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Peptide-protein complex from cattle sclera: Structural aspects and chaperone activity

Anna P. Ilyina [a,*], Egor V. Sidorsky [b], Artem V. Tregubov [a], Valeria M. Chekova [a], Pavel A. Elistratov [a], Viktoria P. Yamskova [a], Igor A. Yamskov [b]

[a] Institute for Bioregulation Problems LLC, Russian Federation, 119991, Leninsky Prospect, 45, Moscow, Russia
[b] A.N.Nesmeyanov Institute of Organoelement Compounds of Russian Academy of Sciences, Russian Federation, St. Vavilova, 28, 119991, Moscow, Russia

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ABSTRACT

The influence of temperature and chaotropic agents on the spatial organization of the peptide-protein complex isolated from cattle sclera at the level of secondary structure was studied by UV, CD spectroscopy, and dynamic light scattering. It is shown that this complex has high conformational thermostability. The point of conformational thermal transition (65 °C) was determined, after which the peptide-protein complex passes into a denatured stable state. It was found that the peptide-protein complex in aqueous solutions forms thermostable nanosized particles. It was shown that the peptide-protein complex isolated from cattle sclera shows the properties of chaperone, an inhibitor of model protein aggregation induced by dithiothreitol.

1. Introduction

Currently, in various mammalian tissues, including eye tissues, a certain group of bioregulators has been identified, which are localized in the intercellular space and ensure the maintenance of adhesive intercellular interactions that can affect migration, proliferation, differentiation, and also contribute to an increase in cell vitality *in vitro*. In addition, their most important property was discovered – they stimulate regenerative and reparative processes in injured and pathologically altered tissues [1,2]. Their activity is characterized by the presence of tissue specificity, but the lack of species specificity. An interesting property is their effect on the cell plasma membrane, changing its permeability, in very low doses – 10^{-15}-10^{-8} mg protein/ml. An important property of bioregulators of this group is their ability to influence the visco-elastic properties of the plasma membrane of cells in low doses [2]. These bioregulators are resistant to various physical and chemical factors: changes in pH, temperature, the action of chelating agents, etc. Their tendency toward intermolecular association with the formation of nanoparticles 50–300 nm in size was also noted, and the nanoscale state determines the nature of their activity [3,4]. The similarity of the physicochemical properties and the nature of the biological action made it possible to separate these bioregulators into a group of membranotropic homeostatic tissue-specific bioregulators (MHTB) [1,2].

MHTBs have a complex composition. Their basis is a peptide-protein complex, which includes biologically active peptides (molecular weight 1–8 kDa) and a protein modulating their biological effect – a protein modulator [4,5], interacting with peptides by the calcium-binding mechanism [2,5]. For MHTBs isolated from sclera, pigment epithelium, and cattle serum, it was found that the modulator protein is *Bos taurus* serum albumin of a certain isoform [4–6]. It should be noted that carbohydrates and lipids were detected in the peptide-protein complex, however, their functional significance in the composition of MHTB remains unknown [3,7,8].

Previously, MHTB as a biologically active fraction, ASK-53, was isolated from the sclera of the cattle's eye by extraction with Ringer's solution at 4°C, followed by fractionation by precipitation of proteins with a saturated solution of ammonium sulfate and reversed-phase HPLC in a water-acetonitrile gradient. Its composition included the isoform of serum albumin *B. taurus* (under the number gi|1351907 in the Uniptrot database) and a set of peptides with molecular weights of 1300–5100 Da [6]. To study the specific activity of ASK-53, an experimental model of culturing the posterior part of the eye of the newt *Pleurodeles waltl* [9] was developed, which was possible due to the fact that the biological effect of MHTB is characterized by the presence of tissue specificity, but the lack of species specificity [10]. The addition of

Abbreviations: MHTB, membranotropic homeostatic tissue-specific bioregulator; PPC, peptide-protein complex.

* Corresponding author.
E-mail address: a.ilyina@gmail.com (A.P. Ilyina).

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ASK-53 to the culture medium at low concentrations helped to preserve the spatial organization of the tissues of the posterior part of the eye and increase the viability of fibroblasts. This was expressed in maintaining the interaction between the vascular and scleral membranes, and the preservation of compact adhesive interactions was noted within the choroid. The monolayer of pigment epithelium was also in better condition compared to the control: the pigment was distributed evenly in the cells, adhesive interactions remained between the cells. In the sclera, a more compact and dense arrangement of fibers with less noticeable bundles was observed than in the control. The calculation of fibroblasts in the tissue of the sclera of cultures of the experimental and control series showed that when exposed to ASK-53, the number of viable fibroblasts was 2.5 times greater than in the control [6,10].

In the present work, the investigation of the ASK-53 fraction was continued and attempts were made to study the conformational dynamics to explain its stability. Also, the ability of this fraction to intermolecular association with the formation of nanosized particles was studied. The spatial organization of ASK-53 was analyzed at the secondary and tertiary structure levels by UV and CD spectroscopy, and its association in the native state and when exposed to chaotropic agents and temperature by dynamic light scattering. In addition, taking into account the report that serum albumin exhibits chaperone-like activity, based on this review [11], the ability of ASK-53 to inhibit the aggregation of certain proteins *in vitro* was studied.

2. Materials and methods

2.1. Isolation and purification

The peptide-protein complex, which is part of the MHTB sclera, was isolated from the freshly enucleated eyes of young bulls obtained at meat processing plants in Moscow and the Moscow Region. The sclera was separated and cleaned from other tissues of the eye and placed for 3 h at 4 °C in an extracting solution of the following composition: 1 mM $CaCl_2$, 0.15 M NaCl, 1.0 mM HEPES, pH 7.0–7.2. The resulting tissue extract was filtered through four layers of cheesecloth and centrifuged at 3000 g for 15 min. The supernatant was collected and the proteins precipitated with ammonium sulfate (~70% saturation) for 96 h at 4 °C. The resulting protein suspension was centrifuged at 15,000 g for 45 min, the supernatant and precipitate were collected separately. The supernatant was dialyzed against distilled water, the pellet was dialyzed against 0.01 M Tris buffer until all traces of salt were removed. The precipitate fraction was separated by reversed-phase HPLC on an "Agilent 1260" liquid chromatograph (United States) with a Kromasil C4 (4.6 × 250 mm) "AkzoNobel" column (Sweden) equilibrated with an aqueous solution of 0.1% trifluoroacetic acid (pH 2.2). The material bound to the sorbent was eluted with a 0–60% acetonitrile concentration gradient in 0.1% trifluoroacetic acid (pH 2.2) for 60 min at an elution rate of 1 ml/min. Detection was performed spectrophotometrically at 210 nm.

The peptides and albumin of the ASK-53 complex were separated in a solution containing 6.0 M guanidine hydrochloride, 3.0 mM ethylenediaminetetraacetic acid and 0.3 M NaCl, at 45°C for 24 h. Then five volumes of 95% ethanol were added to the solution and left on 72 h at 4 °C. The precipitate and supernatant obtained after precipitation with alcohol were separated by reversed-phase HPLC to obtain two fractions containing peptides and albumin. All fractions were assayed for membranotropic activity.

2.2. Protein content

At all stages of the study, the protein content in all studied fractions was determined spectrophotometrically by the method of Warburg and Christian [12].

2.3. Membranotropic activity assessment method

The study was performed on male F1 C57BL/CBA mice (weighing 18–20 g) contained in standard vivarium conditions of the Koltzov Institute of Development Biology Russian Academy of Sciences and all animal experiments should comply with the ARRIVE guidelines. The membranotropic activity was investigated by a method previously developed for the identification of MHTB [13]. The method is based on the determination of the viscoelastic properties of liver tissue under conditions of shear strain after organotypic cultivation. Data were statistically processed with the Student's *t*-test.

2.4. The effect of temperature on the activity of the peptide-protein complex

The temperature dependence of the membranotropic activity of ASK-53 was studied after pre-incubated the complex aqueous solution at a concentration of 100 μg/ml for 20 min at temperatures of 20, 60, 80, and 100°C. Then, membranotropic activity was studied by the method previously developed for the identification of MHTB [13]. Data were statistically processed with the Student's *t*-test.

2.5. UV spectroscopy

UV spectra were recorded on a "Jasco V-780" spectrophotometer (Japan) in quartz cells with the pathlength of 1 cm and the bandwidth of 1 nm, and the concentration of the analyzed samples was 100 μg/ml. The final spectrum was obtained by averaging the data of three scans and subtracting the spectrum of the baseline (control).

2.6. Circular dichroism spectroscopy

Circular dichroism spectra were recorded on a "Jasco 1500" CD spectrometer (Japan) in quartz cells with the pathlength of 0.1 cm for the peptide region of the spectrum (190–260 nm) and 1 cm for the aromatic region (240–340 nm). A cell with a 1.5 μM ASK-53 solution in 10 mM sodium phosphate buffer solution was thermostated at temperatures of 20, 30, 40, 50, 60, 70, 80, 90, 100°C for 15 min until the CD spectrum was taken in a "ThermoMixer F1.5" thermostat ("Eppendorf", Germany). All CD spectrum were collected continuously at a scan speed 100 nm/min, the step in 0.1 nm in a continuous mode, the concentration of the analyzed samples is 100 μg/ml. The final spectrum was obtained by averaging the data of three scans and subtracting the spectrum of the baseline (control) [14]. The content of secondary structure elements was evaluated using the program of the manufacturer CD Multivariate SSE "Jasco" (Japan).

2.7. Laser dynamic light scattering

The method of laser dynamic light scattering (photon correlation spectroscopy) was used to determine the hydrodynamic radius of ASK-53 particles dispersed in an aqueous solution at a concentration of 100 μg/ml. The measurement was carried out on a "Photocor Compact-Z" analyzer (Russia) equipped with a thermostabilized AlGaInP diode laser with a wavelength of $\lambda = 637.4$ nm and a power of 30 mW, with a built-in "Photocor-FC" multichannel correlator, obtaining the correlation function of the fluctuations of the scattered light intensity and the integral scattering intensity. The correlation function was processed using DynaLS software from "Alango Technologies Ltd" (Israel) [15,16]. The range of acceptable measured sizes of nanoparticles is in the range from fractions of a nanometer to 5–10 μm. The measurements were carried out at a scattering angle of 90° in the temperature range from 20 to 100°C. The aqueous solution containing the studied PPC was preliminarily cleaned of dust by filtration through Durapore membranes from "Millipore" (USA) with a pore diameter of 0.45 μm.

2.8. Inhibition of DTT-induced aggregation of BSA and lysozyme

DTT-induced BSA aggregation was studied in water at 50°C, lysozyme in 1 mM borate buffer (pH 9.0) at 50°C. The buffer was placed in a cylindrical vial with an inner diameter of 6.3 mm and pre-incubated for 5 min at 50 °C. The vials were corked to prevent evaporation. The aggregation process was initiated by adding an aliquot of DTT to the protein solution to a final volume of 0.5 ml. The measurement was carried out on a "Photocor Compact-Z" analyzer (Russia) equipped with a thermostabilized AlGaInP diode laser with a wavelength of $\lambda = 637.4$ nm and a power of 30 mW, with a built-in "Photocor-FC" multichannel correlator, obtaining the correlation function of the fluctuations of the scattered light intensity and the integral scattering intensity. The correlation function was processed using DynaLS software from "Alango Technologies Ltd" (Israel) [15,16]. The range of acceptable measured sizes of nanoparticles is in the range from fractions of a nanometer to 5–10 μm. The measurements were carried out at a scattering angle of 90°. To study the effect of PPC on the DTT-induced aggregation of BSA and lysozyme, it was added in a wide range of concentrations to a preheated solution of the model protein before the introduction of DTT.

The effect of ASK-53 on the secondary structure of BSA and lysozyme was studied using CD spectroscopy in the far-UV region (190–260 nm) on a "Jasco 1500" CD spectrometer (Japan). Samples were preliminarily prepared in an appropriate buffer, DTT and/or ASK-53 were added for aggregation, after incubation at 50°C for 75 min, measurements were made in quartz cells with the pathlength of 0.1 cm. Spectra were recorded from 3 to 10 times, averaged and subtracted the buffer spectrum.

3. Results and discussion

Earlier, we obtained a bioregulator from cattle sclera tissue according to the developed method for the isolation and purification of MHTB, which includes: extraction with Ringer's solution at 4 °C, precipitation in a saturated solution of ammonium sulfate, separation of the precipitate on reversed-phase HPLC. After separation by HPLC, an ASK-53 fraction was obtained, having an activity consisting of peptides with molecular weights of 1300–5100 Da and one of the isoforms of serum albumin *B. taurus* [6]. This fraction also contained carbohydrates - 1:25 relative to the protein, such as glucose, glucosamine, mannose, galactose, xylose, fucose, N-acetylglucosamine and the components that make up glycerides, phospholipids, glycolipids and saturated fatty acids ($C_{6:0}$, $C_{8:0}$, $C_{9:0}$, $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{17:0}$, $C_{18:0}$) and monoenoic fatty acid ($C_{16:\ 1}$).

3.1. Comparative analysis of the spatial structure of ASK-53 by optical spectroscopy methods

In the near-UV region (230–350 nm), the absorption spectrum of the ASK-53 fraction has a minimum at 255 and a maximum at 276 nm, as well as a pronounced inflection in the spectrum of the peptide-protein complex in the region of 290 nm (figure is not shown). These data, as well as the presence of a negative absorption band at 291 and a positive at 295 nm characteristic of tryptophan residues in the second derivative of the ASK-53 UV absorption spectrum (Fig. 1, green line) [17], indicate the presence of residues of this amino acid in ASK-53. The presence of a negative band at 283 nm and two positive bands – 279 nm and 288 nm, indicates the presence of tyrosine residues in ASK-53 [17]. Also in the second derivative of the absorption spectrum of ASK-53, seven distinct negative and positive bands were detected in the UV region of 250–270 nm, which belong to phenylalanine residues (Fig. 1, green line) [17,18]. The bands shown correspond to the second derivative of the UV absorption spectrum of an albumin solution.

The ASK-53 CD spectrum in the far UV region (190–250 nm), the region of absorption of peptide bonds, is characterized by the presence of two extremes at 208 and 222 nm and a maximum in the region of 190–195 nm (Fig. 2, red line) and is characteristic of α-proteins [19]. According to the calculated data, the ASK-53 fraction contains 47.3% α-helices, 14.6% β-sheets, 10.5% β-turn, and 27.6% of the sections of the random coil (Table 1). These data are consistent with the results of the study of an inactivator protein isolated from cattle serum (48% α-helices and 21% β-structure) [5].

After processing ASK-53 with 6.0 M guanidine hydrochloride and subsequent separation of the reaction mixture on a reverse phased-column, we were able to obtain an active fraction containing peptides with mol. masses 1300–5100 Da and the fraction containing albumin [6]. The calculation of the secondary structure elements of both fractions showed a decrease in the content of α-helices and an increase in the β-structure and sections of the random coil (Fig. 2, Table 1). It can be assumed that after the interaction of the peptides with the albumin molecule in ASK-53, they come to a more stable state, which is confirmed by the higher content of α-helices in the fraction of the ASK interaction of peptides and albumin in ASK-53, they are in a more stable state, which is confirmed higher content of α-helices in the ASK-53 fraction. The same was observed when studying the secondary structure of a serum albumin solution in work [20], that is, a high content of α-helices in the secondary structure of albumin is a criterion for the stability of the molecule. In addition, in the second derivative of the absorption spectrum of the fraction of peptides from ASK-53 mol masses

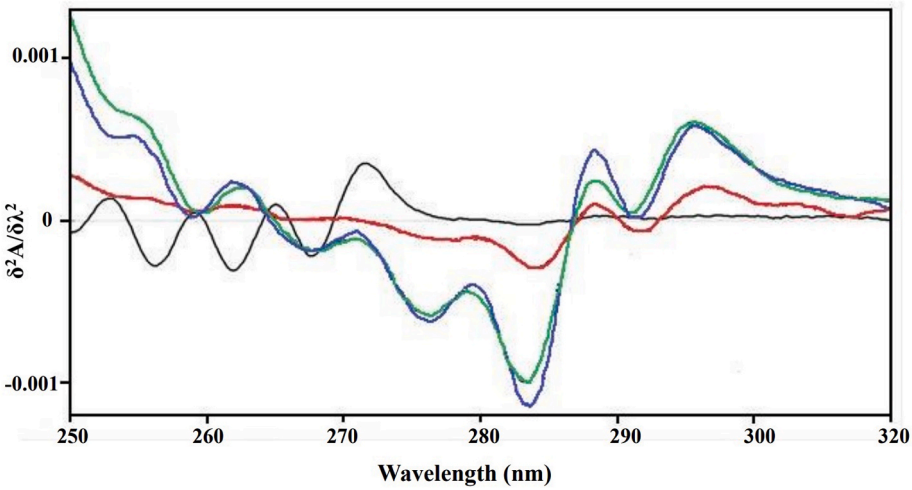

Fig. 1. The second derivative of the UV absorption spectrum ASK-53 (green line), ASK-53 after heating at 100 °C (blue line), ASK-53 after treatment with chaotropic agents and separation by HPLC - fraction containing peptides (red line) and the fraction containing albumin (black line).

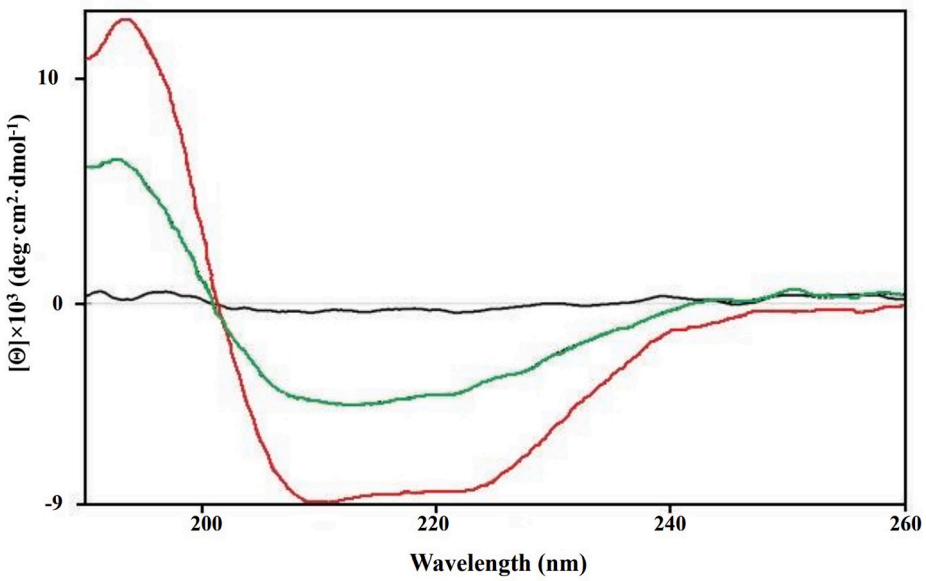

Fig. 2. CD spectrum in 10 mM sodium phosphate buffer in the far-UV region ASK-53 (red line) and ASK-53 after treatment with 6.0 M guanidine hydrochloride and separation by HPLC-fraction containing peptides (black line) and albumin fraction (green line).

Table 1
The content of the secondary structure of the peptide-protein complex ASK-53 at various temperatures, as well as fractions of peptides and albumin isolated from ASK-53.

Sample and temperature	α-helix,%	β-sheets, %	β-turn, %	random coil, %
20°C	47.3 ± 0.8	14.6 ± 0.2	10.5 ± 0.2	27.6 ± 0.5
30°C	48.7 ± 0.8	13.8 ± 0.2	10.5 ± 0.2	27.0 ± 0.5
40°C	46.7 ± 0.8	14.8 ± 0.3	10.6 ± 0.2	27.9 ± 0.5
50°C	44.8 ± 0.8	15.7 ± 0.3	10.7 ± 0.2	28.8 ± 0.5
60°C	40.1 ± 0.7	18.5 ± 0.3	11.0 ± 0.2	30.4 ± 0.5
70°C	30.8 ± 0.5	24.0 ± 0.4	11.6 ± 0.2	33.6 ± 0.6
80°C	22.2 ± 0.4	29.7 ± 0.5	12.0 ± 0.2	36.1 ± 0.6
90°C	21.4 ± 0.4	30.1 ± 0.5	12.2 ± 0.2	36.3 ± 0.6
100°C	19.3 ± 0.3	30.7 ± 0.5	12.2 ± 0.2	37.8 ± 0.6
Peptide fraction	20.0 ± 0.3	19.6 ± 0.3	11.4 ± 0.2	49.0 ± 0.8
Albumin fraction	21.9 ± 0.4	30.7 ± 0.5	11.9 ± 0.2	35.5 ± 0.6

1300–5100 Da, only seven negative and positive bands were detected in the UV region of 250–270 nm related to phenylalanine residues [17]; in the region from 270 nm to 320 nm, other bands are absent (Fig. 1, red line). While the second derivative of the UV absorption spectrum of the albumin fraction included in ASK-53, in comparison with the second derivative of the absorption spectrum of ASK-53, acquired a more smoothed appearance (Fig. 1, black line). When analyzing the second derivative absorption spectra of ASK-53 when heated to 100 °C, it was shown that the temperature effect led to a reversible and insignificant decrease in the intensity of the signal values corresponding to aromatic amino acids (Fig. 1, blue line).

A change in the secondary structure of the ASK-53 molecule occurs with increasing temperature, as evidenced by the CD spectra of the complex in the region of absorption of peptide bonds (Fig. 3). When the ASK-53 is heated in a temperature range of 20–60°C, no special changes are observed and only after 70°C the conformational transition of the ASK-53 fraction occurs, and at 80°C and above it acquires a denatured stable conformational state. To assess the ASK-53 thermal stability, the point of the conformational transition at the level of the secondary structure of the complex was determined from the temperature dependence of the ellipticity at 222 nm (Fig. 4). It was found that the temperature transition point for ASK-53 was ≈65°C. The data obtained are consistent with the data obtained by studying the aggregation of native BSA under the influence of temperature. There are several works in which, using various methods, it was found that 65°C is the transition point of the BSA molecule to the state of aggregation [21,22].

Also, for the ASK-53, elements of the secondary structure were calculated depending on the temperature value (Table 1). From the table it follows that the secondary structure of ASK-53 at 70–100 °C differs from the structure of the native molecule ASK-53 in a lower content of the α-helix and a larger content of the random coil and β-structure. According to the calculated data, the secondary structure of ASK-53 treated with chemical reagents and separated from peptides is almost identical in terms of the content of canonical elements in the structure of ASK-53, heated to 80°C and higher (Table 1). This is another confirmation that the peptides that make up the complex are a kind of natural stabilizer of the conformational structure of albumin in ASK-53 and about the stability of the existing peptide-protein complex ASK-53.

3.2. The study of ASK-53 intermolecular association by dynamic light scattering

Along with the conformational thermal stability of the ASK-53, its intermolecular association was studied using the dynamic light scattering method. The data obtained by measuring the hydrodynamic radius (R_h) of the particles present in the ASK-53 solution using the dynamic light scattering method are shown in Table 2, the aggregation of ASK-53 was analyzed in the temperature range from 20 °C to 100 °C and of albumin from ASK-53 after treatment with 6.0 M guanidine hydrochloride. It was established that in the ASK-53 aqueous solution there are two groups of particles with sizes – 33.3 ± 9.0 nm and 129.9 ± 32.5 nm. Analysis of the ASK-53 particle size under the influence of temperature showed that they are also polydisperse (Table 2), but their size decreases slightly with increasing temperature while remaining in the

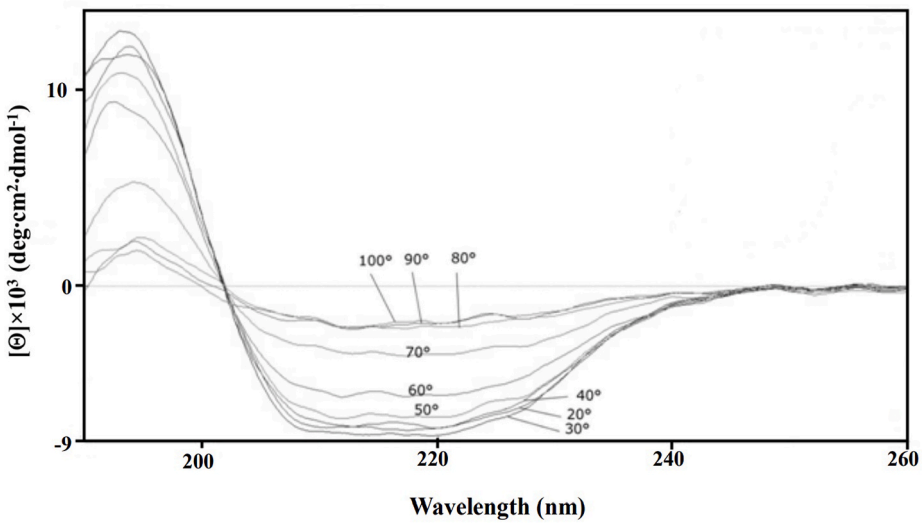

Fig. 3. CD spectra in 10 mM sodium phosphate buffer in the far UV region of ASK-53 after heat treatment.

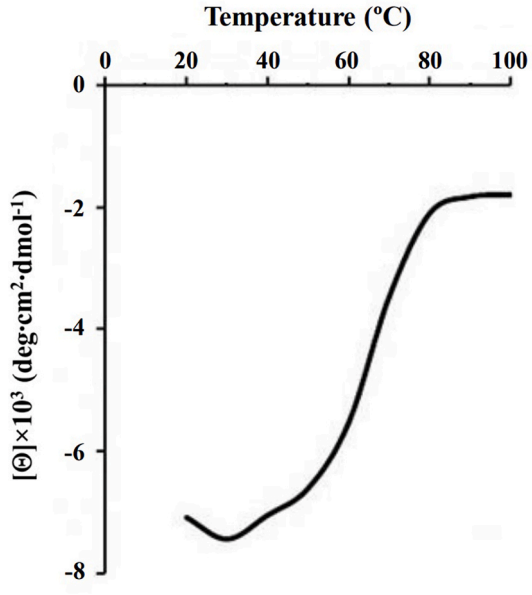

Fig. 4. The dependence of ellipticity at 222 nm ASK-53 on temperature.

Table 2
Hydrodynamic radius of the ASK-53 peptide-protein complex at various temperatures.

Sample	Intensity upon heating, kHz	Hydrodynamic radius (R_h), nm	Intensity after heating and cooling to 20 °C, kHz	Hydrodynamic radius (R_h) after heating and cooling to 20 °C, nm
20°C	179	33.3 ± 9.0 129.9 ± 32.5		
30°C	174	27.5 ± 6.9 145.4 ± 33.8	177	28.6 ± 8.9 111.8 ± 36.2
40°C	168	21.9 ± 5.5 116.0 ± 33.5	176	33.3 ± 11.4 130.0 ± 38.8
50°C	161	23.2 ± 5.5 122.6 ± 32.2	170	28.6 ± 7.6 111.7 ± 35.8
60°C	147	18.2 ± 5.3 130.2 ± 37.1	159	38.8 ± 10.4 130.2 ± 34.6
70°C	137	21.1 ± 4.7 111.8 ± 26.5	145	28.6 ± 6.5 111.9 ± 27.3
80°C	121	24.8 ± 5.3 131.2 ± 27.0	135	24.6 ± 5.8 96.1 ± 34.8
90°C	109	21.2 ± 5.1 112.2 ± 28.3	125	21.1 ± 4.3 96.0 ± 20.5
100°C	77	23.8 ± 2.9 108.1 ± 17.6	98	13.4 ± 3.6 95.9 ± 27.9

standard deviation range, while the intensity slightly but irreversibly decreases at each temperature point. As a result, at 100°C the intensity fell by half, and the size of the hydrodynamic radius of ASK-53 nanoparticles became 23.8 ± 2.9 nm and 108.1 ± 17.6 nm.

The performed treatment of ASK-53 with 6.0 M guanidine hydrochloride followed by separation of the reaction mixture on a reversed-phase column made it possible to obtain components of ASK-53 – peptides that do not form any aggregates in the aqueous solution and albumin, which forms nanoparticles with a clearly defined monomodal peak without scatter of 103.0 ± 20.2 nm (Table 2). This corresponds to the literature data that described the preparation of BSA aggregates with a hydrodynamic diameter of 224 nm after the treatment of its molecules with cationic surfactants [23].

A study of the effect of conformational changes occurring during temperature exposure on the membranotropic activity of ASK-53 showed that heating it to 100 °C for 20 min leads to a loss of activity. This can be explained by the fact that since the active principle of ASK-53, as was shown earlier [6], is its components in the form of peptides, and the second derivative of the spectrum, which was shown by the presence of phenylalanine in them, when heated, there is a spatial rearrangement of aromatic amino acids, and possibly all peptides that are redistributed inward to the more hydrophobic part of the ASK-53 associate; as a result, ASK-53 is not active. The presence of activity at 65 °C, in the region of the conformational transition of the ASK-53 molecule, is explained by the fact that 65 °C is an intermediate state of the ASK-53 molecule, retains compactness and a pronounced internal structure as in the native state, but there is no rigid packing of side chains, which possibly contributes to the manifestation of the activity of peptides that did not pass into the internal hydrophobic part of the associate of the ASK-53 molecule.

3.3. The effect of ASK-53 on the thermal DTT-induced aggregation of BSA

It is known that treatment of BSA molecules with dithiothreitol restores S–S bonds to –SH groups [24]. As a result, the α-helical structure of the BSA molecule is disrupted and, after unfolding, a β-structure is formed [25]. With increasing temperature from 35 to 55 °C, the number

of reduced disulfide bonds also increases [26]. To study the effect of ASK-53 on *in vitro* BSA aggregation, a model was chosen based on BSA aggregation at 50 °C in an aqueous solution of 10 mM DTT using the dynamic light scattering method, which allows to detect changes in the intensity of scattered light and the hydrodynamic radius of protein aggregates during aggregation. Upon incubation of an aqueous BSA solution in the presence of 10 mM DTT at 50 °C for 75 min, multimodal particles with sizes 114 ± 29 nm and 517 ± 129 nm are formed. The dependences of light scattering intensity (Fig. 5) and hydrodynamic radius (R_h) on the time of BSA aggregation in the presence of ASK-53 in the concentration range from 0 to 15 nM were obtained and analyzed (Fig. 6). As can be seen, ASK-53 at a concentration of 7.5 and 15 nM suppresses the DTT-induced aggregation of BSA. It was found that in an aqueous BSA solution in the presence of 10 mM DTT and incubated at 50 °C for 75 min, two groups of particles with sizes of 114.0 ± 29.0 nm and 517.0 ± 132.5 nm are formed. After addition of 1.5 and 3.0 nM ASK-53, they also remain polydisperse and their R_h value remains in the error range with respect to the BSA solution without ASK-53. When 7.5 nM ASK-53 is added to the BSA solution with DTT, although the DTT-induced aggregation of BSA is inhibited, a group of particles is retained in the solution, but the R_h value drops to 10 ± 2 nm and 90 ± 45 nm, respectively (Fig. 6). And only when 15 nM ASK-53 is added to the reaction mixture, 100% inhibition of DTT-induced BSA aggregation occurs (Figs. 5 and 6).

The effect of ASK-53 on the thermal aggregation of BSA induced by DTT was also studied by CD spectroscopy. Fig. 7 shows the CD spectrum of native BSA (turquoise line) in the far-UV region (190–250 nm) and BSA with 10 mM DTT (dark blue line) after heating at 50 °C for 75 min. After incubation of the solution without DTT, the BSA CD spectrum is characterized by the presence of two extremes at 208 and 222 nm and a maximum in the region of 190–195 nm (Fig. 7, turquoise line) and is characteristic of α-proteins [19], after heating in the presence of DTT, the BSA CD spectrum is characterized by the presence of one extremum at 225 nm and a not significant maximum of 190–200 nm (Fig. 7, blue line), which is comparable for β-structured proteins and is confirmed by calculations of secondary structure elements, aggregated and non-aggregated BSA molecules (Table 3). The spectrum of a BSA solution without DTT incubated in the presence of ASK-53 (Fig. 7, orange line) practically does not differ from the spectrum in the absence of an effector. It can be assumed that the addition of ASK-53 does not change the secondary structure of BSA, which is confirmed by calculations of the elements of the secondary structure of the protein (Table 3).

Although incubation of BSA with 10 mM DTT in the presence of 1.5 nM and 3 nM ASK-53 leads to the preservation of polydisperse particles in the solution, nevertheless, ASK-53 affects the secondary structure of the aggregated BSA molecule. In the CD spectrum of BSA in the presence of 1.5 nM ASK-53, in addition to the extremum at 225 nm, an extremum at 206 nm appears (Fig. 7, red line), the ellipticity of which increases in the presence of 3 nM (Fig. 7, black line). With an increase in ASK-53 concentration to 7.5 nM and 15 nM (Fig. 7, green and brown lines) in an aqueous BSA solution with 10 mM DTT, the ellipticity of the extremum at 206 nm approaches the extremum at 208 nm of the CD spectrum of the native BSA molecule, but elliptic at 222 nm it is not completely restored, indicating a partial unfolding of the α-helical structure at the initial stage of protein denaturation. These spectra represent the transition state between the dominant α-helix and the fully unfolded conformation of the protein molecule. This assumption is confirmed by calculations of the elements of the secondary structure (Table 3).

3.4. The effect of ASK-53 on thermal DTT-induced lysozyme aggregation

The large amount of information obtained by inducing/inhibiting lysozyme aggregation under various *in vitro* conditions makes lysozyme an ideal protein model for studying the mechanisms of aggregation and fibrillation [27], as well as for testing new pharmacological drugs to fight with amyloidosis [28]. When studying the effect of pH and temperature on lysozyme fibrillogenesis, it was shown that for the formation of human lysozyme fibrils and chicken eggs, it is necessary to increase the temperature to 55–65 °C and lower the pH to 2.-2.5 [29]. However, we achieved optimal lysozyme aggregation at a concentration of 100 μg/ml protein at 0.5 mM DTT in borate buffer (pH 9.0) at 50°C for 75

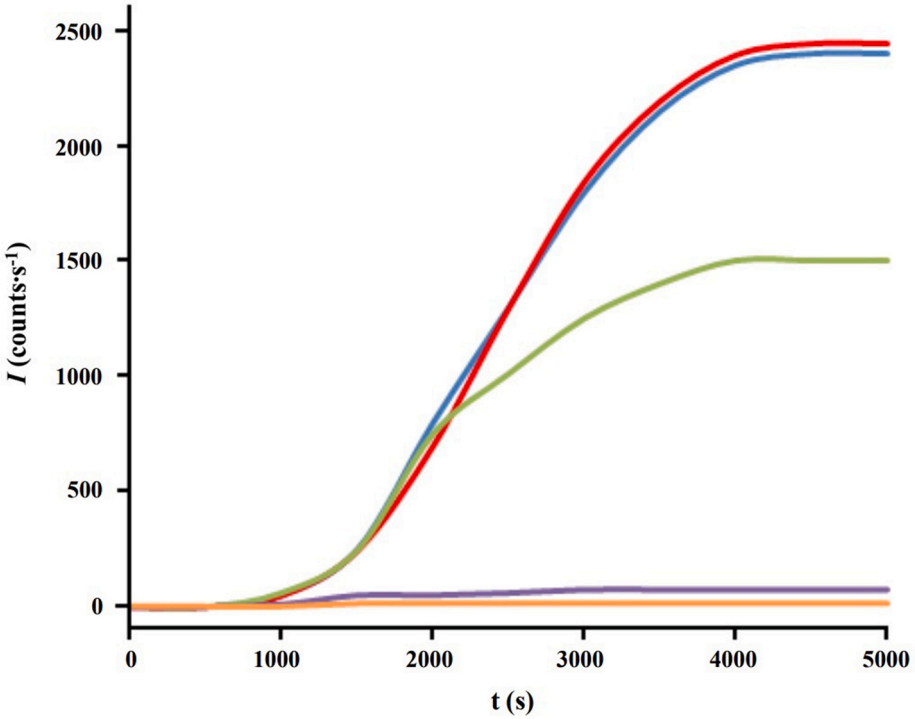

Fig. 5. Dependence of light scattering intensity (*I*) on the incubation time of BSA (100 μg/ml) in the presence of 10 mM DTT, without ASK-53 (blue line) and with ASK-53 at concentrations of 1.5 nM (red line), 3 nM (green line), 7.5 nM (purple line), and 15 nM (orange line).

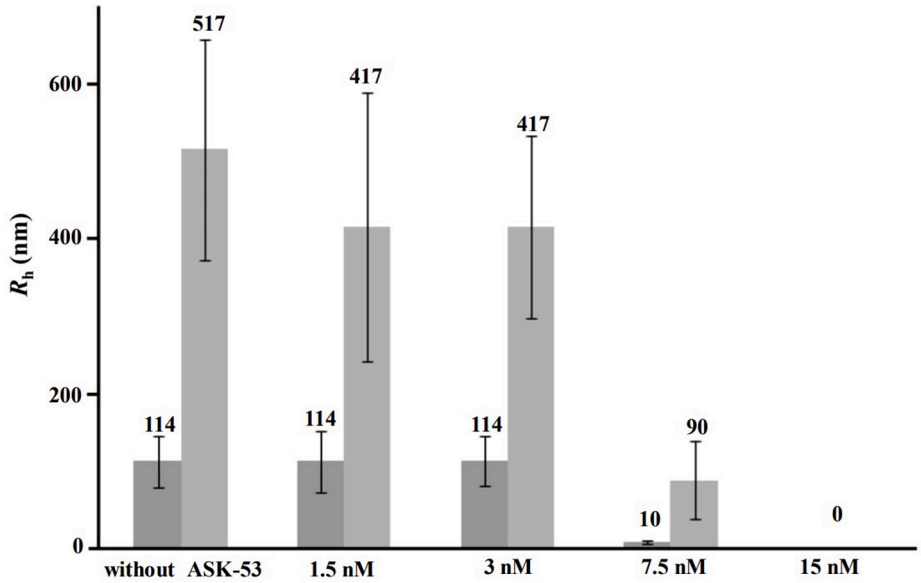

Fig. 6. Dependence of the hydrodynamic radius (R_h) of BSA (100 μg/ml) in the presence of 10 mM DTT on the concentration of ASK-53.

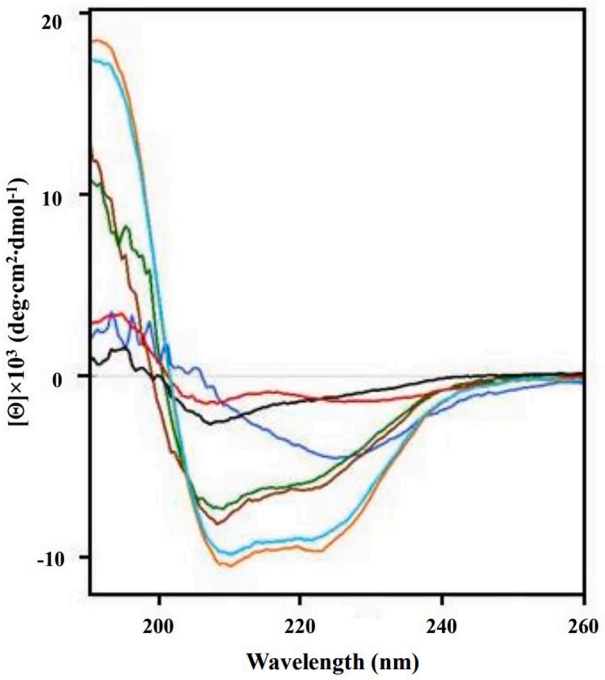

Fig. 7. The action of ASK-53 on the secondary structure of BSA. CD spectrum of native BSA (100 μg/ml) (turquoise line) and incubated with 10 mM DTT (dark blue line); with 15 nM ASK-53 (orange line); with 1.5 nM ASK-53 and 10 mM DTT (red line); 3.0 nM ASK-53 and 10 mM DTT (black line), 7.5 nM ASK-53 and 10 mM DTT (green line); 15 nM ASK-53 and 10 mM DTT (brown line).

min. When lysozyme is incubated under these conditions, monomodal particles with a size of 876 ± 159 nm are formed. The dependences of light scattering intensity (Fig. 8) and hydrodynamic radius (R_h) on the time of lysozyme aggregation in the presence of ASK-53 in the concentration range from 0 to 150 nM were obtained and analyzed (Fig. 9). After adding 1.5 nM ASK-53 to the reaction mixture, the particle size of the aggregated lysozyme remains unchanged; in the presence of 15 nM and 75 nM ASK-53, they are halved. Only when 150 nM ASK-53 is added to the reaction mixture, 100% inhibition of DTT-induced lysozyme aggregation occurs (Figs. 8 and 9).

Table 3
The content of the elements of the secondary structure of the BSA solution incubated with/without ASK-53 and/or 10 mm DTT at 50 °C for 75 min.

Sample	α-helix,%	β-sheets, %	β-turn, %	random coil, %
BSA	44.8 ± 0.8	15.2 ± 0.2	11.2 ± 0.2	28.8 ± 0.5
BSA + DTT	5.2 ± 0.1	38.7 ± 0.6	10.5 ± 0.2	45.6 ± 0.8
BSA+1.5 nM ASK-53	45.6 ± 0.8	15.6 ± 0.2	9.2 ± 0.1	29.6 ± 0.5
BSA+1.5 nM ASK-53 + DTT	10.6 ± 0.2	33.3 ± 0.6	13.9 ± 0.2	42.2 ± 0.7
BSA+3 nM ASK-53 + DTT	17.1 ± 0.3	29.2 ± 0.5	14.1 ± 0.2	39.6 ± 0.6
BSA+7.5 nM ASK-53 + DTT	20.2 ± 0.5	33.5 ± 0.6	12.6 ± 0.2	33.7 ± 0.6
BSA+15 nM ASK-53 + DTT	28.4 ± 0.5	22.2 ± 0.5	12.1 ± 0.2	37.3 ± 0.6

Similarly, the effect of ASK-53 on the thermal aggregation of lysozyme induced by DTT was studied by CD spectroscopy. Changes in the secondary structure of lysozyme were studied in the absence or presence of ASK-53. The lysozyme spectrum after incubation in borate buffer (pH 9.0) at 50 °C for 75 min shows negative extremes in the 208–222 nm region with a maximum in the 190–195 nm region (Fig. 10, brown line). The heating of lysozyme in the presence of DTT in a borate buffer of pH 9.0 leads to a decrease by one and a half times of negative ellipticity in the range of 208–222 nm and by more than a half in the range of 190–195 nm (Fig. 10 red line). The spectrum of lysozyme without DTT in the presence of ASK-53 in a borate buffer (pH 9.0) is similar to the CD spectrum of native lysozyme. This indicates that ASK-53 does not affect the lysozyme structure (Fig. 10, lilac line).

The CD spectrum of incubated lysozyme with 0.5 mM DTT in the presence of 150 nM ASK-53 in borate buffer (pH 9.0) (Fig. 10, orange line) is most similar in appearance to the spectrum of heated lysozyme in borate buffer (pH 9.0) with only a slight decrease in ellipticity at 222 nm. Whereas the CD spectrum of incubated lysozyme with 0.5 mM DTT in the presence of 75 nM ASK-53 (Fig. 10, black line) is close in value to the CD spectrum of aggregated lysozyme. Of particular interest are the CD spectra of incubated lysozyme with 0.5 mM DTT in the presence of 1.5 and 15 nM ASK-53 (Fig. 10, blue and green lines, respectively), they acquire a smoothed appearance in the region of 208–222 nm, although

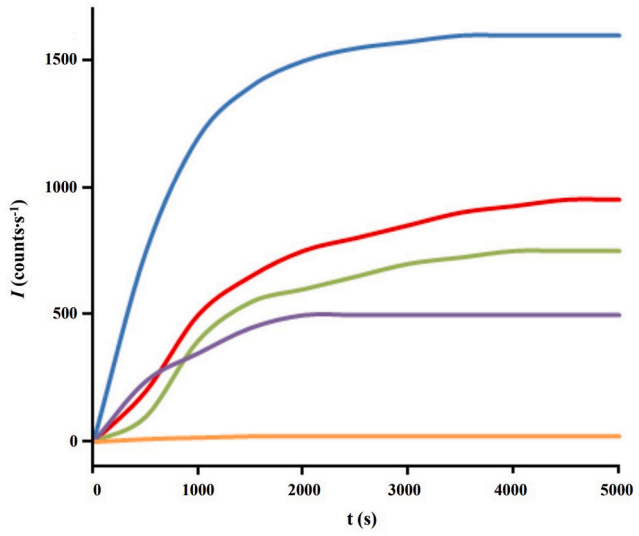

Fig. 8. Dependence of light scattering intensity (I) on the time of lysozyme incubation (100 μg/ml) in the presence of 0.5 mM DTT, without ASK-53 (blue line) and with ASK-53 at concentrations of 1.5 nM (red line), 15 nM (green line), 75 nM (purple line), and 150 nM (orange line).

the ellipticity values in this region are close to the CD spectrum of incubated lysozyme with 0.5 mM DTT in the presence of 75 nM ASK-53 (Fig. 10, black line) and indicate a transitional phase between aggregated and native lysozyme molecules under the influence of chaperone. The calculations of the secondary structure elements (Table 4) show that neither the creation of the DTT-initiated lysozyme aggregation, nor the inhibition of lysozyme aggregation using ASK-53, leads to any significant changes in the content of the secondary structure elements of the lysozyme molecule.

The results obtained by CD spectroscopy using two model proteins are similar. The addition of ASK-53 in the presence of DTT prevented the unfolding of α-helices of BSA and lysozyme molecules and their transformation into a β-structured state, which is a precursor of protein aggregation. Thus, the ASK-53 peptide-protein complex isolated from cattle sclera maintains the native structure of model proteins and prevents their association during thermal DTT-induced aggregation.

4. Conclusion

It has been shown that the previously unstudied ASK-53 peptide-protein complex consisting of isoforms of serum albumin, peptides, as well as carbohydrates and lipids is present in the sclera tissue of the cattle's eye. The spatial structure of ASK-53 is characterized by the presence of an α-helical secondary structure with a conformational thermal transition point of 65 °C, which indicates its significant stability. It has been established that ASK-53 in aqueous solutions forms nano-sized particles that are thermostable and resistant to the effects of chaotropic agents. That is, the albumin contained in ASK-53 forms nanoparticles used to transport active peptides, while the peptides stabilize the albumin molecule while maintaining its ability to form labile/mobile aggregates (associates). This property is characteristic of molecular chaperones, since the lability of chaperone oligomers is important for the recognition and binding of substrates. We have shown that ASK-53 exhibits the properties of chaperone, an inhibitor of DTT-induced aggregation of BSA and lysozyme. While 100% inhibition of BSA aggregation is achieved by 10 times less ASK-53 than inhibition of lysozyme aggregation. It is possible that stabilization of the three-dimensional structure of BSA is achieved faster due to the affinity for the ASK-53 structure as a result of interaction with hydrophobic regions on the surface of the deployed BSA molecules. Since the mechanism of action of molecular chaperones is based on their interaction with hydrophobic regions of non-native proteins and interfere with the binding of these proteins to each other, preserving their folding-competent state [30]. We assume that under normal conditions, the ASK-53 substrate-binding sites are hidden inside the multimer, and an increase in temperature causes a shift in the equilibrium conformation of the ASK-53 molecule, increasing the availability of hydrophobic chaperone sites and binding to the substrate and ASK-53 inhibits subunit exchange between aggregation protein oligomers showing chaperone-like properties.

One of the fundamental directions of modern biology and biotechnology is the study of the association and aggregation of proteins. Due to mutations, post-translational modifications, oxidative damage, changes in environmental conditions (pH, UV radiation, temperature), the conformation of the protein molecule is disrupted, leading to its aggregation. As a result, various structures are formed: soluble oligomers, amorphous aggregates and amyloid-like fibrils, which cause so-called "conformational diseases". The results obtained in this work show that

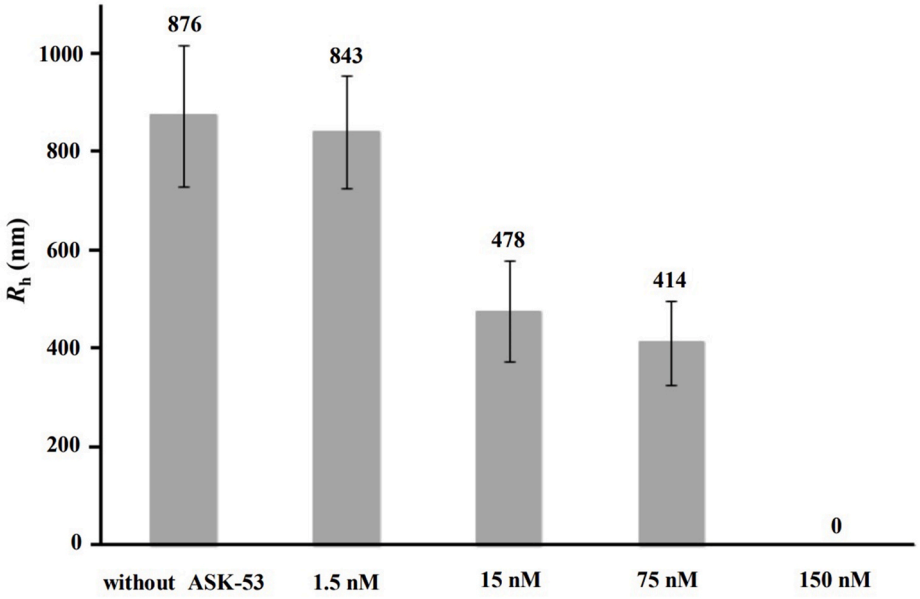

Fig. 9. The dependence of the hydrodynamic radius (R_h) of lysozyme (100 μg/ml) in the presence of 0.5 mM DTT on the concentration of ASK-53.

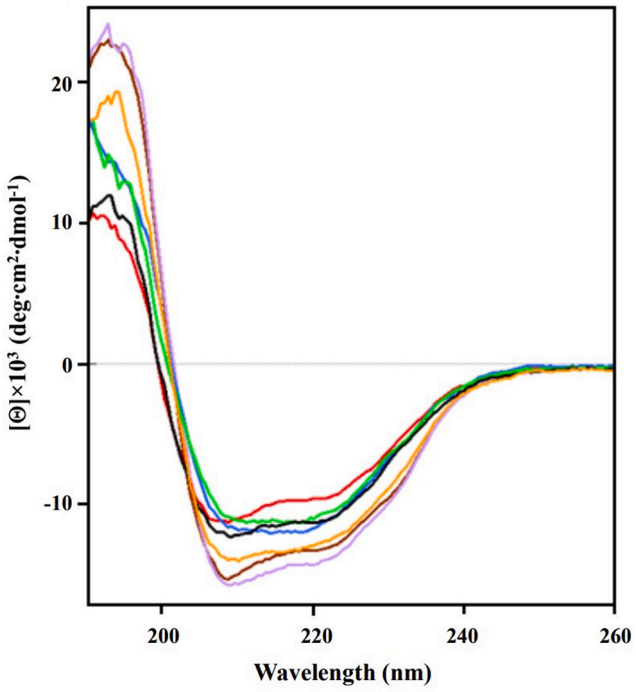

Fig. 10. The effect of ASK-53 on the secondary structure of lysozyme. CD spectrum of lysozyme (100 μg/ml) (brown line), incubated with 0.5 mM DTT (red line); with 150 nM ASK-53 (lilac line); with 1.5 nM ASK-53 and 0.5 mM DTT (blue line); 15 nM ASK-53 and 0.5 mM DTT (green line); 75 nM ASK-53 and 0.5 mM DTT (black line); 150 nM ASK-53 and 0.5 mM DTT (orange line).

Table 4
The content of the secondary structure elements of the lysozyme solution incubated with/without ASK-53 and/or 0.5 mM DTT in borate buffer (pH 9.0) at 50 °C for 75 min.

Sample	α-helix,%	β-sheets, %	β-bend, %	random coil, %
lysozyme	19.5 ± 0.4	31.9 ± 0.6	12.5 ± 0.2	36.1 ± 0.6
lysozyme + DTT	16.9 ± 0.4	33.2 ± 0.6	12.6 ± 0.2	37.3 ± 0.6
lysozyme +1.5 nM ASK-53	19.4 ± 0.4	32.1 ± 0.6	12.4 ± 0.2	36.1 ± 0.6
lysozyme +1.5 nM ASK-53 + DTT	17.1 ± 0.4	33.6 ± 0.6	12.5 ± 0.2	36.8 ± 0.6
lysozyme +15 nM ASK-53 + DTT	16.7 ± 0.4	33.7 ± 0.6	12.5 ± 0.2	37.1 ± 0.6
lysozyme +75 nM ASK-53 + DTT	17.1 ± 0.4	33.0 ± 0.6	12.5 ± 0.2	37.4 ± 0.6
lysozyme +150 nM ASK-53 + DTT	18.9 ± 0.4	32.2 ± 0.6	12.5 ± 0.2	36.4 ± 0.6

ASK-53 naturally occurring in a biological system in the form of nanoparticles can influence and control protein aggregation. This indicates the possibility of fine regulation of the processes of association and aggregation of proteins, which is important in the development of drugs. Therefore, drugs developed on the basis of ASK-53 can be used in ophthalmology. But not only in the pathophysiology of the eyes of animals (bulls, dogs, cats, and so on), but also in the ophthalmology of the human eye, since MHTBs have tissue-specific action, but not species-specific.

CRediT authorship contribution statement

Anna P. Ilyina: Methodology, Validation, Writing - original draft, Data curation. **Egor V. Sidorsky:** Investigation, Visualization. **Artem V. Tregubov:** Software, Formal analysis, Investigation. **Valeria M. Chekova:** Investigation, Visualization. **Pavel A. Elistratov:** Writing - review & editing. **Viktoria P. Yamskova:** Conceptualization, Supervision. **Igor A. Yamskov:** Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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