Lens Protein Composition, Glycation and High Molecular Weight Aggregation in Aging Rats

M. S. Swamy and E. C. Abraham

Because of minimal or no turnover, lens proteins are subjected to substantial post-translational modifications which in turn disrupt lens architecture and change the optical properties leading to senile cataract formation. Progressive glycation is believed to have the potential to initiate the changes that are conducive to lens opacification. Fisher344 rats were systematically followed from juvenile to older and aged phases of their life to study the relationship between lens glycation and high molecular weight (HMW) aggregate formation as well as quantitative and qualitative changes in lens crystallins. Levels of glycated proteins were quantified by affinity chromatography. Changes in lens crystallin composition and HMW aggregate formation were monitored by molecular sieve HPLC, further confirmed by SDS-PAGE and IEF techniques. As the age advances HMW and insoluble proteins increase with a concomitant disappearance of γ-crystallins from soluble fraction. This disappearance of γ-crystallins coincided with increased glycation (approximately 2-fold higher in insoluble fraction) and decreased sulfhydryl groups from soluble fraction. It appears that lens protein glycation, disappearance of γ-crystallins and sulfhydryls from soluble fraction and increase of insoluble fraction and HMW aggregate are interrelated. Invest Ophthalmol Vis Sci 28:1693–1701, 1987

In spite of high protein content, approximately 35% of wet weight,1 a normal rat lens is remarkably transparent. However, with aging there is a deterioration of the lens architecture leading to definite and steady loss of transparency which eventually leads to senile cataract formation.2 Interestingly, in many types of opacities insolubilization of soluble crystallins and formation of high molecular weight (HMW) aggregates are responsible for light scattering effect.3 Lieu-The and Hoenders4 suggested that HMW proteins might be an intermediate in conversion of water-soluble to insoluble proteins.2 Individual lens crystallins undergo quantitative and/or qualitative changes as a result of aging.5–7 Contrary to other crystallins, low molecular weight monomeric γ-crystallins show progressive decrease in the water-soluble fraction during development and aging.8–14 In our earlier work15 on streptozotocin-induced diabetic cataract formation, a possible correlation was made between lens glycation vis-a-vis insolubilization of crystallins and HMW aggregate formation. The present study was designed to probe into the formation of HMW aggregates and lens protein insolubilization during both development and aging of rat lenses and to see if factors such as lens protein glycation, sulfhydryl oxidation, and changes in crystallin composition, γ-crystallin in particular, have any relationship to these events. Such a systematic study, with all these factors considered together, has not been conducted before during development and aging of rats. A recently introduced high performance liquid chromatographic (HPLC) method has enabled us to quantitate HMW aggregates and individual crystallins with relative ease and with small amounts of protein.

Materials and Methods

Animals

Male, Fisher344 rats (Harlan Sprague-Dawley Inc., Indianapolis, IN, and National Institute of Aging, Bethesda, MD) of age 1, 4, 8, 12, 16, 20, 23, 25, and 28 months (three animals in each group) were used in the present study. Based on information provided by Harlan Sprague-Dawley Inc., our experimental animals fall in the following age groups. One-month-old rats are juvenile, by about month 4 they become young adults, 8 to 12-month-old rats can be considered as adults of middle age, and 16 to 20-month-old rats and 23 to 28-month-old rats represent older and aged groups, respectively. The median survival life span is approximately 28 months. Animals were sacrificed under ether anesthesia. Glycated hemoglobin

From the Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, Georgia. Contribution number 1029 from the Department of Cell and Molecular Biology. Supported by a grant from American Diabetes Association, Inc. Submitted for publication: November 25, 1986. Reprint requests: E. C. Abraham, PhD, Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, GA 30912-3331.
and plasma glucose levels (data not presented) were estimated immediately after the sacrifice to screen for diabetes. Lenses were quickly removed, rinsed in 50 mM sodium phosphate buffer (pH 7.0), and gently rolled on blotting paper. Paired lenses were weighed and processed as follows. Animal experiments were performed in conformity with the recommendations of the ARVO Resolution on the Use of Animals in Research and of the Institutional Committee for Animal Care.

Crystallin Preparation

The water-soluble (WS) and the urea-soluble (US) protein fractions were prepared by the modified procedure of Herbrink and Bloemendal\textsuperscript{16} described by Perry and Abraham,\textsuperscript{17} whereas SDS-soluble fraction (SS) was prepared according to the method described by Russell.\textsuperscript{18} Paired lenses were homogenized under nitrogen, in 3 ml of 50 mM sodium phosphate buffer (pH 7.0) (buffer A) using a Dounce ground-glass tissue homogenizer (Wheaton Scientific, Millville, NJ). The resulting homogenate was centrifuged for 1 hr at 6000g at 4°C. The supernatant which comprised WS fraction was then dialyzed for 24 hr against 500 volumes of buffer A at 4°C. The pellet was washed with ten volumes of buffer A and centrifuged for 10 min at 10,000g. three times. The pellet was then resuspended in 2 ml of buffer B containing 7 M urea, 100 mM HEPES, 25 mM NaCl, 1 mM EDTA (pH 8.5) and allowed to stand for 2 hr at 4°C under nitrogen with occasional shaking. This suspension was centrifuged for 30 min at 10,000g at 4°C and the resultant supernatant was the US fraction. The pellet was washed in ten volumes of buffer B and centrifuged at 10,000g for 10 min, twice, and once in buffer C containing 50 mM sodium phosphate, 100 mM KCl, 5 mM EDTA and 10 mM β-mercaptoethanol, followed by a wash with buffer B, once with 3 ml of 0.1 N NaOH and again twice with buffer B. The resulting pellet was boiled in 0.1% SDS for 20 min, cooled and centrifuged at 10,000g for 30 min and the resultant supernatant, termed SDS-soluble (SS) fraction, mainly contained membrane polypeptides.

HPLC Analysis of Lens Proteins

Beckman HPLC system, having model 421 controller, dual 110 pumps and model 160 detector with 10 μl flow cell, and Hewlett Packard (Avondale, PA) 3390-A recording integrator were used to separate and quantify the constituent proteins in both WS and US fractions. The separations were achieved according to the method of Perry and Abraham\textsuperscript{17} using TSK 3000 SW, 7.5 mm × 600 mm, coupled with TSK 4000 SW, 7.5 mm × 300 mm, columns for soluble crystallins and TSK 2000 SW, 7.5 mm × 600 mm, coupled in series with TSK 3000 SW, 7.5 mm × 300 mm, for insoluble proteins. The absorbance was monitored at 280 nm with isocratic flow of 1 ml/min. For analytical runs sample size was 120 μg, whereas during semi-preparative runs sample up to 5 mg in 200 μl was applied and effluent fractions collected with an Eldex Universal fraction collector (Eldex Laboratories Inc., San Carlos, CA).

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done on Bio-Rad (Richmond, CA) Protein\textsuperscript{®} II slab gel according to the method of Laemmli.\textsuperscript{19} Approximately 20 μg protein was loaded on a slab, 20 × 16 × 0.5 mm, with 12% acrylamide for WS fraction and 15% acrylamide for SS fraction and run at 40 mA for about 4 hr. The molecular weights were determined by molecular weight markers from Sigma Chemical Co. (St. Louis, MO).

Isoelectric Focusing

WS fraction as well as constituent crystallins of WS fraction after HPLC molecular sieve separation were subjected to isoelectric focusing on Resolve\textsuperscript{®} Omega\textsuperscript{®} system with CWS-2000 power supply unit using pre-cast Gel Bond\textsuperscript{®} PAG\textsuperscript{®} 5 film having 5% ampholytes and 5% glycerol (all from Isolab Inc., Akron, OH). The pH range of the gel was 3–10. Isoelectric focusing markers, pH range 3.55–9.3 (from Sigma Chemical Co.) were used for calibration. After 10 min of prefocusing at 5 W, focusing was accomplished by graded power supply for 1 hr, 15 min according to the procedure described by the manufacturer. After precipitating with 20% TCA for 30 min, the bands were visualized with Coomassie Brilliant Blue R-250 and destained in 25% ethanol and 8% acetic acid.

Determination of Protein Content

The protein concentration was determined using Bio-Rad protein assay based on the dye-binding principle developed by Bradford.\textsuperscript{20} Bovine serum albumin was used as a standard.

Quantification of Glycated Proteins

The glycated proteins in WS and US fractions were quantified using Glyc-affin micro-affinity columns (Isolab Inc.), after suitable modifications as described by Perry et al. Briefly, the columns were prepacked with phenylboronate agarose in a column size of 0.5 cm × 4 cm. The column was equilibrated with 2 ml
of column preparation buffer containing 100 mM asparagine and 10 mM MgCl₂, pH > 9.0. The sample containing approximately 0.5 mg of WS fraction was made basic (roughly pH 9) by the addition of 15 μl of 0.1 M NaOH and applied to the top of the microcolumn. Once the sample had flowed into the gel, it was allowed to equilibrate with the gel for 10 min before the non-glycated protein fraction was eluted by adding 2 ml of first fraction elutant (100 mM glycine, 10 mM MgCl₂, pH > 9.0). The glycated proteins were eluted by the addition of 1 ml of second fraction elution buffer consisting of 100 mM sorbitol. Protein concentrations of both the fractions were determined using Bio-Rad protein reagent and the percentage of the glycated proteins was calculated. The recovery of WS proteins from affinity columns was about 90%.

The glycated proteins from the US fraction were quantified by a procedure similar to that used for WS fraction except a 7 M urea was added to the eluting buffers. The recovery of US proteins from the affinity column was about 86%.

**Sulphydryl Titration**

The concentration of free and total protein sulphydryls (—SH) in WS fraction was determined by titration with parachloromercury benzoic acid (PCMB) according to the modified method of Boyer. Briefly, two sets of measurements were taken, one in the absence and the other in the presence of 0.01% SDS to determine free and total reactive sulphydryl groups, respectively. Sulphydryl determination of the US fraction was not technically feasible by this spectrophotometric method.

**Results**

**Changes in Lens Weight and Protein Subfractions**

As previously shown by Hockwin, lenses initially had exponential growth, and later the growth curve remained in a stationary phase. After a steep increase in the weight of lenses during the initial 4 month period, the increment was subsequently slowed down up to 16 months, which was followed by a plateau period (Fig. 1A).

With aging there is a deterioration of lens architecture leading to loss of transparency. Changes in protein structure, both quantitative and qualitative, can be linked to this alteration in optical properties. Age-related changes in protein content in WS, US and SS fractions are given in Figure 1B. The WS proteins increased with age up to 20 months; however, the later ages were characterized by a slow decrease. In contrast, the US proteins showed a continuous increase during the experimental period. There was a dramatic increase from 1.07 mg/paired lenses at 1 month to 11 mg by 16 months, followed by a gradual increase to approximately 13 mg as noted in 28-month-old rats. The proteins that would solubilize in the presence of SDS, the SS fraction, also showed an age-related increase. The increase was slow but gradual up to 25 months followed by a steep increase between 25 and 28 months.

**Changes in Soluble Lens Crystallins**

**Molecular sieve HPLC:** Figure 2 represents a typical molecular sieve HPLC chromatogram of pooled WS fraction from 1 through 28 months of age. As is evident from Figure 2, we could get a clear separation of HMW, α, β, and γ-crystallins. β-Crystallin could be further separated into βH, β1, and β2, whereas γ-crystallins could be separated into γH and γL components. The identity of each of the crystallin components was confirmed by amino acid analysis and reported earlier. Further analyses of these peaks are discussed elsewhere in the text.

**Changes in HMW aggregate:** The peak eluting before the α-crystallin with a molecular weight of >1.5 × 10⁶ was considered a HMW component. The
HMW aggregate from the soluble fraction increased from almost undetectable levels up to 16-month rats to 8% in 28-month-old rats (Table 1 and Fig. 6C).

Changes in α- and β-crystallins: As shown in Table 1, the relative proportion of α-crystallins also showed an increase from 13% at 1 month to 26% at 16 months and thereafter showed a slight drop during the rest of the period. Changes in β-crystallins were clearly evident with the advancement of age. βH fraction, which was clearly distinguishable from the rest of β fractions in 1-month and 4-month-old rats and which contributed about 4% of total lens soluble proteins at those ages, showed a decrease and appeared as a shoulder on βL, peak (thus not able to quantitate) from 8th month onwards. In contrast, βL2 that was resolved as a clear major peak in the chromatogram, constituting 26% of the total area, showed a steep increase to 41% by 4th month and thereafter maintained almost steady level. βL1, at the same time, showed a gradual increase from about 1% in 1-month-old animals to about 24% in 20-month-old animals.

Changes in γ-crystallins: The monomeric γ-crystallin showed interesting age-related changes. Table 1 shows that 1-month-old rats showed only one peak, constituting 56% of the total crystallins. There was a steep decline in γ-crystallin content to 22% when the rats attained 4 months of age. Also interestingly, two distinct γ peaks could be clearly separated at this age (4 months), each having different retention times. These two peaks were designated as γH, having longer retention time (33 min) on HPLC columns, whereas γL has relatively shorter retention time (31 min) (Fig. 2). Using molecular weight standards we determined that the γL peak has a molecular weight in the range of 19,000–21,000 daltons whereas γH peak has a molecular weight of 23,000–24,000 daltons. Fractions collected by semi-preparative molecular sieve HPLC were subjected to SDS-PAGE to confirm the molecular weights. As shown in Figure 3 the γL consisted of two bands having molecular weights of approximately 20,000 and 21,000, respectively, whereas γH yielded one major band of molecular weight 24,000 daltons. Furthermore, to check their purity, both γ fractions after semi-preparative molecular sieve HPLC were subjected to isoelectric focusing (Fig. 4) which demonstrated that γL is focused into three major bands having pI(s) of 7.45, 7.55, and 8.0, whereas γH resulted in two major bands having pI(s) of 7.25 and 7.3. However, there were about nine (five major and four minor) bands discernible from total γ fraction distributed in the pH range of 7.0 to 8.0. The age-dependent changes in lens crystallin components were also studied by subjecting the WS fraction from 1 to 28-month-old animals to isoelectric focusing. As shown in Figure 5, γ-crystallins were separated between pH 7.0 to 8.0, whereas β-crystallins occupied pH range between 6.0 and 7.0, and α-crystallins were

Table 1. Age-dependent changes in water-soluble proteins determined by molecular sieve HPLC

<table>
<thead>
<tr>
<th>Age in months</th>
<th>HMW</th>
<th>α</th>
<th>βH</th>
<th>βL1</th>
<th>βL2</th>
<th>γH</th>
<th>γL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13.0 ± 2.70</td>
<td>4.00 ± 1.08</td>
<td>1.11 ± 0.50</td>
<td>26.13 ± 4.79</td>
<td>56.20 ± 5.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>18.07 ± 2.58</td>
<td>4.70 ± 0.99</td>
<td>10.43 ± 2.77</td>
<td>41.17 ± 2.03</td>
<td>6.53 ± 0.21</td>
<td>22.23 ± 1.68</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>20.93 ± 1.80</td>
<td>12.53 ± 0.43</td>
<td>42.61 ± 1.28</td>
<td>6.63 ± 0.38</td>
<td>17.30 ± 0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>24.07 ± 0.68</td>
<td>14.43 ± 0.42</td>
<td>43.21 ± 2.64</td>
<td>7.33 ± 0.64</td>
<td>10.90 ± 2.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>25.73 ± 1.97</td>
<td>17.90 ± 2.08</td>
<td>43.83 ± 1.31</td>
<td>8.77 ± 2.47</td>
<td>9.90 ± 0.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>25.17 ± 1.07</td>
<td>22.17 ± 3.61</td>
<td>41.13 ± 4.09</td>
<td>8.17 ± 2.16</td>
<td>4.00 ± 0.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>24.23 ± 1.91</td>
<td>22.37 ± 4.11</td>
<td>41.81 ± 3.95</td>
<td>8.21 ± 0.56</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>23.40 ± 2.85</td>
<td>22.51 ± 3.41</td>
<td>41.99 ± 3.68</td>
<td>7.80 ± 0.26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>20.54 ± 2.05</td>
<td>24.05 ± 4.15</td>
<td>41.2 ± 2.95</td>
<td>6.05 ± 1.01</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each value is an average of three paired lenses ± SD.
Fig. 3. SDS-PAGE pattern of HPLC peaks shown as in Figure 2. Twenty μg protein was loaded on 12% acrylamide gels and run for 4 hr. Note the constituents of γH peak resolved into one major band, whereas γL resolved into two major bands, shown by arrows. α and β are shown for comparison.

more anodic. The changes in γ-crystallins were in perfect conformity with the observed HPLC profile (Table 1). The γ-crystallin band (pI about 7.5) constituting one of the two major components of γL crystallin showed a dramatic decrease from 1 month to 4 months and a slow but steady decrease thereafter up to 16 months, and finally disappeared. γ-Crystallin with pI 8.0, which is the second major constituent of γL, also showed a decrement, but slowly, and by 28 months a faint band was discernible. However, band with pI 7.25, a major constituent of γH, maintained fairly constant intensity until 25 months of age. The oldest rats (25 and 28 months) in this group showed very faint γ-crystallin bands.

Age-Related Changes in Urea-Soluble Proteins

Changes in HMW aggregate: Increased quantity of US protein (shown in Fig. 1B) alone may not be a sufficient condition to cause opacity since some older normal human lenses have a greater amount of water-insoluble protein than some cataracts obtained in younger lenses. It involves not simply a quantitative but a qualitative change related to refractive index. In human senile cataracts urea-soluble HMW aggregates are believed to be responsible for light-scattering units, and with the recently developed HPLC methodology the HMW aggregate and other proteins could be clearly separated and quantified from the US fraction. The increase in total US protein (Fig. 1B) was paralleled by an increase of this peak from 1 month (9%) to 8 months (20%) followed by a plateau up to 20 months and then a slight increase to 24%, as noted in 28-month-old rats (Fig. 6C). Moreover, changes in the proportions of other proteins were apparent. However, we have not focused on these changes due to the fact that the identity of these proteins has not been established; this work is in progress. A preliminary analysis of insoluble HMW aggregate was undertaken to understand its constituents (Fig. 7). Insoluble HMW aggregate from 20–28-month-old rats was collected after molecular sieve HPLC, concentrated and boiled with 0.1 M β-mercaptoethanol and rechromatographed on HPLC.

Fig. 4. IEF pattern of HPLC peaks shown as in Figure 2. Twenty μg protein was loaded on precoated plates as described in Materials and Methods and run for 75 min with a graded power supply. Note γH is constituted by two major bands whereas γL by three major bands, shown by arrows. βL2 shown for comparison.
Fig. 5. Progressive changes in IEF pattern of WS proteins during development and aging.

Fig. 6. Progressive changes in (A) glycated proteins in WS fraction (C---C) and in US fraction (●●●); (B) sulfhydryls in WS fraction, total (Δ---Δ) and free (▲---▲); and (C) HMW aggregates in the WS fraction (■---■) and in US fraction (□---□) during development and aging. Each value is an average of three paired lenses ± SD.

Fig. 7. Preliminary analysis of insoluble HMW aggregate. HMW peak was pooled from US fraction of 20–28-month-old rats and rechromatographed molecular sieve HPLC column before (A) and after boiling with 0.1 M β-mercaptoethanol for 10 min (C). γL crystallins isolated from WS fraction applied to the same columns for comparison (B). All the samples were applied to TSK 2000 SW-TSK 3000 SW columns used for separation of US proteins described in methodology. Note the dissociation of insoluble HMW aggregate by β-mercaptoethanol and that γ-crystallins appear to be the major constituents of this aggregate.

The constituents were a predominant peak (retention time 33 min), a major peak (retention time 22 min) and a minor peak (retention time 19 min). A small portion of HMW aggregate still remained intact and eluted as a small peak (retention time 15 min) (Fig. 7C).
Progressive Changes in the Glycation of Lens Proteins

Glycated proteins in the WS fraction as determined by microaffinity columns showed a slow increase from 2% to 5% as age advanced from 1 month to 23 months followed by a plateau until 28 months. In contrast, glycation of US proteins showed a steep increase from 3% at 1 month to about 7% at 8 months. This level was maintained during the remaining period (Fig. 6A). A strong parallelism exists between the changes in the levels of glycation and in the proportions of HMW aggregates.

Protein Composition of WS and US Glycated Portions

Glycated and non-glycated portions were isolated by affinity chromatography from pooled WS and US fractions of 20–28-month-old rats and further analyzed on molecular sieve HPLC. The results (Fig. 8) showed an enrichment of HMW peaks in the glycated portion of both WS and US fractions as compared to the non-glycated portion from the respective fractions. In WS fraction non-glycated portion had only 19% of HMW peak whereas glycated portion had 30% (Fig. 8A, B). Similarly, in US fraction HMW peak in the non-glycated portion was only 11% as compared to 35% in glycated portion (Fig. 8C, D).

Age-Dependent Changes in Protein Sulfhydryl Groups

Protein sulphydryls (–SH) in the WS fraction as titrated by PCMB showed age-dependent decrease. There was a sharp decline in –SH groups, titrated both in the absence or presence of SDS, from 1-month to 16-month-old animals, indicating depletion of protein –SH groups probably by formation of disulfide groups. Relatively slow decrease in the levels of –SH groups (Fig. 6B) was seen beyond 16 months.

Discussion

The age of proteins constituting the innermost core of the lens nucleus may be older than the post-natal life of the animal. Due to minimal or no turnover these proteins are unique experimental models for the study of post-translational modifications. The WS lens proteins showed an exponential increase during lens growth and development, whereas aging lenses from older animals yielded a steady loss of soluble proteins (Fig. 1B). With increasing age the composition of proteins of rat lens also changed considerably. It has been noted that lens crystallins are subjected to polymerization to HMW aggregates. In the youngest animals there was no noticeable HMW peak (Fig. 6C and Table 1) in the WS fraction, whereas in the lenses from 12-month-old onwards the HMW peak could be clearly separated from the other.
crystallins and this peak increased by 8-fold in the oldest animals (28 months). In rodents, it was proposed that soluble HMW aggregate was formed by polymerization chiefly of α-crystallins. Moreover, there was a dramatic decrease in γ-crystallins from 1 through 4 months, with no concomitant increase in soluble HMW peak. Hence, γ-crystallins may not be contributing significantly to the formation of soluble HMW aggregates.

It was first observed by Zigman et al. that rat γ-crystallins can be further separated into eight protein fractions having different isoelectric points. Vornhagen et al. found that they belong to three distinct immunological groups. By the molecular sieving HPLC procedure we could demonstrate two distinct peaks of γ-crystallins from the WS fraction, designated γyH and γyL. The nomenclature is arbitrary, based on the retention time; H (heavy) eluted faster than L (light). One-month-old animals seem to have predominantly γyL. On SDS-PAGE this peak resolved into two major bands (Fig. 3) having molecular weight 20,000–21,000 while IEF revealed that this peak is formed by three distinct proteins having pI(s) 7.45, 7.55 and 8.0, respectively (Fig. 4). It is clearly evident that the HPLC data (Table 1) complements the IEF patterns (Fig. 5), particularly in emphasizing the changes in γ-crystallin.

It was reported that decreased γy concentration in WS fraction was the result of four processes: it may have leaked out of the lens, it may have been made insoluble, and it may have been cross-linked to a high molecular weight, and less γ-crystallin may have been synthesized. In rat lens γ-crystallin constitutes a major component of albuminoid. Similarly, in the present study disappearance of γ from WS fraction is paralleled by increased US fraction in general and the HMW peak in particular. It was clearly established that the bonds involved in the aggregation of insoluble HMW are mainly disulfide in nature. It was reported that rat γ-crystallin components of high isoelectric points have about 3 times more sulfhydryls than those of low isoelectric points. It is evident from the data that the constituents of γy have higher isoelectric points than those of γyH (Fig. 4), and γyL showed dramatic age-dependent decrease (Table 1). Free sulfhydryl determination yielded data (Fig. 6B) befitting the changes of γyL fraction. Using noninvasive Raman spectroscopy technique, East et al. demonstrated, in rat lens nucleus, a decrease in −SH with a corresponding increase in S-S during aging. Moreover, HPLC analysis of β-mercaptoethanol-dissociated insoluble HMW aggregate revealed two interesting features (Fig. 7). First, the bonds that held together this aggregate were mainly disulfide in nature, hence β-mercaptoethanol could dissociate this aggregate leaving only a small peak which may be covalently linked (Fig. 7C). Second, the major constituents of this aggregate, the last peak in Figure 7C, showed a close similarity with γyL. The retention time on columns of this peak is exactly the same as that of γyL (33 min) (Fig. 7B). SDS-PAGE yielded polypeptides of 20–21 kd and amino acid analysis also showed a close similarity to γyL (data not presented). So it is logical to believe that high thiol-containing γy crystallins which were depleted from WS fraction were aggregated, by thiol oxidation, into disulfide-linked HMW aggregate of US fraction. This seems to be a more plausible explanation for lowered γ-crystallins; nonetheless, other processes may be possible; less γ may have been synthesized, for example.

Even though γ-crystallins with high sulfhydryl groups seem to be potential molecules for aggregation and insolubilization there must be some initiation factors or agents to trigger and maintain this process. Stevens et al. first suggested that nonenzymatic glycation may influence the functional properties of lens crystallins and postulated that glycation of the ε-amino groups of lysine may result in a structural change leading to insolubilization. Similarly, age-related increase in glycation was observed in human lens by Chiu et al. The data on glycated proteins in both WS and US fractions (Fig. 6A) suggest that the proportion of glycated proteins in US fraction is about 2-fold higher than that found in WS fraction and the parallel increase in glycated proteins in US fraction coincides with the increase in insoluble HMW aggregate (Fig. 6C). Preliminary HPLC analysis of glycated and non-glycated portions of WS and US fractions showed an enrichment of HMW peaks in glycated portion as compared to non-glycated portion, with US fraction showing more profound enrichment (Fig. 8). These observations, coupled with two previous reports wherein it was suggested that glycation of crystallins imparts susceptibility to sulfhydryl oxidation resulting in the formation of HMW aggregates, gives more credence to the view that nonenzymatic glycation may be a causative factor for insolubilization and aggregation of lens proteins.

**Key words:** aging, cataract, glycation, crystallins, protein aggregation

**References**


