. The lenses were stored for about 2 years before they were used. Two normal human lenses with no opacity (from a donor 36 years of age) were also used to prepare a WS human lens lysate (WS-HLL). Prestained and unstained protein molecular weight markers were obtained from GE Biosciences (Piscataway, NJ). Unless indicated otherwise, all other chemicals used in this study were purchased from Sigma-Millipore (St. Louis, MO) or Fisher Scientific (Atlanta, GA). Five polypeptides, two αA-crystallins (named αAP1 [N-terminal acetylated] and αAP2) and three αB-crystallins (named αBP3, αBP4, and αBP5), were synthesized at the core facility of the Diabetic Center, Division of Gerontology, School of Medicine, University of Alabama at Birmingham. The purities of the synthetic peptides were > 95%, as determined by their size-exclusion high-performance liquid chromatography (HPLC) analysis using a TSK G2500PW$_{XL}$ column (Tosoh Bioscience GmbH, Griesheim, Germany). Fluorescein isothiocyanate (FITC)-labeled αAP1, αBP4, and scrambled αAP1 polypeptides were synthesized by Think-Peptides (Oxford, England). Insulin was obtained from Sigma-Millipore, and the Proteostat aggregate assay kit was from Enzo Life Sciences (Farmingdale, NY).

Isolation of LMW peptides from human lenses: The peptides (M_r < 5 kDa) were isolated from WS and WI proteins of cataractous and two age-matched normal human lenses with the trichloroacetic acid (TCA) solubilization method, as previously described [38]. TCA-precipitated crystallins were removed with centrifugation as a pellet, and the soluble fractions (supernatants) were extensively dialyzed against water at 5 °C to remove TCA and concentrated to dryness with lyophilization. Then, the peptide preparations were dissolved in the desired buffers as described below.

Identification of peptides with mass spectrometry: The TCA-solubilized peptide preparations from WS- and WI-protein fractions of the cataractous and age-matched normal human lenses were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [39] using a 15% polyacrylamide gel. The peptides with an M_r < 5 kDa were excised from a gel and analyzed with mass spectrometry as described below at the Targeted Metabolomics and Proteomics Laboratory (TMPL), Department of Pharmacology and Toxicology, University of Alabama at Birmingham. Briefly, after fixing in the gel with 7% acetic acid, the excess stain was removed with an overnight wash with 100 mM ammonium bicarbonate/acetonitrile (50%/ 50% v/v). Then, disulfide bonds were reduced with 25 mM dithiothreitol at 50 °C for 30 min. Next, 55 mM iodoacetamide was used in the dark for alkylation of the free thiol groups. After excess alkylating reagent was removed, gel pieces were washed with 100 mM ammonium bicarbonate for 30 min, and the gel pieces were dried in Savant SpeedVac (Thermo Fisher Scientific, Waltham, MA). Trypsin (12.5 ng/µl; Promega Gold Mass Spectrometry Grade) was added to each gel band and incubated overnight at 37 °C. A 1:1 mixture of 1% formic acid and acetonitrile was used for peptide extraction. Extraction

was repeated, and the extracts were pooled and evaporated to dryness. The samples were resuspended in 0.1% formic acid (30 µl) and used for the mass spectrometric analysis.

Nano-cHiPLC–tandem mass spectrometry: For the mass spectrometry (MS) study, a Nano cHiPLC 200 µm × 0.5 mm trap cartridge attached to an analytical column (ChromXP C18-CL 3 µm 120Å reverse-phase column; Eksigent, Dublin, CA) was used. A 5 µl aliquot of each digest was loaded to the column using an Eksigent autosampler with a flow rate of 2 µl/min. Furthermore, the cartridge was washed with 0.1% formic acid in double distilled water (dd$H_2$0) for 4 min. The bound peptides were flushed off the cartridge and resolved on the analytical column with a 45 min linear (5% to 50%) acetonitrile gradient in 0.1% formic acid at 1,000 nl/min using an Eksigent NanoLC-1Dplus (one-dimensional nano-HPLC system). The column was washed for 10 min with 90% acetonitrile–0.1% formic acid and reequilibrated for 10 min with 5% acetonitrile–0.1% formic acid. Eluted peptides were passed through an IonSpray™ interface (voltage set at 2,300 V and a declustering potential of 80 V), and the resulting ions were analyzed on a SCIEX TripleTOF 5600 mass spectrometer (AB SCIEX, Toronto, Canada). The pressure for the Ion Spray™ and curtain gases was 10 psi and 25 psi, respectively, and the interface heater temperature was 120 °C.

To determine the 20 most intense ions for tandem mass spectrometry (MS/MS), time-of-flight survey scans of the eluted peptides from 400 to 1,250 *m/z* of the eluted peptides were performed. To obtain the tandem mass spectra of the selected parent ions, product ion time-of-flight scans at 50 ms were performed over the range of *m/z* 100–1500). Using Analyst software, version TF (AB SCIEX) spectra were centroided and deisotoped. To calibrate the mass accuracy of the mass spectrometer, a β-galactosidase trypsin digest was used. Proteins were identified from tandem mass spectrometry data using the ProteinPilot 4.5 search engine (AB SCIEX) with the Homo sapiens UniProt protein database. The potential candidates (proteins with at least one peptide) were selected based on a confidence score of 95% or greater, and data were exported as an Excel file. Peptides specifically detected only in the cataractous lenses were categorized in the following three groups: (1) detected in the WS-protein fraction, (2) detected only in the WI-protein fraction, and (3) detected in the WS- and WI-protein fractions.

Selection of peptides and prediction of biochemical properties of peptides: Five peptides, two from αA-crystallin (named αAP1 and αAP2) and three from αB-crystallin (named αBP3, αBP4, and αBP5), were detected in the cataractous lenses but not in the age-matched normal lenses. The biochemical properties of these peptides were predicted using INNOVAGEN. Furthermore, the five peptides were commercially synthesized and were characterized for their effects on the chaperone activity of αA- and αB-crystallins and their role in aggregation of crystallins. The αAP2, αBP3, αAP2, and αBP5 peptides were readily soluble in deionized water, whereas the αAP1 and αBP4 peptides were water soluble after incubation at 37 °C for 10 min.

Antichaperone activity of the peptides: To evaluate whether the five selected peptides exhibited chaperone activity, insulin was used as a target protein, as previously described [40,41]. The dithiothreitol (DTT)-induced aggregation of insulin (200 µg) was performed at 37 °C for 60 min in 10 mM sodium phosphate buffer (pH 7.4, containing 100 mM sodium chloride) in a 1 ml reaction volume with and without the peptides. Varying concentrations (5, 25, 50, and 100 µM) of each peptide were used to evaluate chaperone activity. Aggregation was monitored as turbidity at 360 nm over time (60 min) using a Shimadzu ultraviolet -visible (UV-VIS) scanning spectrophotometer (model UV2101 PC, Columbia, MD) equipped with a six-cell positioned cuvette (Shimadzu model CPS-260) and a temperature controller (Shimadzu model CPS 260).

Effects of peptides on the chaperone activity of αA- and αB-crystallins: The effect of each peptide (100 µM) on the chaperone activity of αA- and αB-crystallins (100 µg) was investigated using insulin as the target protein. Change in absorbance at 360 nm was monitored at 10-min intervals for 60 min as described above. For the experiments, recombinant αA- and αB-crystallins were purified from *E. coli* lysates, as previously described [40,41].

Effects of the αAP1 and αBP4 peptides on the secondary structure of αA- and αB-crystallins: The chaperone activity of αA- and αB-crystallins was inhibited by the αAP1 peptide, whereas the activity was not affected by the αBP4 peptide (described in the Results section). The effects of the two peptides (αAP1 and αBP4) on alteration in the secondary structures of αA- and αB-crystallins was analyzed with Circular Dichroism spectroscopy using a Jasco CD spectrometer (Easton, MD). For CD spectral analysis, 10 µM protein (αA-/αB-crystallin) and 25 µM peptide (αAP1/αBP4) in a 200 µl reaction mixtures of 10 mM phosphate buffer (pH 7.4) were incubated at 37 °C for 15 min, and the secondary structures were recorded in the far-UV region (190–260 nm) using the appropriate controls (the αA- or αB-crystallin alone and the αAP1 or αBP4 peptide alone). The spectrum of the buffer alone was subtracted from each of the spectra, and the secondary structure was predicted using the Selcon 3 program (BMB).

Aggregation of lens proteins on incubation of the αAP1 and αBP4 peptides with WS-HLL: To prepare WS-HLL, two lenses from normal humans with no opacity (36 years of age) were homogenized in 1 ml of 10 mM sodium phosphate buffer (pH 7.4, containing 100 mM sodium chloride, protease inhibitor cocktail [Millipore-Sigma]) and centrifuged at 16873 xg for 10 min at 4 °C. Supernatants were collected, and the process was repeated as described above. Both supernatants (collected after centrifugation) were pooled, designated as the human WS-HLL, and the protein concentration was determined at 280 nm. The WS protein (250 µg) was incubated at 37 °C with 100 µM of the selected peptides in a 200 µl reaction mixture in 10 mM phosphate buffer (pH 7.4, containing 100 mM NaCl) for 18 h. Aggregation of the WS protein was analyzed with the Aggresome assay kit (Enzo Life Sciences) using the manufacturer's instructions with slight modifications. The Proteostat reagent was diluted 1,000X using 10 mM phosphate buffer, and 200 µl of the diluted Proteostat reagent was added to the 200 µl of all the WS-protein preparations that had been previously incubated for 18 h. The reaction mixture was kept in the dark for 15 min. Next, the fluorescence spectra (emission spectra between 460 and 700 nm) were recorded by excitation at 450 nm using a Shimadzu RF-5301PC spectrofluorometer with excitation and emission band passes set at 5 and 3 nm, respectively. Then, these samples were centrifuged at 16,000 ×*g* for 10 min, and the pellets were suspended into Laemmli-SDS loading buffer and examined with SDS–PAGE using a 12% polyacrylamide gel [39]. The experiments were performed in triplicate. Additionally, human lens WS protein was incubated with 50, 100, and 200 µM of peptides, and aggregation was determined with the aggregation assay method as described above.

Effects of αAP1 and αBP4 peptide aggregation in human lens epithelial cells: The FITC-labeled αAP1, αBP4, and scrambled αAP1 peptides were commercially synthesized with six arginine residues at the N-terminal region of the peptides (ThinkPeptides). Human lens epithelial cells (HLECs), a kind gift from Dr. Usha Andley (Washington University, St. Louis, MO), were grown in 35 mm plates in minimum essential medium (MEM) GlutaMAX medium supplemented with 15% fetal bovine serum at 37 °C in 5% CO_2 until they reached > 40% confluence. Next, the cells were washed with PBS (1X; 137 mM NaCl, 2.7 mM KCl, 10 mM $NaPO_4$, 1.8 mM KPO_4, pH 7.4), and the peptides were prepared (1 µM peptide and 3 µl dimethyl sulfoxide (DMSO) were mixed with 1 ml serum-free medium Opti-MEM, Gibco, Thermo Fisher scientific) and added to the cells and incubated for 18 h at 37 °C in 5% CO_2. Opti-MEM containing FITC-labeled peptide was removed after 18 h, and 1 ml of complete growth medium was added to the cells, followed by incubation for 6 h at 37 °C in 5% CO_2. Next, the cells were washed 5X with PBS and fixed with 4% paraformaldehyde for 30 min. The cells were then washed in PBS (3X) and permeabilized with 1% Triton X-100 (Thermo Fisher scientific) and 3 mM EDTA for 30 min at 4 °C. The cells were then washed with PBS and stained with Proteostat Aggresome dye and Hoechst nuclear stain for 30 min. The cells were washed 3X with PBS and covered with coverslip. The peptides and aggregates were visualized using a Leica fluorescence microscope (Deerfield, IL) with 488 nm excitation and emission at 594 nm. In each experiment, 100 cells were manually counted using a hemocytometer to calculate the efficiency of aggregate formation in the presence of the peptides. The experiments were repeated three times.

RESULTS

Isolation and identification of LMW polypeptides: LMW polypeptides (M_r < 5 kDa) were isolated with a TCA-solubilization method as previously described [13]. On the SDS–PAGE of the TCA-solubilized fractions from the WS- and WI-protein fractions, peptides with an M_r < 5 kDa were seen (Appendix 1). The number of peptides present in the WS- and WI-protein fractions were identified with mass spectrometry using the Protein Pilot search engine and are summarized in Table 1. These peptides showed sequence homology to α-, β-, and γ-crystallins. Furthermore, in the WS-protein fraction of the normal lenses, 191 αA peptides and 212 αB peptides were identified, whereas the WS-protein fraction of the cataractous lenses showed relatively fewer peptides, that is, 72 αA peptides and 85 αB peptides (Table 1). Similarly, the WI-protein fraction of the normal lenses contained 105 αA peptides and 219 αB peptides, whereas the WI-protein fraction of the cataractous lenses contained 62 αA peptides and 66 αB peptides.

The focus of this study was to characterize those αA and αB peptides in WS- and WI-protein fractions that were present in cataractous lenses but not in normal lenses (Table 1). As shown in Figure 1, a total of 28 αA peptides (Figure 1A) and 38 αB peptides (Figure 1B) were identified in the cataractous lenses, and among the 28 αA peptides, 53% (11 peptides) were from the NTDs, and 25% and 3% were from the NTD-CD and CD-CTE regions, respectively. This suggests that predominately N-terminal domain peptides were identified in the cataractous lenses.

In the cataractous lenses, the contents of the αB peptides differed from those of the αA peptides. Among the αB peptides, 28% were from the NTDs, 26% from the CDs, 31% from the CTE, and smaller numbers (13%) were from the CD-CTE regions. Based on the abundance of the peptides and their presence in the WS- or WI-protein fractions of the

Table 1. Identified αA- and αB-peptides by MS/MS mass spectrometric analysis in TCA-solubilized fractions from WS- *and* WI-protein fractions of catarctous and age-matched normal lenses.

Sample (TCA-solubilized)	Number of peptides of αA-crystallin	Number of peptide of αB-crystallin
WS-Normal lenses	191	212
WI-Normal lenses	105	219
WS-cataractous lenses	72	85
WI-Cataractous lenses	62	66

cataractous lenses, the following five peptides were selected for further studies: two peptides from the NTD region of αA-crystallin (named αAP1 [NTD, residues 1–15] and αAP2 [NTD, residues 38–50]) and three peptides from αB-crystallin (named αBP3 [NTD, residues 30–44], αBP4 [CD, residues 130–137], and αBP5 [CTE, residues 163–175]; Figure 2).

Prediction of biochemical properties of the selected peptides: The biochemical properties of the five selected peptides were predicted by using an available online tool, as described in the Methods section (Table 2). Based on their amino acid sequences, four peptides were predicted to have an M_r of 1.4–1.8 kDa except the αBP4 peptide with M_r of 0.8 kDa. Furthermore, the αAP1 and αAP2 peptides were predicted to be basic with poor water solubility, and the αBP3 and αBP5 peptides acidic and basic, respectively, both with good water solubility.

Antichaperone activity of selected peptides: Before determining the effects of the five selected peptides on the chaperone activity of αA- and αB-crystallins (see below), their chaperone activity at increasing concentrations (5, 25, 50, and 100 µM) were determined by using insulin as a target protein (Figure 3). The αAP1 (Figure 3A) and αBP3 (Figure 3C) peptides showed increasing turbidity (aggregation) at 360 nm of insulin at their increasing concentrations, but the individual peptides alone did not show any light scattering (turbidity) by themselves at 360 nm. The αAP2, αBP4, and αBP5 peptides also exhibited increasing turbidity of incubation with insulin up to the 50 µM concentration (Figure 3B,D,E, respectively). However, 100 µM of the αAP2, αBP4, and αBP5 peptides did not significantly alter the aggregation of insulin compared to 50 µM of peptides (data not shown). None of the peptides individually exhibited light scattering. Together, the results suggest that the five peptides showed antichaperone activity, and among them, the αAP1 and αBP3 peptides had the most antichaperone activity. However, the αBP4 peptide was predicted to be a neutral peptide but with poor water solubility.

Effects of selected peptides on the chaperone activity of αA- and αB-crystallins: With insulin as the target protein, the chaperone activity of αA- and αB-crystallins was examined in the presence of 100 µM concentrations of each of the five peptides (Figure 4). Chaperone activity of αA-crystallin was inhibited in the presence of 100 µM of the αAP1 peptide (Figure 4A). However, the remaining four peptides (αAP2, αBP3, αBP4, and αBP5) did not affect the chaperone activity of αA-crystallin (Figure 4B–E).

Chaperone activity of αB-crystallin with insulin as the target protein in the presence of 100 µM concentrations of individual peptides was also examined (Figure 5). Similar to the results for αA-crystallin, chaperone activity of αB-crystallin was maximally inhibited by the αAP1 peptide (Figure 5A), whereas the αBP3 and αBP5 peptides also inhibited the chaperone activity of αB-crystallin, but at relatively lower levels compared to the αAP1 peptide. Similarly, the αAP2 peptide also slightly inhibited αB-crystallin chaperone activity but to a lesser level than the three other peptides (Figure 5B). However, the αBP4 peptide did not show any effect on the chaperone activity of αB-crystallin. Together, the results showed that among the five selected peptides, the αAP1 peptide inhibited the chaperone activity of αA- and αB-crystallins, whereas the αBP4 peptide did not show any effect on the chaperone activity of αA- and αB-crystallins. Furthermore, the αAP2, αBP3, and αBP5 peptides affected only the chaperone activity of αB-crystallin.

Secondary structural changes in αA- and αB-crystallins in the presence of the αAP1 and αBP4 peptides: The results above showed that although the αAP1 peptide inhibited the chaperone activity of αA- and αB-crystallins, the αBP4 peptide did not show any effect. Therefore, the secondary structural changes in purified recombinant αA- and αB-crystallins in the presence of the αAP1 and αBP4 peptides were examined with CD spectroscopy (Figure 6). Analysis of the far-UV CD spectra of αA-crystallin showed that in the presence of 25 µM αAP1 peptide, the β-sheet of αA-crystallin was reduced from 64.6% to 50.0%, whereas in the presence of

25 µM αBP4 peptide, the β-sheet of αA-crystallin was 61.0% (Table 3). This suggested that the αAP1 peptide significantly changed the secondary structure of αA-crystallin, whereas the αBP4 peptide had a minimal effect. Similarly, the αAP1 peptide reduced the β-sheet of αB-crystallin from 66% to 55% and increased the irregular structure from 20.9% to 42.5%. In contrast, the αBP4 peptide only slightly reduced the β-sheet structure of the crystallin to 60.0% and increased the irregular structure only from 20.9% to 30.9%. Together, the results suggested that the αAP1 peptide significantly altered

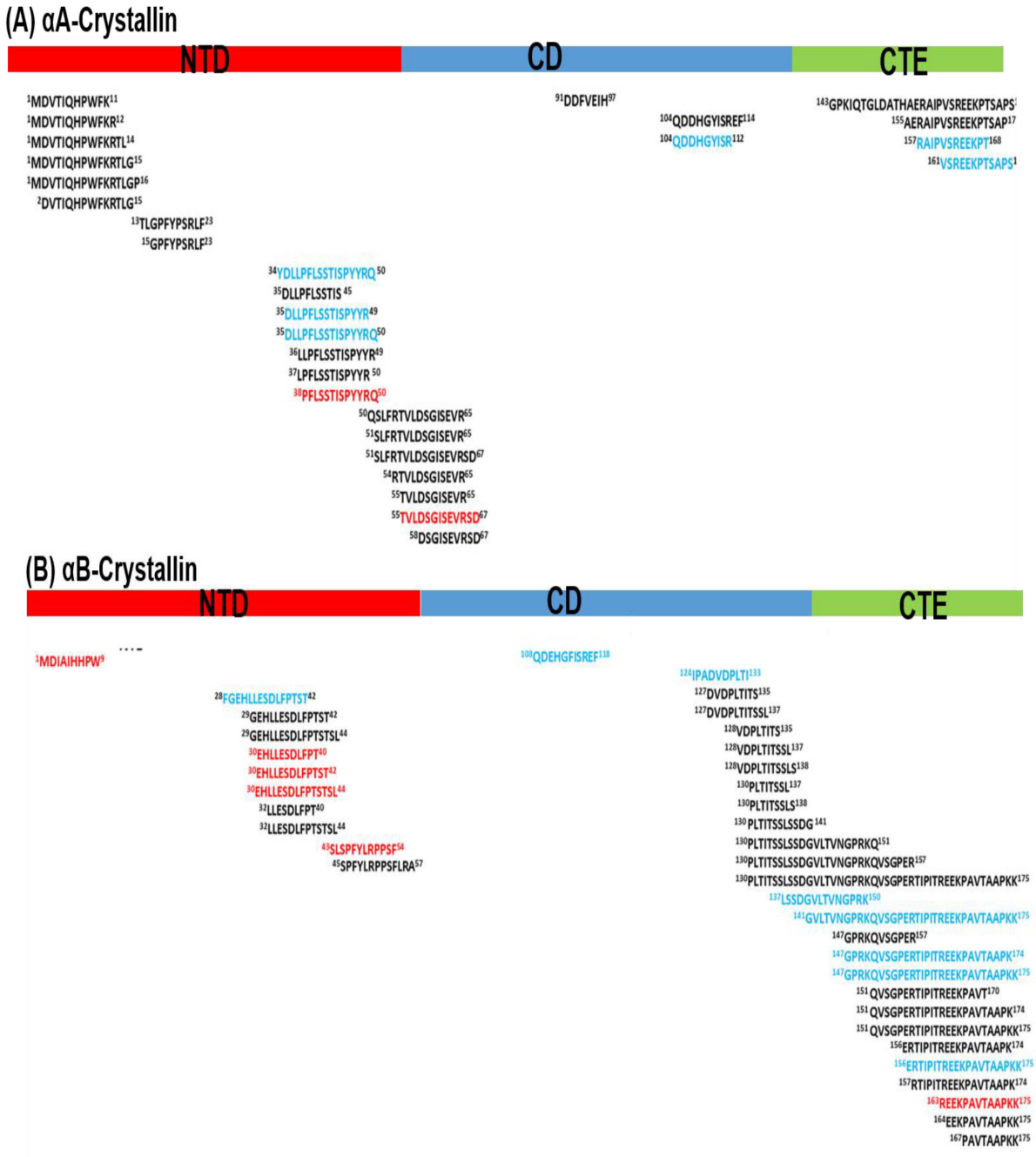

Figure 1. Endogenous LMW peptides isolated from WS- and WI-protein fractions of normal and age-matched cataractous lenses. **A**: Peptides isolated from αA-crystallin. **B**: Peptides isolated from αB-crystallin. Relative to normal lenses, the peptides found in cataractous lenses are as follows: Black present in WS- and WI-protein fractions in normal and cataractous lenses, blue found only in the WS-protein fraction, and ref only in the WI-protein fraction.

Table 2. Predicted properties of five selected peptides of αA- and αB-crystallins.

Peptides	Mr/pI	Charge/Attribute	Amino acid composition (%) Acidic Basic Neutral Hydrophobic	Water Solubility
αA crystallin MDVTIQHP-WFKRTL G (αAP1)	1871.21/8.25	2/ basic	6.67 20 26.67 40	Poor
PFLSSTISPYYRQ (αAP2)	1558.77/9.59	1/basic	0 7.69 38.46 38.46	Poor
αB crystallin EHLLESDLF-PTSTSL (αBP3)	1688.87/3.82	-2/acidic	20 6.67 33.33 33.33	Good
PLTITSSL (αBP4)	830.99/6.01	0/neutral	0 0 50 50	Poor
REEKPAVTAAPKK (αBP5)	1425.68/10.3	2/basic	15.4 30.8 7.7 30.8	Good

the secondary structures of αA- and αB-crystallins, but the αBP4 peptide showed little effect. This could explain the loss of chaperone function of αA- and αB-crystallins by the αAP1 peptide, but not by the αBP4 peptide (Figure 5).

Insolubilization of proteins in HLL by peptides: The aggregation of WS proteins in HLL (at 250 µg) was investigated in the presence of 100 µM of the αAP1-, αBP4-, and scrambled αAP1 peptides. Insolubilization of HLL proteins was determined with an aggregation assay using Proteostat dye, as well as with SDS–PAGE. Upon incubation, the aggregation assay showed that the WS-HLL alone formed aggregates, but the aggregation levels were increased in the presence of the αAP1- and αBP4 peptides (Figure 7A). The maximum levels of the aggregates were observed in the presence of the αBP4 peptide, which was followed by the αAP1 peptide. However, neither of these peptides showed aggregation individually. Following incubation, the aggregates were isolated with centrifugation, and the pellets were examined with SDS–PAGE. Similar to the results obtained with the aggregation assay, as shown in Figure 7A, the SDS–PAGE showed that the concentrations of WI proteins were relatively higher in the presence of the peptides compared to WS-HLL-protein alone (Figure 7B). The results were quantified, as shown in Figure 7C. Together, these results suggested that the peptides facilitated the aggregation of human WS-HLL proteins that led to their insolubility.

To further establish whether the two peptides facilitate aggregation of WS proteins, individual peptides at 50, 100, and 200 µM were incubated with 250 µg WS-HLL-proteins for 18 h, and an aggregation assay was performed as described above using Proteostat dye (Figure 8). Maximum levels of aggregates were observed in the presence of 200 µM αAP1 peptide; however, the 50 and 100 µM αAP1 peptides showed almost equal levels of aggregation (Figure 8A). The levels of aggregates increased with the increasing concentrations of the αBP4 peptide; the maximum aggregation level was observed at the 200 µM concentration (Figure 8B). Together, the aggregation assay results showed that the αBP4 peptide formed greater levels of aggregates at the increasing concentrations compared to the αAP1 peptide.

In vivo effects of the αAP1 and αBP4 peptides on HLECs: To examine the effects of the αAP1 and αBP4 peptides under ex vivo conditions, HLECs in culture were treated with

Figure 2. Diagrammatic representation of the positions of the five selected peptides (αAP1, αAP2, αBP3, αBP4, and αBP5) in αA- and αB-crystallins that were used in the study. These are identified in red, blue, and green.

1 µM FITC-labeled peptides, as described in the Material and Methods section. Fluorescent microscopic analyses showed that the αAP1 and αBP4 peptides were internalized in the majority of the cells (green fluorescence) and formed intracellular aggregates (red fluorescence; Figure 9). On quantification following manual counting of fluorescent versus non-fluorescent cells, about 57% of cells formed aggregates in the presence of the αAP1 peptide, whereas a relatively greater number of cells (about 75%) formed aggregates in the presence of the αBP4 peptide (Figure 9). In the

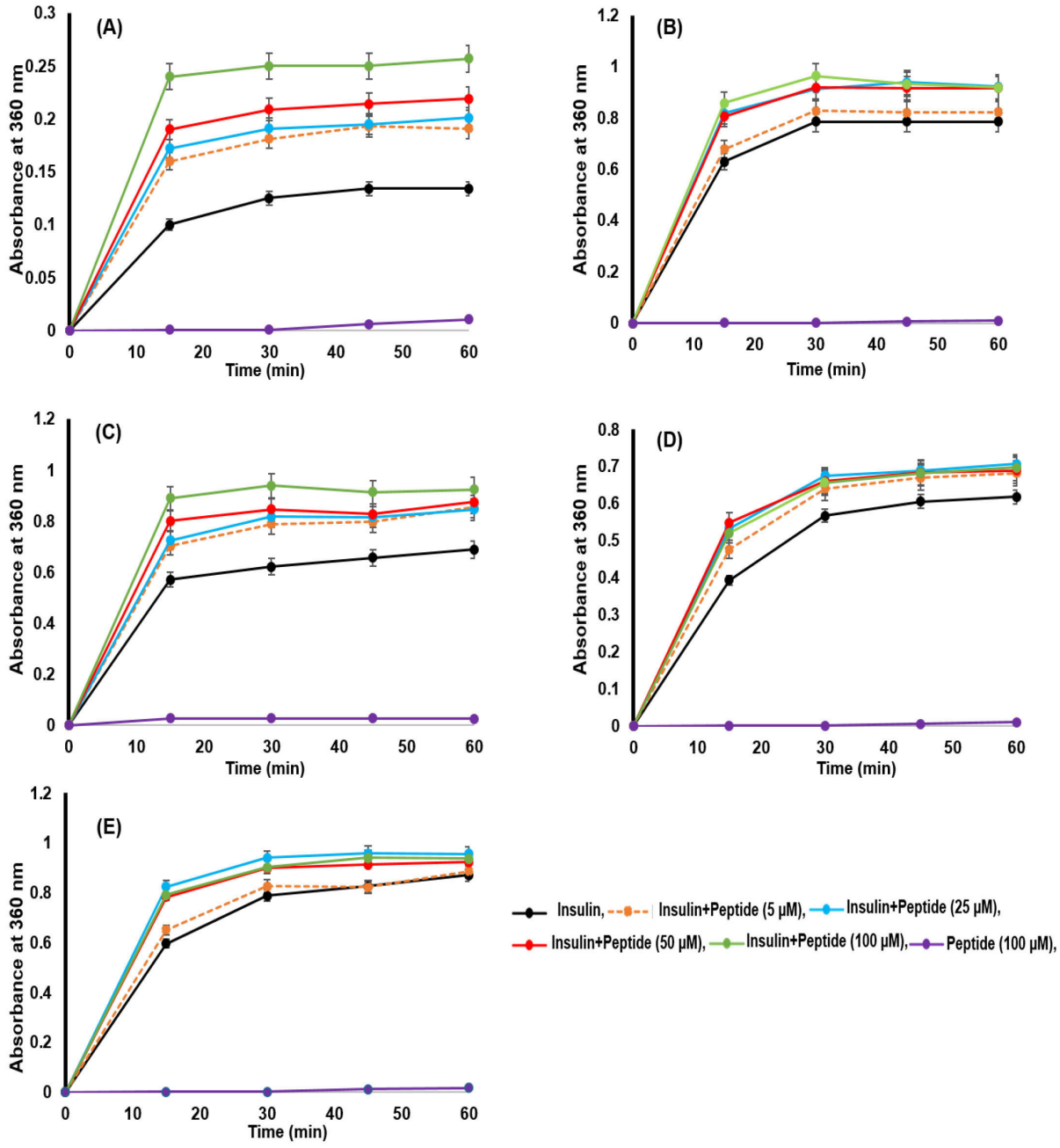

Figure 3. Determination of anti-chaperone activity of selected peptides. Antichaperone activity determination of the five selected peptides—(**A**) αAP1, (**B**) αAP2, (**C**) αBP3, (**D**) αBP4, and (**E**) αBP5—using insulin as the target protein. The effect of various concentrations of the peptides (5, 25, 50, and 100 µM) in aggregation of insulin (200 µg) in the presence of dithiothreitol (DTT) at 37 °C for 60 min was determined. Absorbance at 360 nm was recoded every 15 min.

presence of either peptide, the aggregates were localized around the nuclei of the cells. Additionally, larger aggregates were observed in the presence of the αBP4 peptide relative to the αAP1 peptide. Under similar conditions, the scrambled αAP1 peptide (used as a control) also formed aggregates, but in only 35% of the cells. The results showed that the αAP1- and αBP4 peptides facilitated aggregate formation ex vivo in lens epithelial cells, which further confirmed previous in vitro aggregation results.

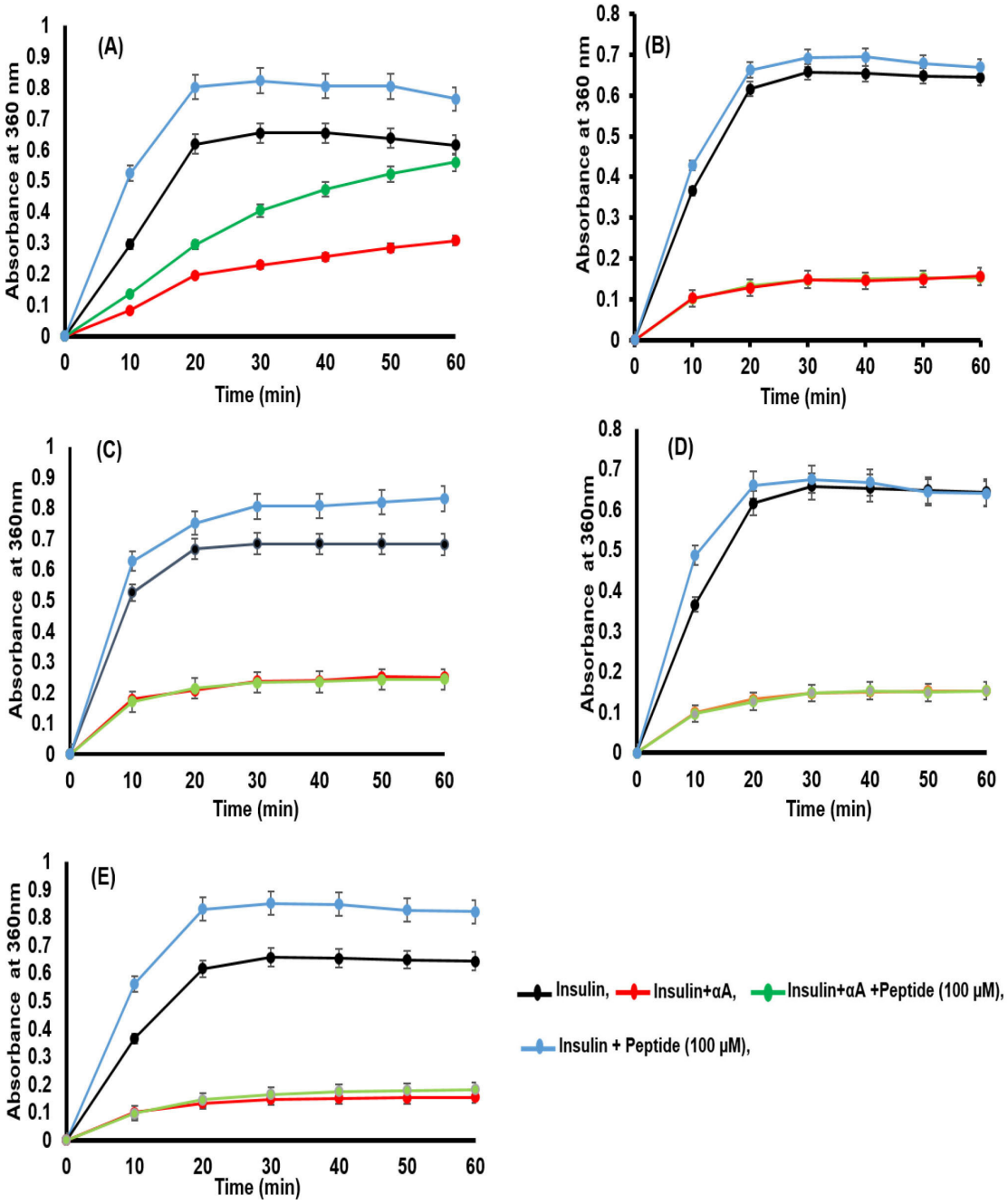

Figure 4. Effect of peptides on the chaperone activity of αA-crystallin using insulin as the target protein. Effect of the five selected peptides—(**A**) αAP1, (**B**) αAP2, (**C**) αBP3, (**D**) αBP4, and (**E**) αBP5—on the chaperone activity of αA-crystallin using insulin as the target protein. In the assay, 100 μM of individual peptides with recombinant αA-crystallin (100 μg each) with insulin (200 μg) as a target protein plus dithiothreitol (DTT) were incubated at 37 °C for 60 min. Absorbance at 360 nm was recoded every 15 min.

DISCUSSION

The major findings of the study are as follows: (1) TCA treatment precipitated all lens proteins in the WS- and WI-protein fractions of normal and cataractous lenses, while peptides with M_r < 5 kDa remained as a soluble fraction. (2) The TCA-solubilized peptides were derived from α-, β-, and γ-crystallins. Among these crystallins, a greater number of αA and αB peptides were solubilized from the 62-year-old normal lenses and from the age-matched nuclear cataractous lenses. (3) A relatively greater number of N-terminally derived αA-peptides were found in the cataractous lenses than those derived from the N-terminal region of αB-crystallin.

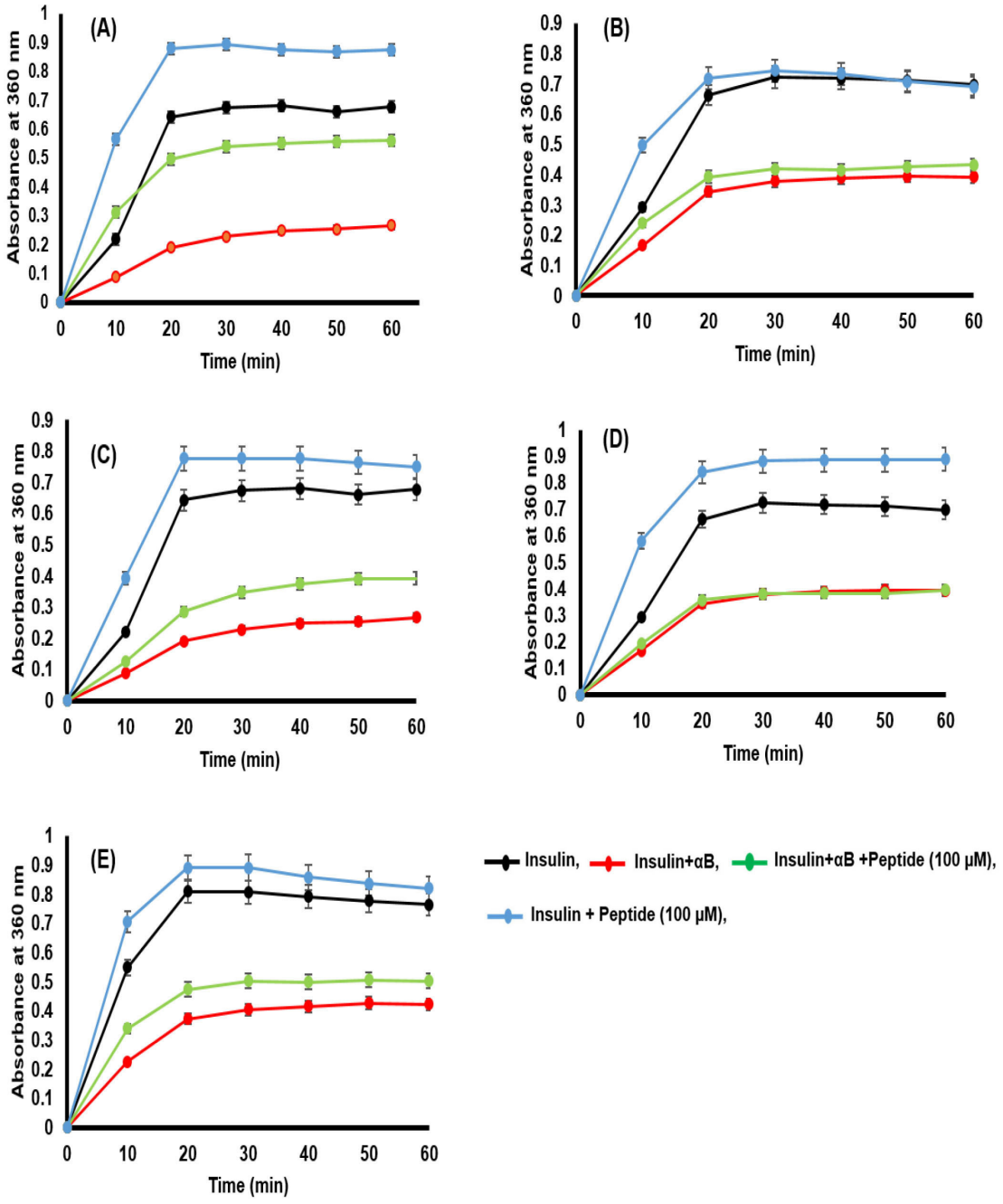

Figure 5. Effect of peptides on the chaperone activity of αB-crystallin using insulin as the target protein. Effect of the five selected peptides—(**A**) αAP1, (**B**) αAP2, (**C**) αBP3, (**D**) αBP4, and (**E**) αBP5—on the chaperone activity of αB-crystallin using insulin as the target protein. The chaperone activity was determined as described in Figure 4 and the Materials and Methods section.

Table 3. Secondary structural changes of αA- and αB- crytsallin in presence of αAP1 and αBP4 during CD-spectroscopy.

Sample	Helix (%)	Sheet (%)	Irregular (%)
αA	7.3	64.6	27.7
αA+ αAP1	8	50.6	37
αA+ αBP4	1.4	61.1	20
αB	7.5	66.8	20.9
αB+ αAP1	6.3	55.6	42.5
αB+ αBP4	6.2	59.8	30.9
αAP1	1.41	45.3	37.7
αBP4	2.3	50.3	35.4

The TCA-solubilized fractions contained relatively fewer peptides derived from the CD and C-terminal region of the αA- and αB-crystallins. (4) Based on the abundance of the peptides, five peptides—two αA peptides (αAP1(1–15) and αAP2(38–50)) and three αB peptides (αBP3(30–44], αBP4(130–137), and αBP5 (163–175))—were commercially synthesized. Among the five peptides, the maximum anti-chaperone and aggregation properties were associated with the αAP1 peptide and the αBP4 peptide, respectively.

Although long-lived crystallins have almost no turnover [18], cleavages in lens crystallins occurs during aging and cataract formation [28-30]. Whether the cleavages of these crystallins occur due to enzymatic or non-enzymatic mechanisms is not well understood [42-44]. The clearance of cleaved crystallin fragments is believed to occur via the proteasome or ubiquitin system [43]. However, the systems operate only in lens epithelial cells and cells in the outer cortical region because of the loss of organelles in the inner cortical and nuclear regions. It has also been shown that the cleavages in crystallins occur at serine residues as a preferred site with involvement of either a yet unidentified serine-type protease or an unidentified mechanism [22,44,45]. However, despite several yet unknown factors that generate crystallin fragments or small peptides in vertebrate lenses, their accumulation during aging and cataract development is well established [8,10,11,24,25,39].

The focus of the present study was to determine the potential roles of the five selected peptides described above on chaperone activity of αA- and αB-crystallins and their aggregation properties. Although previous studies have not

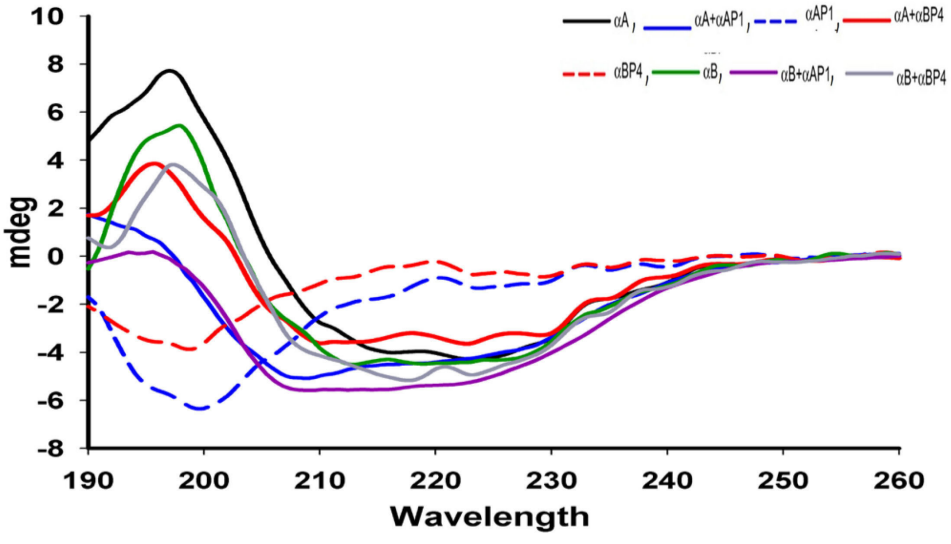

Figure 6. Determination of the effects of the αAP1 and αBP4 peptides on the secondary structure of αA- and αB-crystallins with CD spectroscopy. For circular dichroism (CD) spectral analysis, 10 µM protein (αA- or αB-crystallin) and 25 µM peptide (αAP1 or αBP4) in 200 µl reaction mixtures of 10 mM phosphate buffer (pH 7.4) were incubated at 37 °C for 15 min, and the secondary structures were recorded in the far-ultraviolet (UV) region (190–260 nm) using a Jasco CD spectrometer and appropriate controls (αA- or αB-crystallin alone and the αAP1 or αBP4 peptide alone). The spectrum of the buffer alone was subtracted from each spectrum, and the secondary structure was predicted using the Selcon 3 program.

shown that the five peptides are present in human cataractous lenses relative to age-matched normal lenses, specific cleavages in the regions of crystallins that could produce the peptides have been reported. (1) Similar to the αA1(1–15) peptide, αA(2–12) peptide has been detected [46]. (2) Similar to the αAP2(38–50) peptide, an αA(37–49) peptide was detected [45]. (3) Similar to the αBP3(30–44) peptide, an αB(28–39) peptide was also detected [45,47]. (4) Similar to the αBP4 peptide, an αB(130–137) peptide was seen [47]. (5) Similar to the αBP5 (163–175) peptide, αB peptides, with residue 155–175, 157–155, and 160–175, were also detected [45].

Previous studies have implicated crystallin fragments in the development of lens opacity because they exhibited inhibition of chaperone activity of α-crystallin and enhanced aggregation of lens proteins [25,26]. αA- and αB-crystallins mediated the protection of lens cells against heat and oxidative stress–induced cell death [45]. Similarly, intraperitoneal

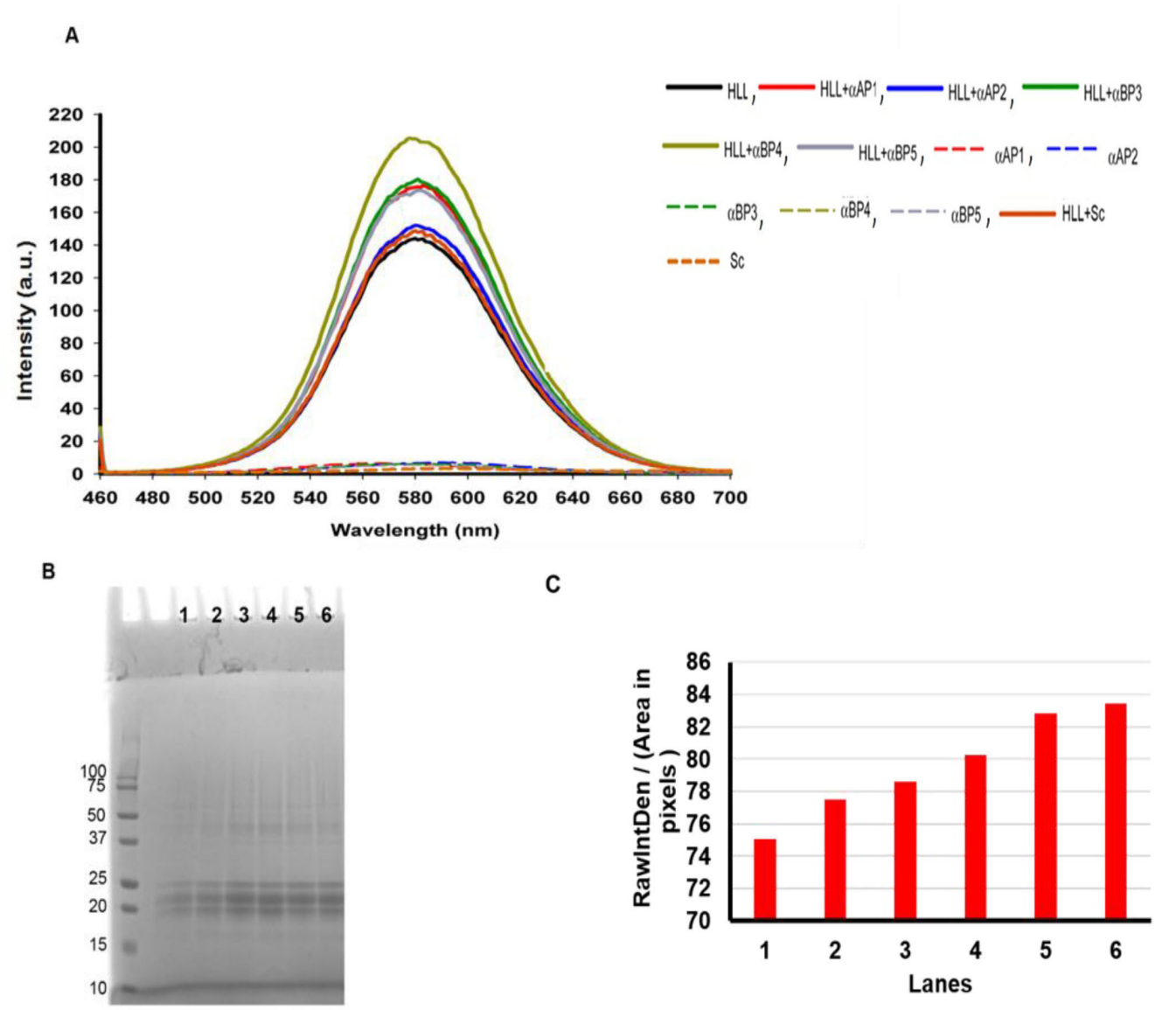

Figure 7. Aggregation of lens proteins on incubation αAP1 and αBP4 peptides with WS-HLL. The water soluble (WS) protein (250 µg) was incubated at 37 °C with 100 µM of the selected peptides in a 200 µl reaction mixture in 10 mM phosphate buffer (pH 7.4, containing 100 mM NaCl) for 18 h. Aggregation of the WS protein was analyzed with the Aggresome assay kit (Enzo Life Sciences) using the manufacturer's instructions as described in the Materials and Methods section. Experiments were performed in triplicate, but the results of only one set are presented.

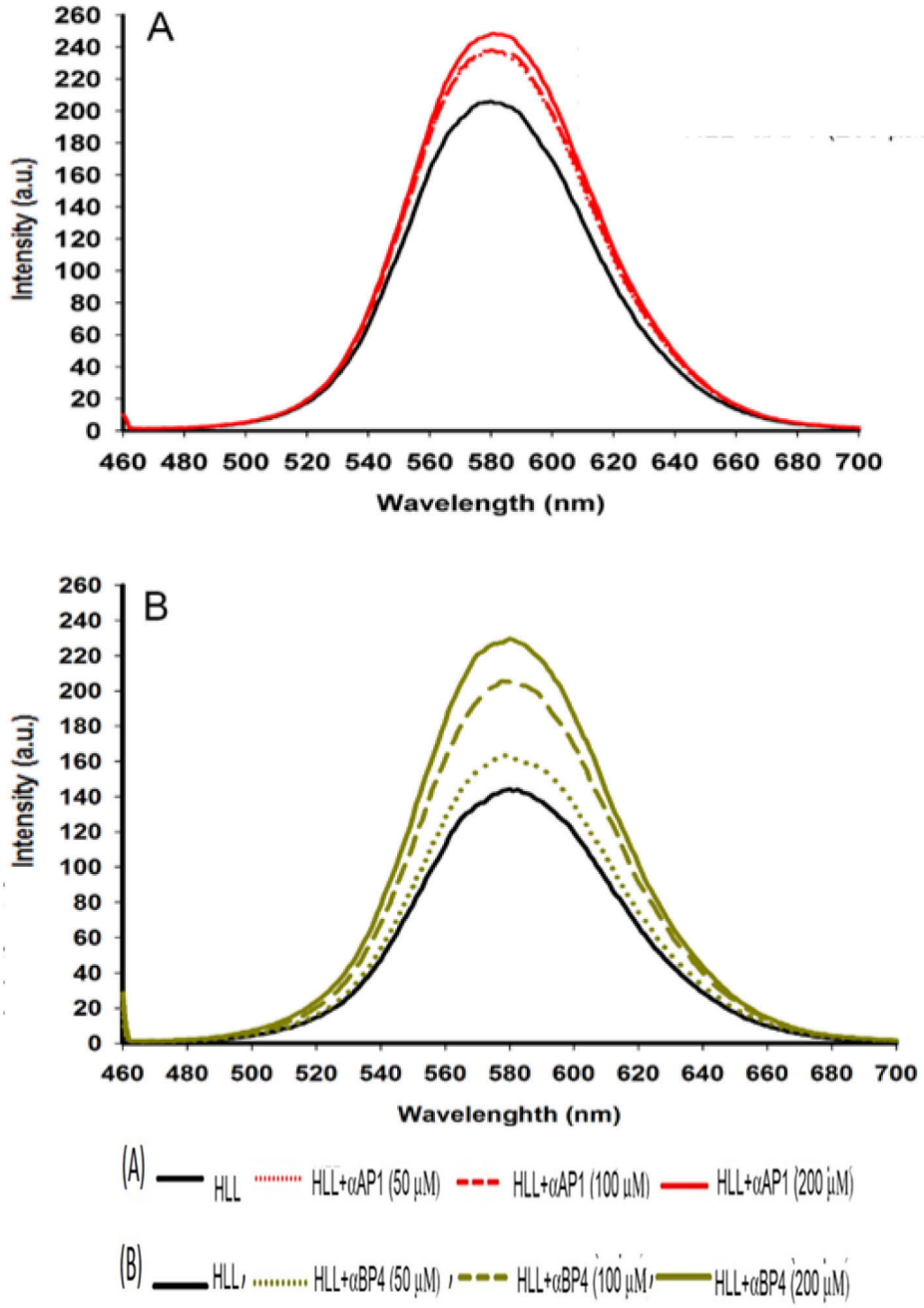

Figure 8. Emission spectra of HLL (250 μg) in the presence of 50, 100, and 200 μM peptides after 18 h incubation at 37 °C. **A**: The αAP1 peptide. **B**: The αBP4 peptide.

injection of the minichaperones of the αA peptide ($_{70}$KFVIFLDVKHFSPEDLTVK$_{88}$) and the αB peptide ($_{73}$DRFSVNLDVKHFSPEELKVK$_{92}$) inhibited cataract development in selenite-treated rats, which was accompanied by inhibition of oxidative stress, protein insolubilization, and induction of caspase activity [47]. Although the independent existence of these minichaperones within the lens has not been demonstrated, if they exist, these peptides would be beneficial for lens cell survival.

In this study, a higher number of peptides (a total of 38) were identified from αB-crystallin compared to 28 peptides from αA-crystallin (Figure 1) that were present in cataractous lenses. This suggested relatively greater susceptibility to cleavage of αB-crystallin compared to αA-crystallin in

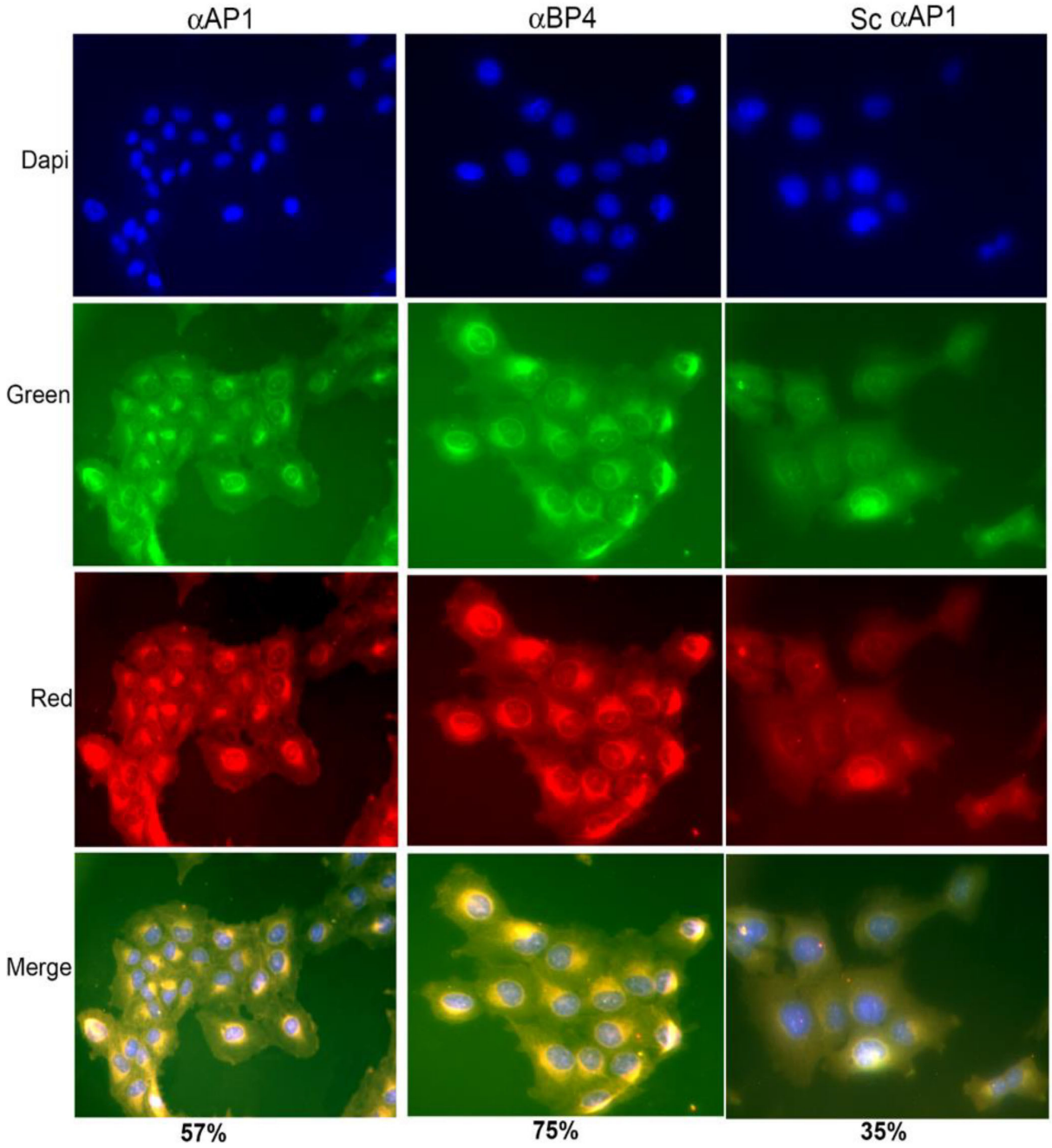

Figure 9. Aggregate formation by internalized FITC-labeled αAP1, αBP4, and scrambled peptides in human lens epithelial cells (HLEB3) in culture. The cells were permeabilized and then treated with Proteostat Aggresome dye. In the cells, individual peptides alone showed green fluorescence, and red fluorescence by aggregated peptides reactive to Proteostat Aggresome dye, along with cellular nuclei (stained blue with Hoechst stain). The merged image shows the overlay of the green and red fluorescence of cells. Each experiment was performed in triplicate, and 100 cells with either red or green fluorescence or both were manually counted with a hemocytometer using a Leica fluorescent microscope.

cataractous lenses, as was reported in our previous study [29]. The majority of the αA peptides (53%) and αB peptides (28%) were generated from the N-terminal region and fewer from the CD and CTE regions, which was also reported by us [22,24]. Only three peptides and 13 peptides were observed from the C-termini of αA- and αB-crystallins, respectively, only in cataractous lenses. This result differed from previous studies by Kumar et al. [25], in which they did not find any peptides from the CTE region of α-crystallin, and that of Su et al. [22], who reported only a few peptides from the CTE region of αA- and αB-crystallins. Similar to other studies, only a few peptides from β- and γ-crystallins were also recovered in the TCA-soluble fractions (data not presented). Although peptides with N-termini from αA- and αB-crystallins were recovered in greater abundance, previous reports have suggested a greater susceptibility of amino acids from the CTE region to proteolytic cleavage [43,44]. A few peptides were reported to increase the surface hydrophobicity of α-crystallin, which might expose the NTD region of α-crystallin and make the region unstable and cleavage susceptible [27]. Su et al. analyzed NTD peptides and suggested that cleavage targeting serine residues is a non-enzymatic process, such as internal splicing and spontaneous peptide bond cleavage [22]. However, this study also suggested that a trypsin-like proteolytic cleavage might also be responsible [22].

All five selected peptides (two from the NTD of αA-crystallin and three from αB-crystallin [one peptide from each NTD, CD, and CTE]) exhibited light scattering with insulin as the target protein. Similarly, the αA(66–80), αB (1–18), and βA3/A1(59–74) peptides were also reported to increase light scattering of α-crystallin with alcohol dehydrogenase as the target protein [24,25,27,48]. In these studies, most of the peptides were hydrophobic, and possibly their interactions with hydrophobic residues of the target protein facilitated aggregation. Among the five peptides, the αAP1 peptide maximally inhibited the chaperone function of αA- and αB-crystallins, whereas the αBP4 peptide did not show any effect. The three remaining peptides (αAP2, αBP3, and αBP5) slightly inhibited the chaperone activity of αB-crystallin; however, they did not show any effect on the chaperone function of αA-crystallin. The CD spectra showed a significant alteration in the secondary structures of αA- and αB-crystallins in the presence of the αAP1 peptide, but only a slight alteration in the presence of the αBP4 peptide (Figure 6), suggesting that the chaperone activities of αA- and αB-crystallins were affected due to their secondary structural changes in the presence of the αAP1 peptide. These results are similar to the earlier reported loss of the chaperone activity of crystallins by the peptides αA(66–80), αB(1–18), and βA3/A1(59–74) [24-26,48].

Presently, the mechanism of the antichaperone activity of crystallin peptides is not well understood. The amyloid fibrils are speculated to play a major role in the chaperone and antichaperone mechanisms of a protein. Amyloids are self-assembled, highly ordered linear aggregates of a soluble protein or peptide. One key factor that describes whether an amyloid structure of a protein or peptide would display chaperone-like or antichaperone activity is its electrostatic interactions with a presented substrate [49]. If the surface charges of amyloid fibrils on binding to a substrate are cancelled, its colloidal stability is reduced, and it facilitates protein aggregation. For example, a recent study showed that Aβ(25–35) amyloid contains a net positive charge at a neutral pH and on interaction with a negatively charged acidic protein by electrostatic interaction facilitated aggregation [49]. This suggested that the amyloid fibrils' property of chaperone-like or antichaperone activity is based on their colloidal stability following electrostatic interaction, with a substrate [49]. Presently, we do not know whether the αAP1 peptide forms amyloid fibrils, and an investigation is planned as future research.

The present study showed that although the αAP1 and αBP4 peptides caused aggregation of proteins in the WS-HLL human lens homogenate (Figure 7), there was greater aggregation by the αBP4 peptide relative to the αAP1 peptide. The increasing peptide levels occur in human lens WS-HLL with age or during cataract development, and these peptides may play a role in the insolubilization of the WS proteins. A recent study showed that αA(66–80) peptide formed aggregates, and the resulting peptide-α-crystallin aggregates were resistant to dissociation with high ionic strength, except in high dissociating agents such as guanidium hydrochloride and urea [27]. The study showed that the peptide formed a hydrophobically driven stable complex with α-crystallin, which reduced the solubility of the latter. Therefore, the crystallins' peptides could play a potential role in the insolubilization of proteins during cataract development [24-26,45].

The present study also showed that 1 μM αAP1 and αBP4 peptides formed aggregates in HLECs under ex vivo conditions. The αAP1, αBP4, and scrambled αAP1 peptides were covalently conjugated with FITC and six arginine residues at their N-termini, as the use of polyarginine is a more efficient method among cell-penetrating homopolypeptides [34-37]. They are highly cationic and therefore easily cross the cell membrane. The αBP4 peptide showed maximal protein aggregates (about 75%) in cells relative to 57% cells by the αAP1 peptide, but only about 35% cells by the scrambled αAP1

peptide (Figure 9). The αBP4 peptide also formed bigger aggregates than the αAP1 peptide, and they were present around the nucleus. We do not know whether the neutral and hydrophobic nature of the αBP4 and αAP1 peptides, respectively, are responsible for the difference in their in vivo aggregation properties. A previous study showed that the αA(66–80) peptide interacted with soluble α-crystallin and induced its aggregation and precipitation, and it was believed that the αA(66–80) peptide formed a hydrophobically driven, stable complex with α-crystallin and reduced its solubility.

In summary, we identified peptides from cataractous lens, which showed antichaperone activity and facilitated aggregation of proteins in ex vivo and in vitro conditions. In the future, we plan to investigate the potential mechanism of αAP1 peptide-induced antichaperone activity and αBP4-induced aggregation of lens crystallins.

APPENDIX 1. SDS–PAGE ANALYSIS OF LMW POLYPEPTIDES ISOLATED FROM WS- AND WI-PROTEIN FRACTIONS OF 62-YEAR OLD NORMAL AND AGE-MATCHED CATARACTOUS LENSES.

To access the data, click or select the words "Appendix 1." Lanes (1) and (2) show TCA-solubilized proteins from WS-protein fraction, and WI-protein fractions of normal lenses, respectively. Lanes (3) and (4) show TCA-solubilized proteins from WS-protein fraction, and WI-protein fractions of cataractous lenses.

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