Quantitative verification of the existence of high molecular weight protein aggregates in the intact normal human lens by light-scattering spectroscopy

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The method of quasi-elastic light-scattering spectroscopy was used to establish quantitatively the concentration of high molecular weight (HMW) aggregates present in the normal human intact lens as a function of age. The concentration of HMW proteins increases monotonically with age. HMW proteins are absent in the infant lens, but represent 3% of the total soluble lens protein at age 60 years. The percent concentration of HMW proteins measured in intact lenses of various ages by quasi-elastic light scattering is in striking agreement with values determined biochemically.

Key words: quasi-elastic light scattering, HMW aggregates, intact human lens

It has been proposed theoretically that cataract is the result of the scattering of light from aggregates of lens proteins of molecular weight exceeding $50 \times 10^6$ gm/mol. From the biochemical point of view, it is now well established that these aggregates—heavy molecular weight (HMW) components—do in fact occur in vitro solutions of water-soluble lens proteins isolated from animal and human lenses. Furthermore, in vitro experiments have shown that the concentration of these aggregates increases with age in the normal lens.

Spector and collaborators first identified HMW proteins in the bovine lens and showed that there is an age-related increase in concentration of HMW proteins as the calf matures to steer age. Aggregation also occurs in individual lenses with the maturation of lens fibers. Liem-The et al. identified aggregates larger than $10^7$ gm/mol in nuclear extracts of rabbit lenses. They also discovered that there is a substantial increase in the content of HMW aggregates in x ray-induced rabbit lens cataract. Jedziniak et al. and Spector et al. made the important discovery that large protein aggregates are present in the human lens and increase in concentration with age and with cataract development. Jedziniak et al. provided the first direct evidence that the concentration of aggregates having a molecular weight greater than $150 \times 10^6$ gm/mol is two to three times higher in cataractous lenses than in normal lenses of the same age. They later demonstrated that the concentration of HMW proteins increases roughly linearly with age. Spector et al. showed that the age-related increase in concentration of HMW proteins occurs primarily in the nuclear region of the human normal lens. In vitro solutions of pro-
teins extracted from the nuclear region of human nuclear sclerotic cataracts also contain a large population of HMW proteins. Clearly, it has become necessary to ascertain whether HMW protein aggregates are present in the intact lens itself or whether they are artifacts associated with biochemical processing.

Recently Tanaka and Benedek discovered that protein diffusivity in the intact lens can be determined from the spectrum of laser light scattered from the lens. Since the diffusivity of a macromolecule in solution is inversely related to its size, this advance has made it possible in principle to measure the distribution of protein sizes in the lens in situ.

Accordingly, there were two main objectives of this study: (1) to use the method of quasi-elastic light-scattering spectroscopy to establish quantitatively the concentration of HMW aggregates present in normal intact lenses as a function of age and (2) to compare these results to the concentration of HMW aggregates established for biochemically separated in vitro solutions of human lens proteins.

Materials and methods

Human lenses were obtained from the Cornea Laboratory at Tufts University. Eyes were enucleated 2 to 12 hr after death. Lenses were removed immediately and stored at −40°C. The lenses were subsequently thawed to approximately 4°C and placed in 10 mm path-length glass cuvettes containing 0.15M saline for light-scattering measurements. The temperature was regulated electronically in the range 4°C to 40°C (±0.1°C) with a thermoelectric heating/cooling block.

The light source for the scattering spectrometer was a Spectra Physics Model 164 argon-ion laser operating at a power of 100 mW at a wavelength of 5,145 Å. The laser light was focused onto the nucleus of the normal human lens, and the scattered light was collected at a scattering angle of 90 degrees. The temporal fluctuations in the photomultiplier photocurrent were analyzed by a 19-channel double-scaled digital autocorrelator with channel times varying from 4 to 200 μsec. Analysis of the correlation function was carried out with the method of cumulants, developed by Koppel and Mazer. This method yields the average diffusivity, D, as well as the variance and higher moments of the distribution of diffusion coefficients.

Results and discussion

The first objective of this study was to determine the concentration of HMW aggregates in the intact human lens by the method of light-scattering spectroscopy. For this purpose, the mean diffusion coefficient, D, in the nucleus of intact human lenses of various ages was measured as a function of
temperature, $T$, from $-2^\circ$ to $35^\circ$ C. Fig. 1 shows the results of $D$ vs. $T$ for lenses of ages 5 months and 2, 13, 20, 26, and 43 years. It is clearly seen in the figure that for each lens the dependence of $D$ on $T$ is essentially linear. Lens age for the individual plots is expressed on the right-hand side of the figure.

The most striking feature that emerges from the data is that at given temperature, $D$ decreases monotonically with increasing lens age. For example, at $25^\circ$ C $D$ decreases from $2.2 \times 10^{-7}$ cm$^2$/sec for the 5-month-old lens to $0.9 \times 10^{-7}$ cm$^2$/sec for the 43-year-old lens. The clear qualitative conclusion is that there is a substantial and steady increase in the mean size of lens proteins with increasing age. It remains to determine from these data the actual concentrations of HMW aggregates which occur in these lenses of various ages.

The data obtained from quasi-elastic light scattering appear in the form of an intensity autocorrelation function. Cumulants analysis of this function by computer yields two key quantities which permit a characterization of the distribution of diffusing proteins. The first is the average protein diffusivity, $\bar{D}$. For macromolecules of uniform hydrodynamic radius, $R$, freely diffusing in solution of viscosity, $\eta$, diffusion coefficient $D$ is inversely proportional to the radius:

$$D = \frac{kT}{6\pi\eta R} \quad (1)$$

The scattered light intensity is proportional to the product of concentration (in grams per cubic centimeter) and molecular weight for a given diffusing specie. Hence the average diffusivity, $\bar{D}$, for a collection of macromolecules, each of molecular weight $M_i$ and $D_i$, and comprising fraction $f_i$ of the total weight of scatterers, is given by

$$\bar{D} = \frac{\sum f_i M_i D_i}{\sum f_i M_i} \quad (2)$$

Cumulants analysis of the light-scattering spectrum yields the average diffusivity $\bar{D}$.

The second quantity obtained from the data analysis is the variance, $V$. The variance is a mathematical measure of the width of the distribution of protein sizes. The more uniform in size the proteins, the smaller the variance. If the second moment of the distribution of diffusion coefficients is given by $D^2$, then $V$ is defined by

$$V = \frac{\sqrt{D^2 - (\bar{D})^2}}{\bar{D}} \quad (3)$$

Any change in the relative proportions of HMW and low molecular weight (LMW) protein aggregates in the lens at any fixed temperature will result in a change in both the average diffusivity and the variance.

The experimental values found for $\bar{D}$ and $V$ at $25^\circ$ C for human lenses of ages 5 months to 43 years are shown in Figs. 2A and 2B, respectively. For convenience, all results in the ensuing analysis will be discussed for a fixed temperature ($25^\circ$ C), thereby avoiding the effects of changing viscosity. Fig. 2A shows that $\bar{D}$ decreases from $2.2 \times 10^{-7}$ cm$^2$/sec at

![Fig. 2A. Average diffusivity vs. age for human normal lenses at fixed temperature (25° C).](image)

![Fig. 2B. Variance of the distribution of diffusivities vs. age for human normal lenses at 25° C.](image)
Fig. 3. Distribution of protein diffusivities assumed for an adult human normal lens. A fraction $f_{\text{HMW}}$ of the total lens protein exists as HMW aggregates, and a fraction $f_{\text{LMW}}$ as LMW aggregates. Each of the two Gaussian distributions is characterized by a mean diffusivity $D$ and by a variance $V$.

It is seen to increase linearly with age—from 0.9 at 5 months to approximately 1.5 at 43 years. The increase of $V$ with age signifies that the over-all distribution of protein sizes in the lens becomes broader with increasing age.

To calculate the extent of protein aggregation which occurs with aging in the lens, it was assumed that at any age the soluble protein distribution in a normal lens consists of only two distinct components, as seen in Fig. 3. This is a plot of the assumed distribution of diffusion coefficients, $g(D)$, as a function of diffusivity $D$. These two peaks correspond to the well-known HMW and LMW components of the water-soluble lens proteins which have been separated by agarose chromatography. Each component is characterized by a mean value of diffusivity, $D_{\text{HMW}}$ and $D_{\text{LMW}}$, and a variance, $V_{\text{HMW}}$ and $V_{\text{LMW}}$. Fraction $f_{\text{HMW}}$ is the fraction of total lens protein contained in the soluble HMW component, and $f_{\text{LMW}}$ is the fraction in the soluble LMW component.

At any age the over-all average diffusivity $D$ of proteins within the lens arises from a linear combination of varying amounts of these two slow-diffusing (HMW) and fast-diffusing (LMW) protein distributions. To calculate $D$ and $V$, the parameters $D_{\text{HMW}}$, $D_{\text{LMW}}$, $V_{\text{HMW}}$, and $V_{\text{LMW}}$ were first established. To measure $D_{\text{HMW}}$ and $V_{\text{HMW}}$, light-scattering measurements were performed on the agarose-separated HMW protein of a 40-year-old normal lens at 25° C. $D_{\text{HMW}}$ was found to be $0.4 \times 10^{-7}$ cm$^2$/sec. This value corresponds to a molecular weight of $300 \times 10^6$ gm/mol. The value measured for $V_{\text{HMW}}$ was 1.40. This relatively large variance indicates the presence of a broad distribution of very heavy molecular weight aggregates comprising the HMW component. To establish $D_{\text{LMW}}$ and $V_{\text{LMW}}$, light-scattering measurements were performed on the agarose-separated LMW fraction from the 5-month-old lens. The elution profile for this fraction showed that no HMW protein existed in this lens. The light-scattering values found were $D_{\text{LMW}} = 2.15 \times 10^{-7}$ cm$^2$/sec, corresponding to a molecular weight of $2 \times 10^6$ gm/mol, and $V_{\text{LMW}} = 0.88$. Interestingly, these parameters obtained for the biochemically separated LMW protein were virtually identical to the values found for the intact 5-month-old lens, a consequence of the absence of heavy aggregates in the infant lens.

With $D_{\text{HMW}}$ and $D_{\text{LMW}}$ determined, the average diffusivity $D$ can now be calculated from equation 2. For the case of the protein distributions, of the HMW and LMW fractions, equation 2 becomes

$$D = \frac{f_{\text{HMW}}M_{\text{HMW}}D_{\text{HMW}} + f_{\text{LMW}}M_{\text{LMW}}D_{\text{LMW}}}{f_{\text{HMW}}M_{\text{HMW}} + f_{\text{LMW}}M_{\text{LMW}}}$$

(4a)

where $M_{\text{HMW}}$ and $M_{\text{LMW}}$ are the average molecular weights of the two distributions. Since it has been determined that $M_{\text{HMW}}/M_{\text{LMW}}$ is approximately 150, equation 4a reduces to

$$D = \frac{150 f_{\text{HMW}}D_{\text{HMW}} + f_{\text{LMW}}D_{\text{LMW}}}{150 f_{\text{HMW}} + f_{\text{LMW}}}$$

(4b)

That is, the contribution to the over-all average diffusivity due to a single HMW molecule must be counted with a weight of 150 relative to that due to a single LMW molecule.
An additional condition is the conservation of the total amount of lens protein, whether soluble or insoluble:

\[ f_{\text{HMW}} + f_{\text{LMW}} + f_{\text{IS}} = 1 \]  

(5)

where \( f_{\text{IS}} \) represents the fraction of lens protein which is insoluble. The insoluble fraction of protein has been presumed to contribute insignificantly to the spectrum of the scattered light, and hence to \( D \), because of its negligible diffusivity.

Using equations 4b and 5, one can show that fraction \( f_{\text{HMW}} \) is directly proportional to \((1 - f_{\text{IS}})\), which is equal to \( f_{\text{HMW}} + f_{\text{LMW}} \). It is therefore possible to compute, independently of the insoluble fraction \( f_{\text{IS}} \), the quantity \( f_{\text{HMW}}/(f_{\text{HMW}} + f_{\text{LMW}}) \), which is the concentration of HMW protein expressed as the fraction of total soluble lens protein. One finds

\[ \frac{f_{\text{HMW}}}{f_{\text{HMW}} + f_{\text{LMW}}} = \frac{D_{\text{LMW}} - D}{149D - 150D_{\text{HMW}} + D_{\text{LMW}}} \]  

(6)

These results are shown in Fig. 4, where \( f_{\text{HMW}}/(f_{\text{HMW}} + f_{\text{LMW}}) \) is plotted as a function of lens age. Clearly, the fraction of soluble protein which has formed soluble HMW aggregates increases markedly with age.

A sensitive test of the validity of our two-component model, Fig. 3, is provided by an analysis of the variance \( V \). In order to compute the variance, the quantity \( D^2 \) is needed, as seen in equation 3. Since \( D^2 \) is the second moment of the distribution of diffusion coefficients, it follows that

\[ D^2 = \frac{f_{\text{HMW}} M_{\text{HMW}} D^2_{\text{HMW}} + f_{\text{LMW}} M_{\text{LMW}} D^2_{\text{LMW}}}{f_{\text{HMW}} M_{\text{HMW}} + f_{\text{LMW}} M_{\text{LMW}}} \]  

(7)

For the two Gaussian distributions, the second moments \( D^2_{\text{HMW}} \) and \( D^2_{\text{LMW}} \) are given simply by

\[ D^2_{\text{HMW}} = (1 + V^2_{\text{HMW}}) (D_{\text{HMW}})^2 \]  

(8a)

\[ D^2_{\text{LMW}} = (1 + V^2_{\text{LMW}}) (D_{\text{LMW}})^2 \]  

(8b)

From equations 7 and 5 it follows that

\[ D^2 = \frac{150 \alpha_{\text{HMW}} D^2_{\text{HMW}} + (1 - \alpha_{\text{HMW}}) D^2_{\text{LMW}}}{149 \alpha_{\text{HMW}} + 1} \]  

(9)

where \( \alpha_{\text{HMW}} = f_{\text{HMW}}/(f_{\text{HMW}} + f_{\text{LMW}}) \), whose values were previously determined for each lens age and plotted in Fig. 4.

Fig. 4. Concentration of HMW aggregates as a function of lens age, as determined by light-scattering spectroscopy. The HMW protein concentration is expressed as the fraction of total soluble lens protein.

Fig. 5. Comparison of experimental to theoretical variance of the distribution of protein diffusivities as a function of lens age. The experimental values (•) were determined directly from the light-scattering spectrum of the intact lens, whereas the theoretical values (o) were computed from the model of Fig. 3 and the results for HMW protein concentration, from Fig. 4.
Fig. 6. Comparison of the percent concentration of HMW aggregate as a function of lens age, as determined by light-scattering spectroscopy (•) and biochemical separation (○).

only the change of $\bar{D}$ with age but also the age dependence of the variance of the distribution of diffusion coefficients. This latter agreement represents an independent verification of the model assumed, which was motivated by the in vitro biochemical protein separation.

It may be concluded from these studies that during the normal aging process protein in the lens is slowly and steadily being converted to large aggregates. Furthermore, only very small concentrations of HMW protein are needed to drastically affect the overall average protein diffusivity, because of the large difference in molecular weights. By the age of 43, at which point the average $\bar{D}$ has fallen by more than a factor of two from the value for a baby lens, only 1.5% of the total soluble protein is in the form of soluble HMW aggregates.

The second objective of this study was to compare the age-dependent concentration of HMW soluble aggregates in the intact human normal lens determined from light-scattering data to the concentration of HMW aggregates previously established by biochemical methods for in vitro solutions of human lens proteins. Wide variations in the concentration of HMW established biochemically from both the normal and cataractous lens have been reported in the literature. Much of this variation is due to operational differences in homogenization and centrifugation and to variations in exclusion limit of the agarose gel used to separate HMW proteins. In this study, the water-soluble lens proteins were separated from the water-insoluble proteins by centrifugation at 14,000 rpm for 20 min. HMW aggregates were isolated by gel chromatography on agarose 150 columns. The average size of these aggregates has been measured to range between 100 and $300 \times 10^6$ gm/mol by optical mixing spectroscopy and by sedimentation velocity. The concentration of HMW for human lenses of various ages was calculated by measuring the absorptivity at 280 nm. In the case of human HMW protein, Spector (personal communication) has established that an optical density measurement of 1.0 corresponds to a protein concentration of 0.4 mg/ml. Thus the measured absorption at 280 nm was corrected by a factor of 2.5.

Fig. 6 compares the percent concentration of HMW protein measured in intact lenses of increasing age by quasi-elastic light scattering to the percent concentration of HMW protein determined biochemically for in vitro solutions of human normal lenses. In both cases the percent of HMW protein represents the ratio of water-soluble HMW proteins to total water-soluble proteins: $\frac{W_{\text{HMW}}}{W_{\text{total}}}$ For $f_{\text{HMW}}$, it was assumed that an optical density of 1 corresponds to 1.0 mg/ml LMW. In the figure, the quasi-elastic light-scattering results are designated by the open circles, with the biochemically determined concentrations given by the closed circles.

It is immediately evident from both sets of data that there is excellent quantitative agreement between the two methods for the absolute concentration of HMW over the age range considered.

It may now be concluded that HMW aggregates are present in the intact human normal lens. The concentration of HMW protein increases monotonically with age. In the infant normal lens, HMW protein is virtually nonexistent. In lenses of 60-year-old persons, HMW protein represents about 3% of the total soluble lens protein.
REFERENCES