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***Nonlinear Laser Spectroscopy,  
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# Study of molecular mechanisms of UV-induced aggregation of crystallins and possibility of maintaining eye lens transparency.

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## ABSTRACT

The effect of D-pantethine and L-carnosine on the rate of UV-induced (XeCl laser  $\lambda = 308$  nm) aggregation of a mixture of  $\beta$ L-crystallin and  $\alpha$ -crystallin is studied. We also demonstrate that the suggested by us combination of short-chain peptides shows better protective properties with respect to UV-induced aggregation than known anti-cataract agents.

**Keywords:**  $\alpha$ -,  $\beta$ -,  $\gamma$ -crystallin, cataract, aggregation, UV laser, fluence, repetition rate, D-pantethine, L-carnosine.

## 1. INTRODUCTION

Cataract development is one of the causes of loss of transparency in the eye lens. In up to 85% of cases, the mechanisms of senile cataract formation are little known. Earlier studies showed relation between irradiation at 290-320 nm and the development of age-related cataract<sup>1</sup>. The onset and progress of some types of cataract, including photocataractogenesis, is related to aggregation of main proteins of the eye lens:  $\alpha$ -,  $\beta$ - and  $\gamma$ -crystallins<sup>2-5</sup>. When exposed to ultraviolet (UV) light the crystallins form high molecular weight aggregates, resulting in lens opaqueness and increased light scattering and absorption<sup>6</sup>. Today there is a lack of information on the molecular mechanisms of UV-induced aggregation of crystallins in the literature. Providing this information is important not only for the crystallin photo-aggregation theory but also in the search for anti-cataract agents. A certain progress in this direction was made in papers<sup>7,8</sup>, in which aggregation processes of model carbonic anhydrase protein irradiated by pulsed XeCl laser light at 308 nm were studied. In earlier papers<sup>9-13</sup> proteins were exposed to UV irradiation at a constant energy density of light pulses  $w$  and a constant pulse repetition rate  $F$ . In papers<sup>7,8</sup> the UV-induced aggregation of carbonic anhydrase was studied for widely varying values of  $w$  and  $F$ . These experiments indicated a strong dependence of the aggregation rate on  $w$  and  $F$ . A simplified theoretical model was developed that took into account only dimer formation. This model, in which protein aggregation occurs at interaction of two light-activated molecules, can qualitatively explain experimental results.

Among all eye lens proteins,  $\beta$ - and  $\gamma$ -crystallins are most susceptible to aggregation<sup>9,10</sup>.  $\alpha$ -crystallin has chaperone-like activity and inhibits aggregation of these proteins when affected by various denaturing factors<sup>14</sup>. The chaperone activity of  $\alpha$ -crystallins decreases with aging, a factor that is thought to be one of the causes of age-related cataract<sup>15</sup>. *In vitro* experiments<sup>16</sup> showed that pantethine and its components inhibit the thermal aggregation rate of a mixture of  $\beta$ L- and  $\alpha$ -crystallins by maintaining protective properties of the  $\alpha$ -crystallin. A. Boldyrev et al. performed experiments on intact animals to reveal carnosine properties as an anti-cataract agent<sup>17</sup>.

In the present communication we investigate photo-aggregation of  $\beta$ L-crystallin using a technique described earlier<sup>7,8</sup>. It is found that, similar to carbonic anhydrase, the aggregation rate of  $\beta$ L-crystallin is strongly dependent on  $w$  and  $F$ . The chaperone activity of  $\alpha$ -crystallin at aggregation of  $\beta$ L-crystallin irradiated by pulsed excimer laser light is investigated. The effect of D-pantethine, D-pantothenic acid, L-carnosine and N-acetylcarnosine on the rate of UV-induced aggregation of a mixture of  $\beta$ L-crystallin and  $\alpha$ -crystallin is studied. We also report results of *in vitro*

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investigations of a combination of short-chain peptides. This combination was specially selected by us and appeared to have better protective properties than known anti-cataract agents.

## 2. MATERIALS AND METHODS

In experiments we used bovine lens crystallins and D-pantethine from "Sigma" company, USA, and L-carnosine from Hamari Chemicals Ltd., Japan. In all experiments the proteins were dissolved in phosphate buffer pH=7.2 and then passed through a membrane filters with pore sizes 0.45  $\mu\text{m}$  and 0.22  $\mu\text{m}$  (Sartorius, Germany). A vial with protein was centrifuged for 15 min at  $5 \cdot 10^3 \cdot g$  to remove minor undissolved materials. Part of the protein solution was left at the vial's bottom and was not treated in further experiments. A source of UV radiation was a XeCl laser LPX-200 (Lambda Physik) at 308 nm with a pulse energy of up to 450 mJ and a pulse repetition rate of up to 80 Hz. Experiments were conducted at temperature  $(22 \pm 1)^\circ\text{C}$ ; heating of the protein solution during irradiation did not exceed  $2^\circ\text{C}$ . To study the dynamics of protein aggregation under UV irradiation we measured kinetic curves, the dependences of the intensity of a test beam scattered in the cuvette with the protein solution, versus UV-dose D. The test beam was from a single-mode (TEM<sub>0</sub>) HeNe laser with a wavelength of 633 nm, power of 10 mW and divergence of  $1.1 \cdot 10^{-3}$  rad. Simultaneously, we measured energy of 308 nm pulses passed through the cuvette. Before and after irradiation by varying UV-doses, we obtained optical transmission spectra with a spectrophotometer Specord M40 and molecular weight distributions (MWD) chromatographically by using a Superose 12HR column. The height of the cuvette made of quartz was 10 mm, its length along HeNe and XeCl laser beams was 10 mm and 5 mm, respectively. The experimental scheme is illustrated in Fig. 1.

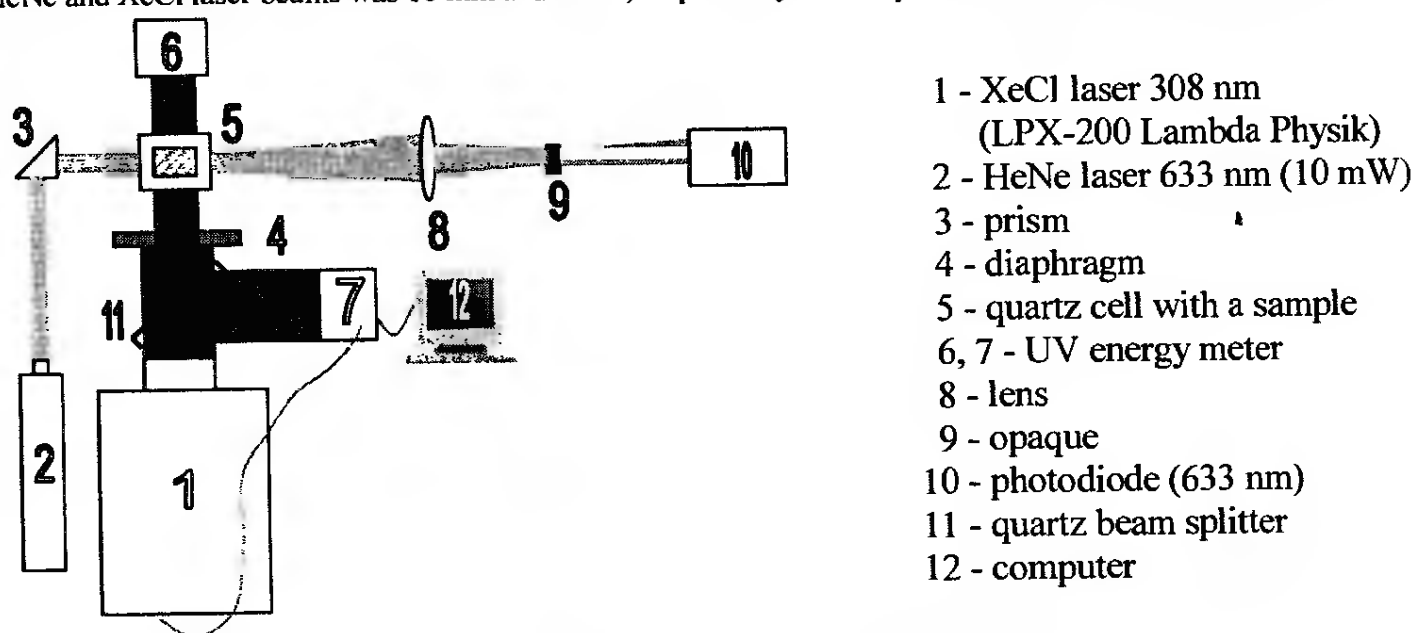


Fig. 1. Experimental scheme for crystallin solution irradiation.

Scattered radiation of the test beam was measured by the dark field method, as earlier<sup>7,8</sup>, thus providing absolute value of scattered power. The energy of the XeCl laser pulses was measured using a patented<sup>18</sup> energy meter (7) and was kept constant during the irradiation process. The energy of radiation at 308 nm passed through the cuvette (5) was registered by using an ED-200 energy meter (Gentec Inc., Canada) (6), and the power of scattered radiation at 633 nm – by using a photodiode (10). An automated system (12) continuously processed the signals from the energy meters 6, 7 and the photodiode 10 and averaged them over a given number of XeCl laser pulses. The results were then displayed graphically on a computer monitor.

## 3. RESULTS AND DISCUSSION

### 3.1. Study of UV-induced aggregation of $\beta\text{L}$ -crystallin.

Intense laser irradiation of protein solutions may result in processes that do not occur in the eye lens under natural sunlight. First of all, this is the possibility of nonlinear, e.g., two-photon, absorption of laser radiation in protein solutions. We measured laser light absorption in  $\beta\text{L}$ -crystallin solution with concentration of 0.5 mg/ml in a 5-cm

cuvette. The absorption coefficient was  $5 \times 10^{-2} \text{ cm}^{-1}$  and remained constant at  $w$  ranging from 2 to 300  $\text{mJ/cm}^2$ , thus indicating the absence of nonlinear absorption. In our experiments maximal value of  $w$  was 90  $\text{mJ/cm}^2$ , so the laser light absorption by crystallins was one-photon, as in the case of sunlight exposure to the eye lens.

The kinetic curves for the  $\beta\text{L}$ -crystallin solution with concentration of 0.5  $\text{mg/ml}$  obtained at varying  $w$  and  $F$  are illustrated in Fig. 2 a, b. Note that for each value of  $w$  there is a characteristic value of  $F^*$ , below which  $D^*$  considerably increases ( $D^*$  is a dose at which the scattering intensity of a test beam starts increasing). If at  $w = 75 \text{ mJ/cm}^2$  and  $F = 4 \text{ Hz}$  the value of  $D^*$  is  $\sim 100 \text{ J/cm}^2$ , then at the same value of  $w$  but  $F = 0.5 \text{ Hz}$   $D^*$  will be  $\sim 1000 \text{ J/cm}^2$ .

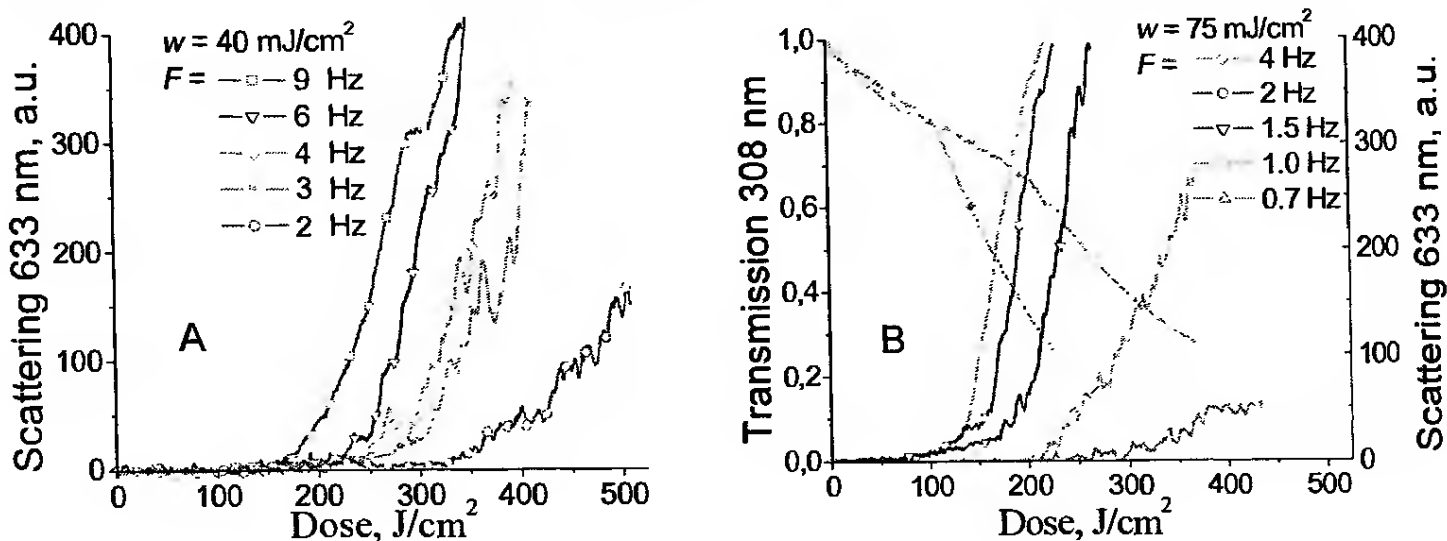


Fig.2. The kinetic curves for solution of  $\beta\text{L}$ -crystallin with  $n = 0.5 \text{ mg/ml}$  for different fluences ( $w$ ) and different repetitions rates ( $F$ ). In fig. 2, B changes in transmission of 308 nm are shown.

In addition, at small repetition rates of laser pulses the slope of the kinetic curves decreases. On the other hand, at larger  $F$ ,  $D^*$  and the slope of the kinetic curves do not depend on  $F$  any longer. These results indicate that at  $F < F^*$  the probability of protein molecules aggregation significantly decreases. Figure 3 shows the dependences  $D^*(F)$  obtained from the analysis of the kinetic curves presented in Fig. 2 a,b.

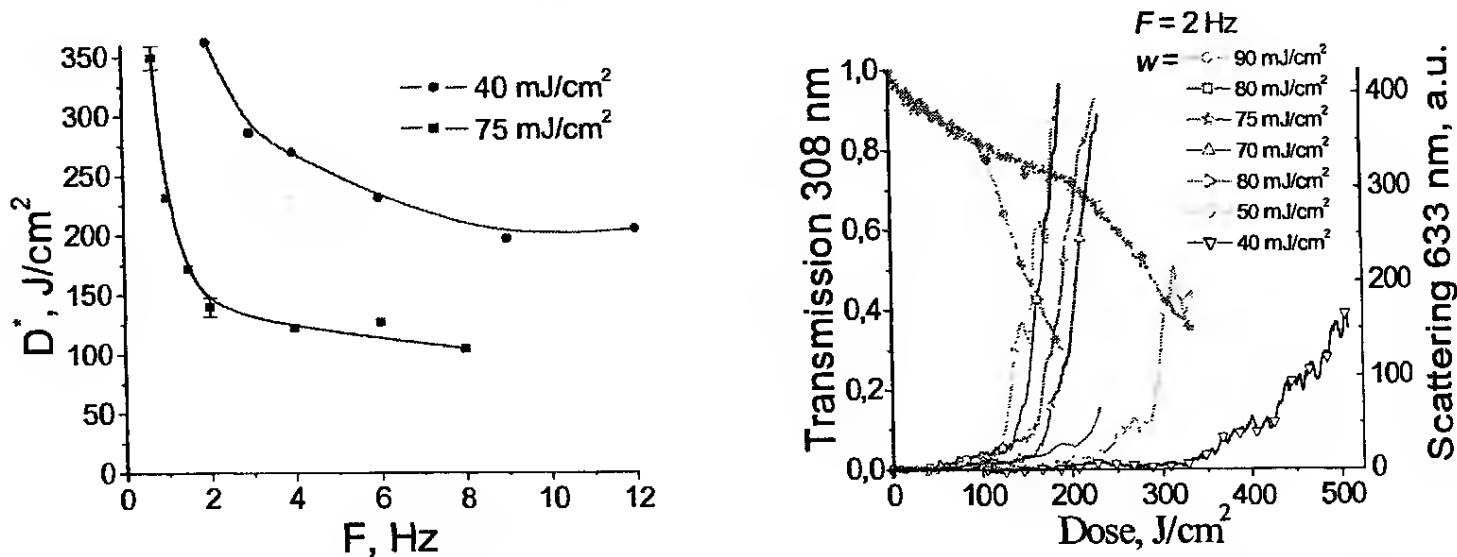


Fig. 3. The dependence of  $\text{DOSE}_{\text{begin scattering}} (D^*)$  vs. laser pulse repetition rate ( $F$ ).

Fig.4. The kinetic curves for solution of  $\beta\text{L}$ -crystallin with  $n = 0.5 \text{ mg/ml}$  for constant repetition rate ( $F$ ) and different fluences ( $w$ ). Changes in transmission of 308 nm are shown.

Besides we measured kinetic curves at  $F = \text{const}$  and at varying  $w$ , see Fig. 4. For larger  $w$  the behavior of the kinetic curves is almost independent of  $w$ , but for smaller  $w$  (beginning with  $w = 75 \text{ mJ/cm}^2$ )  $D^*$  grows and the slope of the curve decreases. The dependence  $D^*(w)$  is presented in Fig. 5.

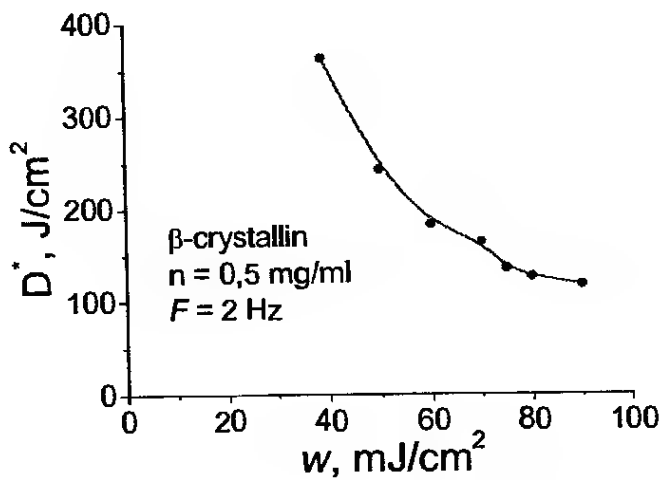


Fig. 5. The dependence of dose  $D^*(w)$

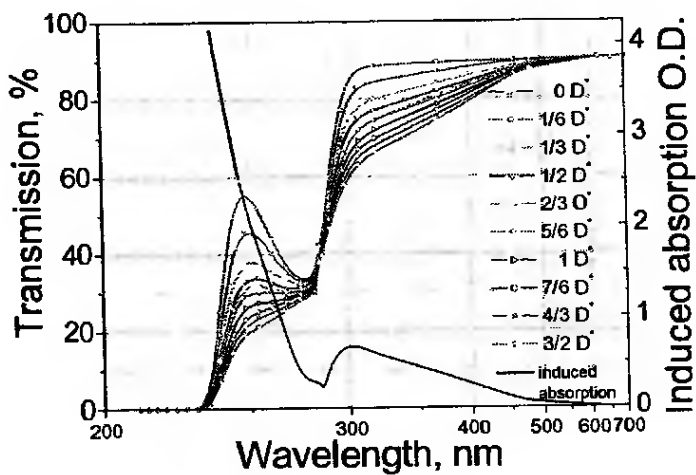


Fig. 6. Optical transmission spectra after irradiation by different doses.

Optical transmission spectra of the protein solution after irradiation by various UV-doses (Fig. 6) appeared to be similar to relevant spectra of carbonic anhydrase<sup>7,8</sup>, and the spectrum of induced absorption was analogous to that reported<sup>2</sup> for a soluble fraction of bovine eye lens. Chromatographic measurements showed that when the irradiation dose grows the amplitude of the monomer peak of  $\beta\text{L-crystallin}$  with molecular weight of  $M_m \sim 45 \text{ kDa}$  monotonically decreases. At the same time, the peak broadens mainly towards higher molecular weights. At  $D \sim 0.5D^*$  a peak with  $M_m = 2000 \text{ kDa}$  appears, which corresponds to the exclusion limit of the Superose 12HR column. Thus aggregates whose effective molecular weights are two orders of magnitude higher than that of a monomer are formed at fairly small UV-doses, at which the scattered intensity of the test beam is not growing yet. As the UV-dose increases, the amplitude of the peak grows.

The above kinetic curves and dependences  $D^*(F)$  and  $D^*(w)$  qualitatively agree with the results obtained in the study of UV-induced aggregation of carbonic anhydrase<sup>7,8</sup>. This suggests that in the first approximation a simplified model can also be used to describe the photo-aggregation of  $\beta\text{L-crystallin}$ , in which aggregation occurs at interaction of two light-activated protein molecules and the growth of the scattered intensity of the test beam is due only to dimer formation. The results that we obtained by UV-irradiation of a recombinant  $r\beta\text{A3-crystallin}$  with various concentrations  $n$  in solution (Fig. 7) also fit this model. When protein concentration decreases by several times, the photo-aggregation rate may decrease by several orders of magnitude. The dependence  $D^*(n)$  is given in Fig. 8.

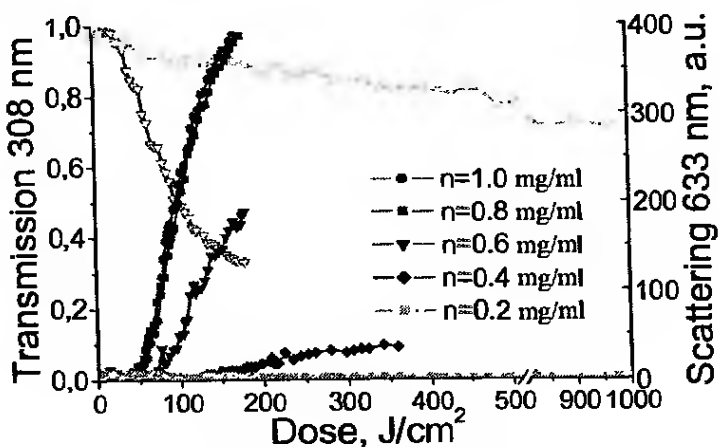


Fig. 7. The dependences of scattering and transmission of solutions with different concentrations  $r\beta\text{A3-crystallin}$  vs. dose of UV-irradiation. Changes in transmission of 308 nm are shown.

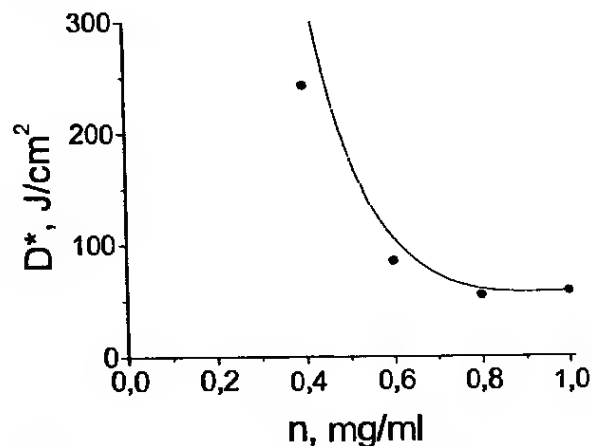


Fig. 8. The dependence of  $\text{DOSE}_{\text{begin scattering}} (D^*)$  vs. concentration ( $n$ ) of recombinant  $r\beta\text{A3-crystallin}$ .

All the results described above can be explained as follows. Since photo-aggregation occurs at pairwise interaction of photo-activated molecules, the probability of photo-aggregation is proportional to the square of concentration of activated protein molecules. That is why the dependences  $D^*(F)$ ,  $D^*(w)$ , and  $D^*(n)$  are nonlinear.

### 3.2. Study of the effect of peptides on UV-induced aggregation rate of a mixture of $\beta$ L- and $\alpha$ -crystallins.

By measuring kinetic curves and using chromatographic techniques we studied, for the first time, the effect of a mixture of  $\beta$ L- and  $\alpha$ -crystallins of several agents<sup>16, 17</sup> on the UV-induced aggregation rate. Concentration of proteins and added agents was 0.5 mg/ml. We investigated the protective properties of D-pantethine, D-pantothenic acid, L-carnosine and N-acetylcarnosine, which were patented by John Clark et al. and A. Boldyrev et al. as anti-cataract agents. We also performed analogues investigations of some other substances to find a new efficient anti-cataract agent. As a result, we found a combination of short-chain peptides which *in vitro* appeared to be a better inhibitor of  $\beta$ L- and  $\alpha$ -crystallins aggregation than known anti-cataract agents. This makes us believe that our combination (we shall call it «protective additive») may be more efficient than patented anti-cataract agents<sup>19, 20</sup>.

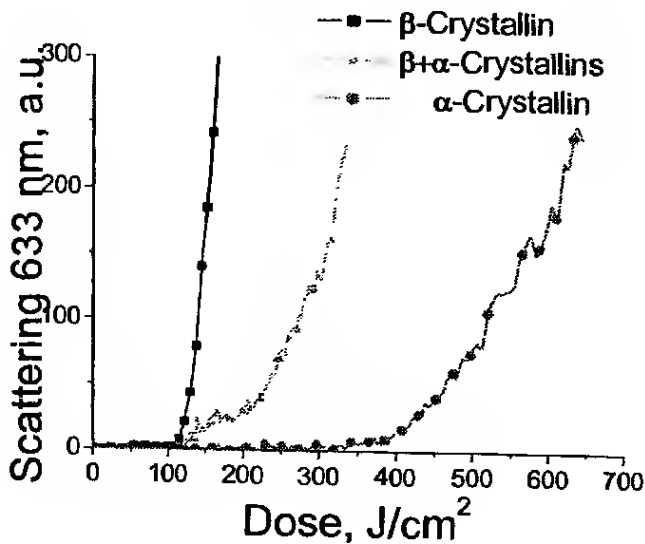


Fig. 9.  $\alpha$ -crystallin chaperone properties.

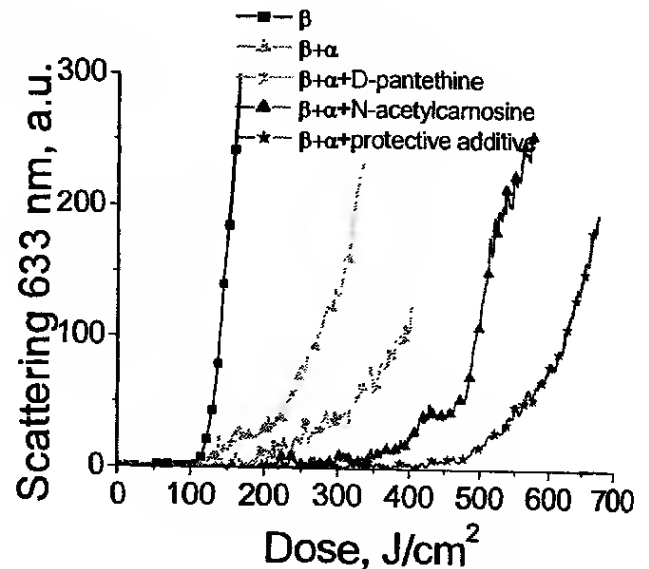


Fig. 10. Effect of the peptides on crystallins solution aggregation.

Figure 9 shows kinetic curves for  $\beta$ L-crystallin,  $\alpha$ -crystallin and a mixture of  $\beta$ L- and  $\alpha$ -crystallins. These dependences were obtained at  $w = 75 \text{ mJ/cm}^2$  and  $F = 2 \text{ Hz}$ , i.e., at the same laser radiation parameters as in our study of  $\beta$ L-crystallin photo-aggregation. It can be seen that the  $\alpha$ -crystallin has chaperone activity with regard to the  $\beta$ L-crystallin and a lower aggregation rate. These results are similar to those obtained earlier<sup>11</sup>, which studied the effect of  $\alpha$ -crystallin on UV-induced aggregation of  $\gamma$ -crystallin.

Of all substances we have studied, the best inhibitor of photo-aggregation of the  $\beta$ L- and  $\alpha$ -cystallins mixture was D-pantethine, N-acetylcarnosine and the new protective additive. Further we shall discuss the results obtained only for these compounds. Figures 10, 11 show kinetic curves for  $\beta$ L-crystallin,  $\beta$ L- and  $\alpha$ -crystallins mixture and for same mixture with added D-pantethine, N-acetylcarnosine and protective additive, as well as changes in transmission of 308 nm radiation for some mixtures. It is seen that all additives have chaperone-like (protective) properties, and maximal inhibition of protein aggregation rate is with the protective additive. Its efficiency is 2.5 times higher than of D-pantethine and almost 1.5 times higher than of N-acetylcarnosine.

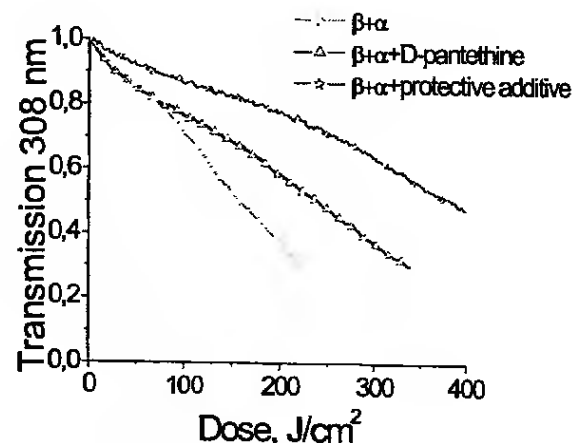


Fig. 11. Changes in transmission of 308 nm.

Figure 12 a, b demonstrates chromatographic profiles of the  $\beta$ L- and  $\alpha$ -crystallin mixture with and without addition of the new protective additive after irradiation of protein solutions by different UV-doses. For illustration purposes, we do not indicate here the peak corresponding to the  $\alpha$ -crystallin but rather show changes of the peak of  $\beta$ L-crystallin with  $M_m \sim 45 \text{ kDa}$  and of peak in the region of exclusion limit of the Superose 12HR column (with  $M_m = 2000 \text{ kDa}$  and

higher), which is due to higher molecular weight protein aggregates. As the UV dose increases, the amplitude decreases, the peak with  $M_m \sim 45$  kDa broadens and the peak of higher molecular weight aggregates grows. When the protective additive is added, these changes are significantly reduced, indicating inhibition of the aggregation rate of the  $\beta$ L- and  $\alpha$ -crystallin mixture.

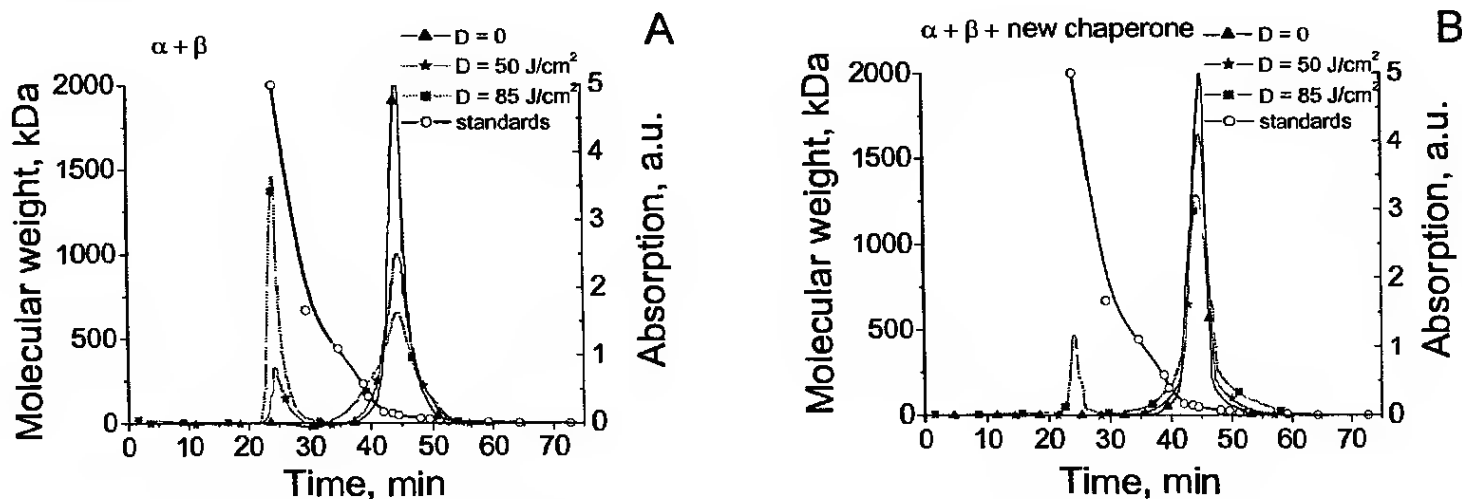


Fig.12. Chromatographic profiles. Only changes of  $\beta$ -monomer and high molecular fraction are shown.

Figure 13 a, b presents the results of analysis of chromatograms obtained after irradiation of the  $\beta$ L- and  $\alpha$ -crystallin mixture and same mixture with added D-pantethine, N-acetylcarnosine and new protective additive. Changes in amplitudes of peaks corresponding to  $\beta$ L-crystallin and higher molecular weight aggregates are shown. The figure demonstrates that inhibition of the aggregation rate of the two proteins mixture is most efficient when the protective additive is added.

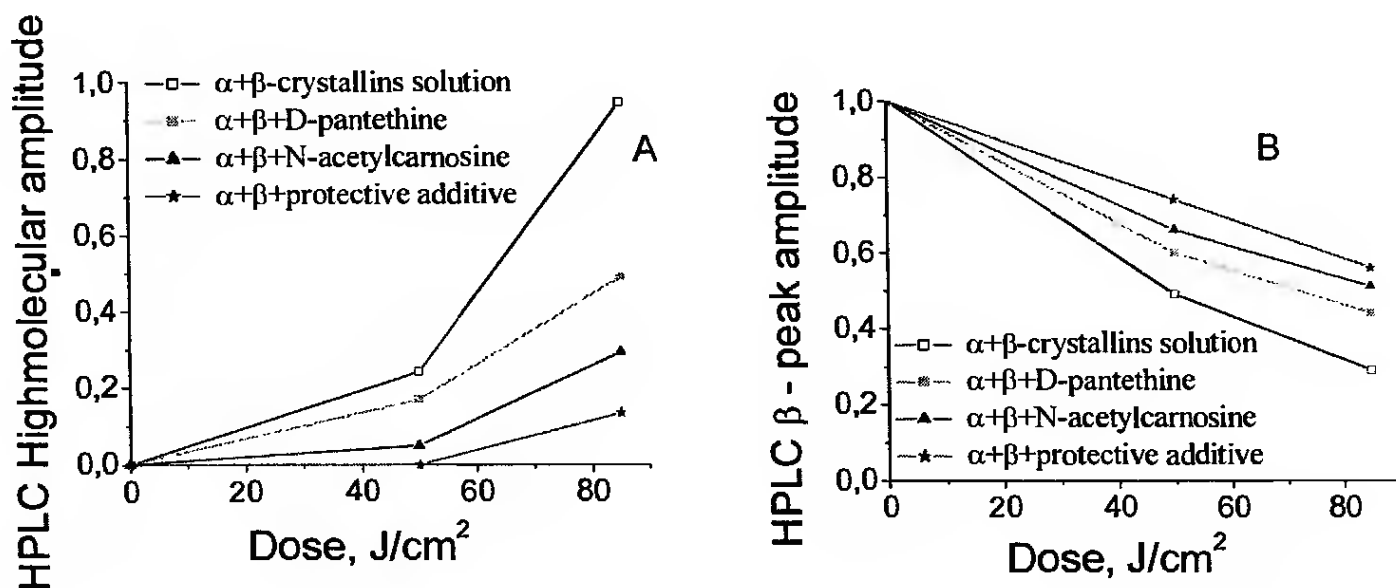


Рис. 13. Changes of molecular weight distribution peaks for  $\alpha+\beta$  solution: A -  $\beta$ -crystallin (monomer) and B - highmolecular fraction.

It should be noted that the chromatographic results were obtained at fairly small UV-doses – several times smaller than those needed for the intensity of scattered radiation of the test beam to start increasing.

In our studies of  $\beta$ L-crystallin aggregation and the effect of peptides on aggregation rate of the mixture of  $\beta$ L- and  $\alpha$ -crystallins, we measured not only kinetic curves and MWD but also energy of laser pulses ( $\lambda = 308$  nm) passed through the cuvette with protein solution. The dependences of 308 nm light transmission versus UV-dose in different irradiation regimes and for different protein solutions are presented in Figs. 2 b, 4, 7 and 11. First of all, we should note

that in the experiments where growth of test beam scattering was observed, the transmission curves have two characteristic regions. An initial region (at small values of D) is well approximated by the exponential function. One may suggest that this region is caused by to the growth of 308 nm light absorption in solution, which is due to some reorganization of the energetic structure of protein molecules. The absorption growth is observed until the intensity of scattered light at 633 nm starts increasing. With increasing UV-dose the exponential region terminates and the transmission curve declines more rapidly. This decline is due to the growth of scattered light at 308 nm in protein solution. During irradiation this effect manifests itself by appearance of scattered UV in different directions from the protein cuvette. Under conditions of our experiment, the dose at which the transmission curve has a sharp kink, is somewhat smaller than the dose at which the growth of test beam scattering starts. As Fig. 7 shows, for  $\beta$ A3-crystallin concentration of 0.2 mg/ml the intensity growth of scattered 633 nm light was not observed until  $D = 1000 \text{ J/cm}^2$ . In this case, the transmission curve has only an exponential region without any sharp kink associated with scattering of 308 nm light in the cuvette.

It is important to note that the transmission curves correlate both with corresponding kinetic curves and with chromatographic measurements, i.e., they provide an additional source of information on processes of UV-induced aggregation of the proteins being studied.

#### 4. CONCLUSION

In this communication the results of an investigation of UV-photoaggregation of pure  $\beta$ L-crystallins and a  $\beta$ L- and  $\alpha$ -crystallins mixture are reported. Similarity between kinetic curves behavior of bovine eye lens protein ( $\beta$ L-crystallin) and model protein carboanhydrase was found. The chaperone effect of  $\alpha$ -crystallin on  $\beta$ L-crystallin UV aggregation was shown.

For the first time the effect of D-pantethine and L-carnosine on the UV-induced aggregation of  $\alpha$  and  $\beta$ L-crystallins solution was demonstrated and studied. A protective additive with better anticataract properties than those of other known low-molecular compounds was found.

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