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Similarity of the yellow chromophores isolated from human cataracts with those from ascorbic acid-modified calf lens proteins: evidence for ascorbic acid glycation during cataract formation

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Abstract

Chromatographic evidence supporting the similarity of the yellow chromophores isolated from aged human and brunescant cataract lenses and calf lens proteins ascorbylated *in vitro* is presented. The water-insoluble fraction from early stage brunescant cataract lenses was solubilized by sonication (WISS) and digested with a battery of proteolytic enzymes under argon to prevent oxidation. Also, calf lens proteins were incubated with ascorbic acid for 4 weeks in air and submitted to the same digestion. The percent hydrolysis of the proteins to amino acids was approximately 90% in every case. The content of yellow chromophores was 90, 130 and 250 A_{330} units/g protein for normal human WISS, cataract WISS and ascorbate-modified bovine lens proteins respectively. Aliquots equivalent to 2.0 g of digested protein were subjected to size-exclusion chromatography on a Bio-Gel P-2 column. Six peaks were obtained for both preparations and pooled. Side by side thin-layer chromatography (TLC) of each peak showed very similar R_f values for the long wavelength-absorbing fluorophores. Glycation with [U - ^{14}C]ascorbic acid, followed by digestion and Bio-Gel P-2 chromatography, showed that the incorporated radioactivity co-eluted with the A_{330} -absorbing peaks, and that most of the fluorescent bands were labeled after TLC. Peaks 2 and 3 from the P-2 were further fractionated by preparative Prodigy C-18 reversed-phase high-performance liquid chromatography. Two major A_{330} -absorbing peaks were seen in peak 2 isolated from human cataract lenses and 5 peaks in fraction 3, all of which eluted at the same retention times as those from ascorbic acid glycated calf lens proteins. HPLC fractionation of P-2 peaks 4, 5 and 6 showed many A_{330} -absorbing peaks from the cataract WISS, only some of which were identical to the ascorbylated proteins. The major fluorophores, however, were present in both preparations. These data provide new evidence to support the hypothesis that the yellow chromophores in brunescant lenses represent advanced glycation endproducts (AGEs) probably due to ascorbic acid glycation *in vivo*. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Human lens; Brunescant cataract; Ascorbic acid; Advanced glycation endproduct; Maillard reaction; Yellow chromophore; Fluorescence; Aging

Abbreviations: AGE, advanced glycation endproduct; DTPA, diethylenetriaminepentaacetic acid; HFBA, heptafluorobutyric acid; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; WI, water-insoluble fraction; WISS, water-insoluble sonicate supernatant; WS, water soluble fraction

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1. Introduction

The aging of the human lens is characterized by an increase in water-insoluble (WI) proteins associated with high levels of yellow chromophores and non-tryptophan fluorescence [1–4]. These biochemical changes are all enhanced in senile and brunescant cataractous lenses. The glycation of lens proteins has been suggested to be one of the major protein modifications present in older lenses, and indeed diabetic patients are at higher risk for cataract formation [5]. Due to the possibly life-long stability of the lens crystallins, they can accumulate damaging modifications continuously. In time, these modifications may be responsible for the protein aggregation, protein crosslinking seen in age-onset cataract [6–8]. A dramatic, visible increase in protein-bound chromophores occurs during brunescant cataract formation especially in tropical countries [9,10].

Ascorbic acid is present in the human lens in relatively large amounts (up to 2 mM), which is higher than the normal glucose levels [11,12]. This is potentially significant, because ascorbic acid has been shown to participate in both glycation reactions [13] and the metal-dependent generation of oxygen free radicals *in vitro* [14]. *In vitro* incubation experiments have revealed that the oxidation products of ascorbic acid can rapidly react with lens proteins via a glycation mechanism and to form cross-linked proteins with characteristic browning and fluorescence [15,16]. These changes resemble those occurring in the lens during normal aging and cataract formation. The structures of several advanced glycation end-products (AGEs), such as pentosidine [17,18], carboxymethyllysine [19] and LM-1 (vesperlysine A) [20,21] are known, and have been identified in human lens proteins. Similar compounds can be formed by the reaction of lens proteins with ascorbic acid *in vitro*, however, the exact glycating agent responsible for forming these AGEs *in vivo* still remains largely unknown.

In order to prove a role for ascorbate glycation in lens aging and cataract formation, it will be necessary to isolate the AGEs from ascorbylated proteins, and show that several of these are identical to the yellow, fluorescent compounds isolated from both aged human and brunescant cataract lenses. The identity of the major yellow chromophores bound

to human lens proteins has been pursued by many investigators, but with little success. Several purified compounds have been isolated from human cataract, but these have largely been tryptophan oxidation products [22,23] or filter compounds such as 3-hydroxykynurenine glycoside, which form adducts with proteins during aging [24]. While the presence of AGEs has been demonstrated in human lenses, the quantities, when measured, were very low. Therefore, the major adducts have not as yet been isolated or identified. In this report we describe the extensive enzymatic digestion of human cataract lens WI proteins, and the chromatographic profiles of these digests by size exclusion Bio-Gel chromatography. The components of the various peaks were then compared by reversed-phase high-performance liquid chromatography (RP-HPLC) in order to determine the complexity of this population and to show the similarity of the yellow chromophores in human cataract lens with those produced by ascorbic acid glycation *in vitro*. It is also anticipated that these procedures may provide the basis of a reproducible procedure to isolate the yellow chromophores present in aged human lenses and cataracts for further study.

2. Materials and methods

2.1. Materials

Ascorbic acid (99.6% pure) was obtained from Fisher Chemical Co. (St. Louis, MO). All other reagents were obtained in the highest purity available from Sigma Chemical Co. (St. Louis, MO). L-[U-¹⁴C]ascorbic acid was prepared from D-[U-¹⁴C]glucose by the method reviewed by Crawford and Crawford [25] as described in detail previously [26]. This labeled ascorbic acid was synthesized in crystalline form by Valeri V. Mossine at this university. De-ionized water (18 M Ω resistance or greater) was used for all experiments. Phosphate buffers were treated with 10 g/l Chelex resin (200–400 mesh, Bio-Rad Laboratories, Richmond, CA) to remove trace metal ion contaminants as described by Beyer and Fridovitch [27], and filtered through a 0.2- μ m nitrocellulose filter before use.

Aged normal human lenses were obtained as a kind gift from the Heartland Lions Eye Tissue

Bank following cornea removal. Brunescant cataracts were collected from hospitals in Rajkot, India and stored frozen at the Patney Eye Clinic. After transport by hand to Columbia, MO, they were stored individually frozen at -70°C until use.

2.2. Preparation of lens proteins

Newborn calf lenses (1.0–1.2 g) were obtained from Pel-Freeze Biologicals, Rogers, AR, shipped in dry ice and stored at -70°C . The water-soluble (WS) fraction was prepared from the outer cortex of thawed, decapsulated lenses by removing the outer 1.0 cm of tissue with a cork borer. This tissue was homogenized in deionized water with a hand Dounce homogenizer and the resulting homogenate was centrifuged at $27\,000\times g$ for 20 min. The supernatant was dialyzed extensively against water and used directly for glycation experiments. The protein content of these calf lens protein preparations was determined using the BCA assay as described by the manufacturer (Pierce, Rockford, IL).

Type I and II brunescant lenses (dark yellow to tan in color) were decapsulated, pooled and homogenized in deionized water. The $27\,000\times g$ pellet was obtained and washed three times with an equal volume of deionized water. The final pellet was resuspended once again and sonicated in ice for 5 min at a power setting of 4 and a duty cycle of 40%. The solubilized protein was recovered after centrifugation at $27\,000\times g$, and the pellet was resuspended and sonicated again. The second supernatant, when combined with the first, represented 95% of the total water-insoluble (WI) fraction and was designated the WI sonicate supernatant (WISS) as described previously [28,29].

2.3. Glycation reactions

Dialyzed calf lens cortical proteins (10 mg/ml) were incubated with 20 mM L-ascorbic acid in 0.1 M Chelex-treated phosphate buffer (pH 7.0) containing 1.0 mM DTPA. Each reaction mixture was sterile-filtered through a 0.22- μm nylon syringe filter into a sterile plastic culture flask. Each flask was wrapped in aluminum foil and incubated in the dark at 37°C for 4 weeks. The reaction mixtures were then dia-

lyzed extensively against 5 mM Chelex-treated phosphate buffer, pH 7.0 at a ratio of 1 to 8 (v/v) for several changes.

2.4. Proteolytic digestion of ascorbylated calf lens proteins and cataract lens proteins

Proteolytic digestion was carried out by a modification of the method of Luthra et al. [23]. Solutions containing 10 mg/ml of either pooled cataract WISS fractions or dialyzed 4-week ascorbylated lens proteins were prepared in 5.0 mM Chelex treated phosphate buffer (pH 7.0), and 3.6 mg of porcine intestinal peptidase (Sigma P7500) was added per 100 mg protein. The digestion mixture was then sterile filtered into a sterile tissue culture flask, bubbled with argon, and incubated for 24 h at 37°C in the dark. A solution of Pronase (Sigma P5147) was then added through a syringe filter after 24 h to a level of 3.6 mg Pronase, and digestion was continued for 72 h with two more additions of Pronase after 24 and 48 h, respectively. Following adjustment of the digest to pH 8.5 with 1.0 M NaOH, leucine aminopeptidase (Sigma L 5658) was added to a final concentration of 40 units per 100 mg protein. The digest was sterile filtered, bubbled with argon and incubated for 24 h at 37°C . After adjusting the pH of the digest to 7.6 with 1.0 M HCl, it was sterile filtered again, bubbled with argon, and further proteolysis carried out by the sequential sterile addition of 5.0 mg of trypsin per 100 mg protein (Sigma T8642), 5.0 mg chymotrypsin (Sigma C4149) and finally 3.6 mg Proteinase K (Sigma P6556) per 100 mg protein with 24 h between each addition. Corresponding enzyme blanks incubated without added protein were also incubated and analyzed on several occasions. Amino acid analysis was used to evaluate the efficiency of the enzyme digestion by comparing the total free amino acids in a 12% sulfosalicylic acid supernatant to that of an equivalent aliquot acid hydrolyzed in 6.0 N HCl, for 20 h.

2.5. Bio-Gel P-2 size exclusion chromatography

Digests from either cataract WISS or ascorbylated proteins were concentrated and subjected to gel filtration chromatography on a Bio-Gel P-2 column

(5×76 cm, bed volume 1.5 l), with 25 mM formic acid as eluant. Samples equivalent to 2 g of digested protein (40 ml) were applied to the column and 14.5-ml fractions were collected at a flow rate of 1 ml/min. The collected fractions were monitored for absorbance at 330 nm, for fluorescence at excitation/emission = 350/450 nm, and amino acid content by the fluorescamine assay [30]. An enzymatic digest of calf lens protein (control) was run under the same conditions. Peak fractions were pooled according to the A_{330} readings. Six peaks (designated P1–6) were obtained.

2.6. Thin-layer chromatography (TLC)

Two hundred fluorescence units of each fraction from the P-2 column were subjected to thin-layer chromatography on a preparative silica gel plate (Whatman, Hillsboro, OR, 20×20 cm, 1000 μ m) with ethanol/ammonia (7:3, v/v) as irrigant A for peaks P1, P2 and P3; and with *n*-butanol/methanol/ammonia (7:3:2, v/v/v) as irrigant B for peaks P4, P5 and P6. The fluorophores were detected by irradiation with a Wood's lamp at the long wavelength (360 nm) setting and photographed.

2.7. Incorporation of [U - 14 C]ascorbic acid into calf lens proteins

One hundred mg of dialyzed WS calf lens proteins were incubated with 200 μ mol [U - 14 C]ascorbic acid (1.0 μ Ci/ μ mol) in 10 ml of Chelex-treated 0.1 M phosphate buffer (pH 7.0) containing 1.0 mM DTPA as described in Section 2.3. After a 2-week incubation, the sample was extensively dialyzed against 5.0 mM Chelex-treated phosphate buffer (pH 7.0), digested, concentrated and then subjected to a smaller Bio-Gel P-2 column (2.5×87 cm, bed volume 430 ml) with 25 mM formic acid as eluant. Fractions of 4.2 ml each were collected and a portion of each fraction was measured for absorbance at 330 nm, fluorescence at 350/450 nm and radioactivity by liquid scintillation spectrometry using a Packard model 2100 instrument. Aliquots from each peak were spotted on TLC plates, developed with irrigant A and the pattern of radioactivity in each lane determined with an AMBIS imaging system.

2.8. High-performance liquid chromatography

Fractions P2, P3 and P6 were isolated from the Bio-Gel P-2 column and injected onto a preparative C18 reversed phase preparative column (Prodigy 21.2×250 mm, Phenomenex, Torrance, CA) using an SSI HPLC system (Scientific System, Fisher Scientific, St. Louis, MO). The column was eluted for 2 min with 5% (v/v) acetonitrile in water containing 0.1% (v/v) heptafluorobutyric acid (HFBA) at a flow rate of 10 ml/min, and then with a linear gradient from 5% to 45% (v/v) acetonitrile in 0.1% HFBA over 40 min, followed by a linear increase to 100% acetonitrile over the next 5 min. The eluant from the column was monitored with an on-line fluorescence detector at 450 nm (excitation at 350 nm) and a dual-wavelength absorption detector set at 280 nm and 330 nm.

Fractions P4 and P5 were similarly isolated from the Bio-Gel P-2 column and further fractionated on a semi-preparative reversed-phase Prodigy column (10×250 mm) with the same solvents. The column was eluted for 2 min with 5% acetonitrile in water and 0.1% heptafluorobutyric acid (HFBA) at a flow rate of 1.8 ml/min, and then with a linear gradient from 5% to 25% acetonitrile in 0.1% HFBA over 20 min. The column was eluted isocratically with 25% acetonitrile in 0.1% HFBA, for 15 min, followed by a linear increase to 100% acetonitrile over the next 20 min. The eluant from the column was monitored with an on-line fluorescence detector at 450 nm (excitation at 350 nm) and a dual-wavelength absorption detector at 280 nm and 330 nm.

Table 1

Total yellow color (A_{330}) of several lens protein preparations following proteolytic digestion

Preparation	<i>n</i>	A_{330} /g protein
Indian cataract WISS (type I and II)	5	130 ± 13
Aged human lens WISS	4	90 ± 20
4-Week ascorbate-modified CLP	3	250 ± 66
4-Week incubated CLP w/o ascorbate	3	24 ± 13
Enzymes alone ^a	1	11

^aEnzymes equivalent to those used to digest 100 mg protein were added in sequence to buffer alone and the final A_{330} multiplied by 10.

3. Results

3.1. Lens protein digests

The washed WI fraction from Indian cataracts and from aged normal eye bank lenses was solubilized by sonication in water and digested with a battery of proteolytic enzymes. An aliquot of each digest was taken to determine the total yellow color present in these fractions. These data are expressed as the absorbance at 330 nm/g of lens protein and shown in Table 1. Approximately 2 g of protein was proteolyzed in each preparation. The Indian cataracts (type I and II) were a mixture of dark yellow and tan-colored lenses, and these preparations contained 44% more A_{330} -absorbing compounds than the aged normal lenses. Calf lens proteins ascorbylated for 4 weeks under air gave variable results, but the A_{330} absorbance/g protein was almost twice as high as that for the Indian cataract proteins. Similar incubations in the absence of ascorbic acid did exhibit some yellow color, but was only 10% of the preparations with ascorbic acid. Most of this color development occurred during the concentration step after the digestion was complete. Blank digestions with no lens proteins were also carried out, but very little A_{330} was contributed by the enzymes alone.

Samples of the digest were submitted for amino acid analysis directly after precipitating any remaining proteins or large peptides with 12% sulfosalicylic acid. A similar aliquot was hydrolyzed with 6.0 N HCl to determine the total amino acid content of each preparation. These data, when corrected for the values obtained with enzymes alone showed that the proteolytic procedure hydrolyzed the proteins at least 90% to the level of free amino acids.

3.2. Bio-Gel P-2 chromatography of the lens protein digests

Fig. 1 shows the Bio-Gel P-2 elution profile of the proteolytic digest of a water-insoluble sonicate supernatant fraction from Indian cataract (Fig. 1A), and for comparison, a digest of calf lens proteins modified for 4 weeks with ascorbic acid (Fig. 1B). The elution profiles for the absorbance at 330 nm, the fluorescence at 350 nm excitation/450 nm emission and the amino groups by the fluorescamine assay

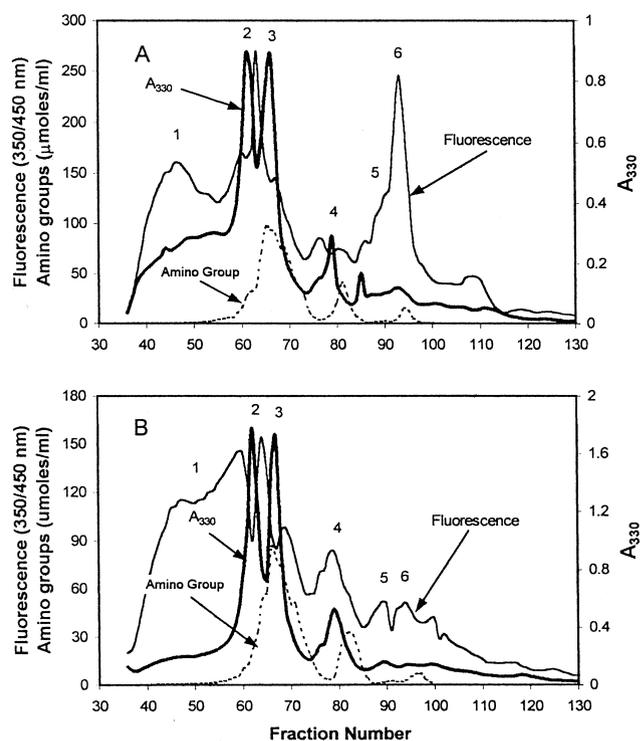


Fig. 1. Bio-Gel P-2 elution profiles of the proteolytic digest of a water-insoluble sonicate supernatant (WISS) fraction from Indian cataract (A) and a 4-week ascorbic acid modified calf lens protein preparation (B). The profiles for absorbance at 330 nm, 350/450 nm fluorescence and amino groups by the fluorescamine assay [30] are labeled in each panel. The numbers shown identify the peaks which were pooled based upon 330 nm absorbance.

are labeled in each panel. The numbers shown above the peaks identify those peaks, which were pooled based upon 330 nm absorbance. A similar elution profile was seen for both preparations in the early part of the chromatogram. Later peaks were less distinct at 330 nm, but the cataract digest contained a major late-eluting fluorescence peak, which was not present in the ascorbate-modified proteins. The fluorescence profile showed a sharp peak between peaks 2 and 3. When similar fractions were diluted 10-fold, however, a broader peak of fluorescence was seen. Apparently the high level of absorbance at 350 nm in peaks 2 and 3 diminished the light able to excite the fluorophores by a filtering effect on both sides of the fluorescent peak.

Peak 1 contained all of the material eluting before the first major peak. This peak is likely composed of mainly incompletely-digested peptides due to their

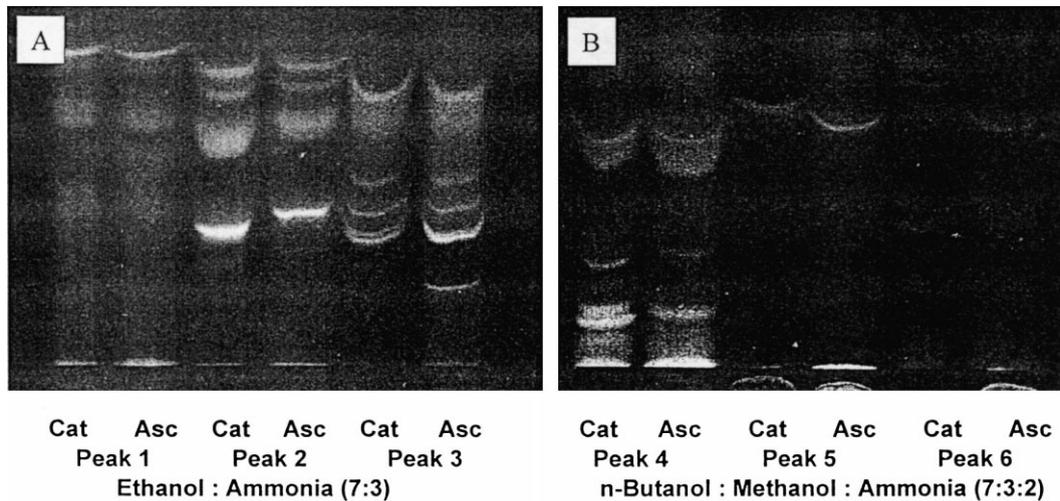


Fig. 2. TLC comparison of the fluorophores present in peaks P1–P6 isolated by Bio-Gel P-2 chromatography. Aliquots equivalent to 200 units of fluorescence (350/450 nm) were subjected to preparative TLC separation. (A) Comparison of the fluorophores present in peaks P1, P2 and P3. Aliquots were spotted side by side and the plate was developed with irrigant A. (B) Preparative TLC separation of peaks P4, P5 and P6 with irrigant B. The fluorophores were detected by irradiation with a Wood's lamp at the long wavelength setting.

elution position, and because amino acid analysis of this peak showed only 14% free amino acids. Peak 2 eluted slightly ahead of the main amino acid peak. It could contain dipeptides or crosslinks, but only 40% of the peak was present as free amino acids. Peak 3 co-eluted with the amino acid peak, while peaks 4 (fractions 72–85), 5 (fractions 86–91) and peak 6 (fractions 92–100) likely represented hydrophobically modified amino acids, which bound to the column matrix during elution. Amino acid analysis of all of these peaks gave values of greater than 80% free amino acids. Amino acid analysis also showed that the hydrophobic amino acids Phe, Tyr and Trp eluted in fractions 78–84, 93–97 and 110–114, respectively.

3.3. TLC separation of the fluorophores present in the fractions from the Bio-Gel P-2 column

The peaks showed in Fig. 1 were pooled, concentrated and the aliquots equivalent to 200 units of fluorescence (350/450 nm) were subjected to preparative TLC separation. Fig. 2A shows a comparison of the fluorophores present in peaks 1, 2 and 3. Aliquots were spotted side by side and the plate was developed with irrigant A. Fig. 2B shows the preparative TLC separation of peaks 4, 5 and 6. In this case the plate was developed with irrigant B. The

fluorophores were detected by irradiation with a Wood's lamp at the long wavelength (360 nm) setting and photographed. The fluorophores in all six peaks were almost identical for the Indian cataract and the 4-week ascorbic acid modified proteins. As expected, peak 1 contained a smear of fluorescence throughout the lane with only one prominent band. Peaks 2, 3 and 4 gave complex patterns with the majority of the peaks co-migrating. Peak 5 had only one major fluorescent band, and peak 6 contained no obviously similar fluorophores. The faster-moving bands at the top of the plate with peak 6 from the cataract preparation are likely responsible for the higher fluorescence seen only in the cataract digests. By and large, however, ascorbate glycation appears to produce the same fluorophores as those seen in the cataract digest. Similar results were seen for five different cataract digests and three different digests of ascorbylated lens proteins. Similar bands were also present in the aged normal lens digests (data not shown).

3.4. Incorporation of [U - ^{14}C]ascorbic acid into lens proteins

The data in Figs. 1 and 2 argue that ascorbate glycation produces modified amino acids, which are very similar to those present in cataract WI proteins.

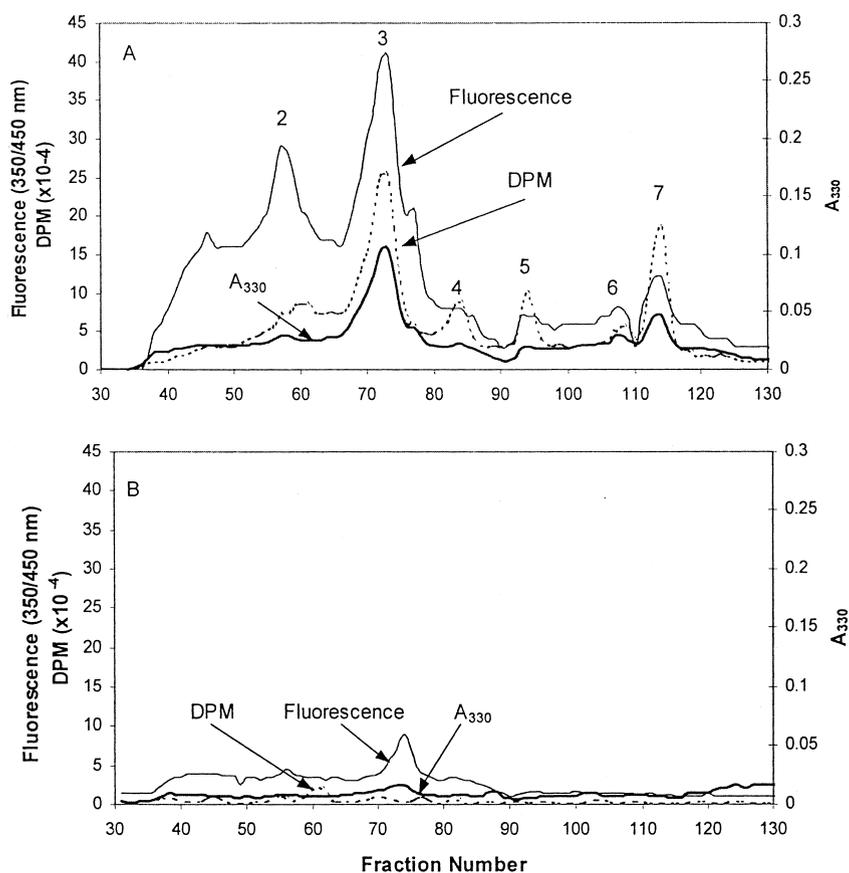


Fig. 3. Incorporation of [$U\text{-}^{14}\text{C}$]ascorbic acid to calf lens protein. The incorporation reaction was carried out as described in detail in Section 2. Digested protein was subjected to a small Bio-Gel P-2 gel filtration chromatography (2.5×87 cm). (A,B) Profiles of the proteins incubated with and without ascorbic acid. The profiles for absorbance at 330 nm absorbance, 350/450 fluorescence and radioactivity (DPM) are labeled.

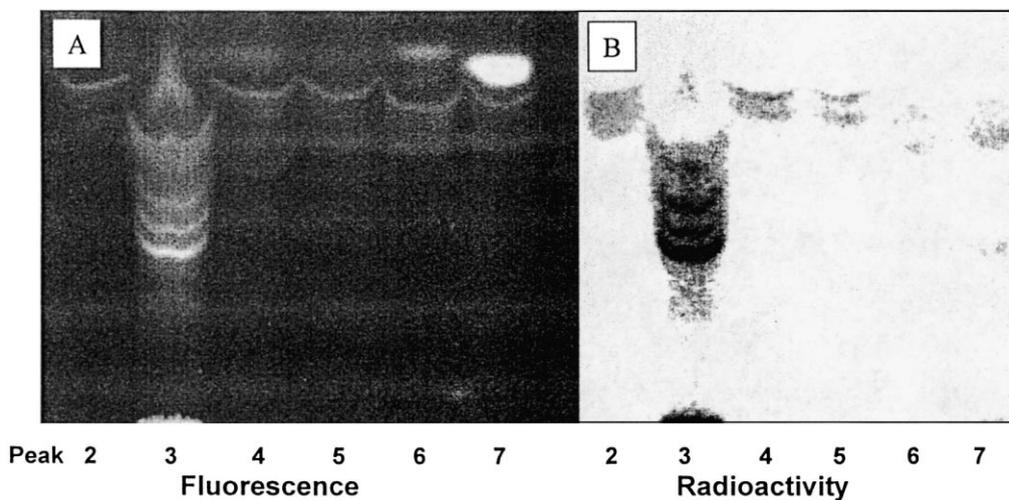


Fig. 4. The TLC profiles of labeled peaks isolated from [$U\text{-}^{14}\text{C}$]ascorbic acid-modified calf lens protein obtained from the Bio-Gel P-2 chromatography shown in Fig. 3A. Peaks 2–7 were spotted on a preparative TLC plate and developed with irrigant A. The fluorescence in each lane was detected by irradiation with a Wood's lamp at 360 nm and the pattern of radioactivity was determined by scanning the plate with an AMBIS imaging system.

However, it remained possible that the length of the glycation reaction and the digestion could have led to a direct modification or oxidation of the Trp residues in the protein. This could lead to the formation of yellow fluorophores by mechanisms other than the formation of AGEs on lysine residues in the protein. To determine whether ascorbic acid incorporation is responsible for the yellow fluorophores, calf lens proteins (10 mg/ml) were incubated with 20 mM [U- 14 C]-ascorbic acid for 2 weeks, digested and subjected to a Bio-Gel P-2 gel filtration chromatography. Radioactivity measurements after dialysis gave a value of 0.03 μ Ci incorporated per mg protein. This preparation was compared to lens proteins incubated for 2 weeks in the absence of ascorbic acid. Fig. 3 shows the profiles of the proteins incubated with (Fig. 3A) and without (Fig. 3B) [U- 14 C]ascorbic acid. The 330 nm absorbance, 350/450 fluorescence and radioactivity of each fraction (4.2 ml/fraction) were measured, and plotted. The smaller column did not resolve peaks 2 and 3, but otherwise the profile of the labeled digest was similar to that in Fig. 1. The major

fluorophores eluted as an early peak at tube 55, a peak which co-eluted with the major amino acid peak at tube 72 and several smaller peaks later in the chromatogram. The incorporated radioactivity was largely present in peak 3, and mirrored the A_{330} profile. The control digest without ascorbic acid displayed some yellow color after concentration, but no major peaks were seen. The digest of the control sample of calf lens protein incubated without [U- 14 C]ascorbic acid was fractionated immediately after the [U- 14 C]ascorbic acid modified sample on the same column. Fig. 3B shows that no radioactivity remained on the column to contaminate the next run, and little or no A_{330} or fluorescence was present in the control proteins. A 3-fold increase in A_{280} was seen following glycation, which eluted with peak 3 (data not shown). Since all of the hydrophobic amino acids eluted later from the column, the absorbance in peak 3 was likely also due to the AGEs formed.

To confirm that the fluorophores formed were due to ascorbate modification, each peak from Fig. 3A

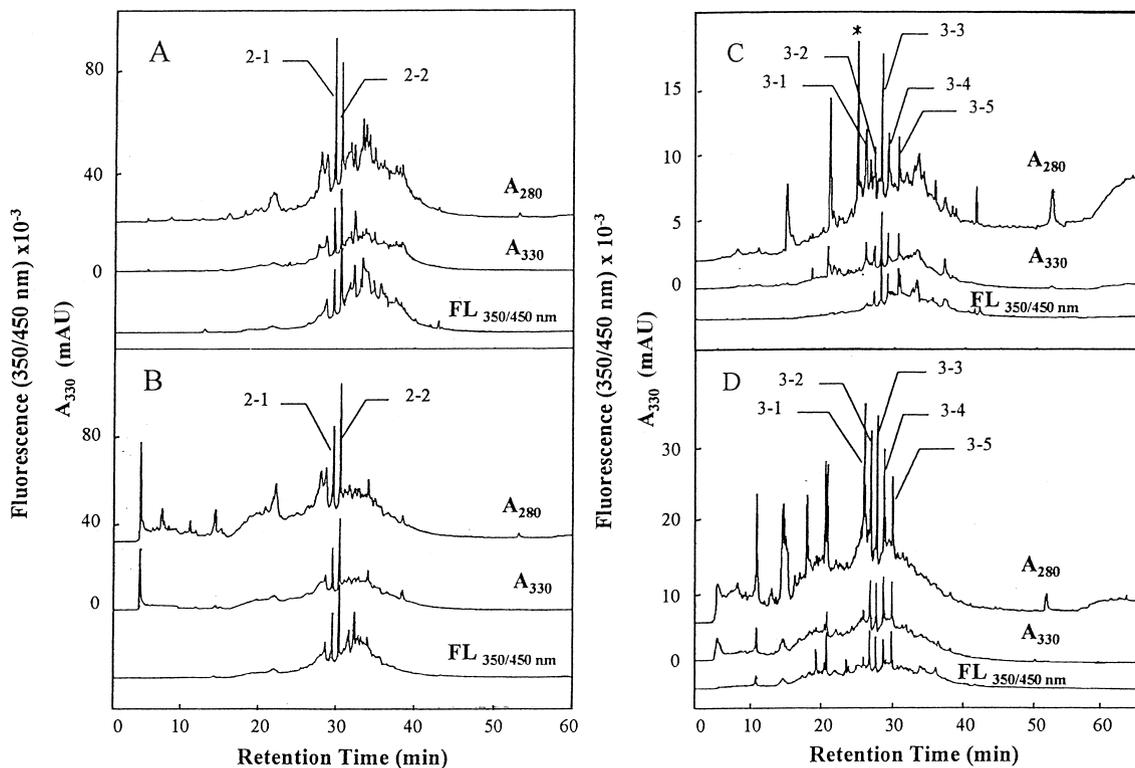


Fig. 5. Reversed-phase HPLC profiles for peaks P2 and P3 from the Bio-Gel P-2 column shown in Fig. 1. Peaks P2 and P3 from both Indian cataract (A,C) and ascorbic acid modified calf lens protein (B,D) are shown. The 280 and 330 nm absorbance, as well as fluorescence at 350/450 nm, were monitored and labeled in each panel.

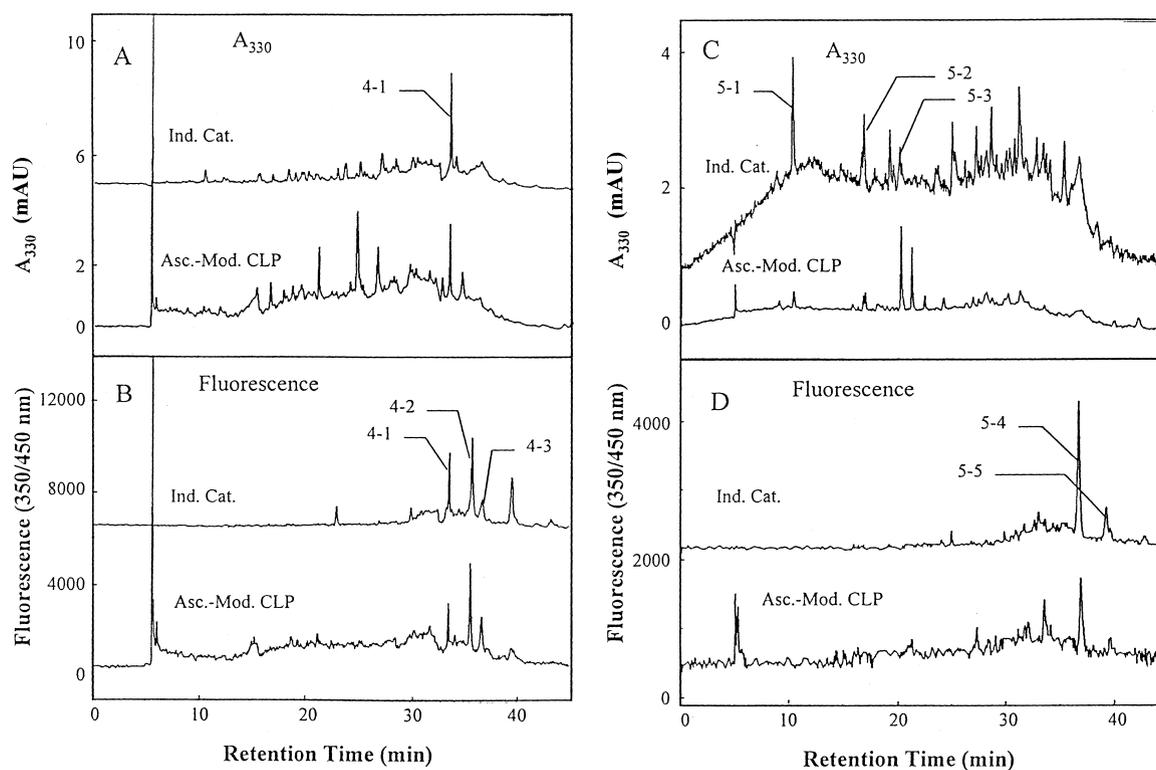


Fig. 6. Reversed-phase HPLC profiles for peaks P4 and P5. Peaks P4 and P5 from both Indian cataract and ascorbic acid modified calf lens protein were further fractionated by semi-preparative RP-HPLC. The 330 nm absorption (A,C) and the fluorescence at 350/450 nm (B,D) for both Indian cataract and ascorbic acid modified calf lens proteins were monitored and are directly compared in each panel.

was pooled, concentrated and spotted on a preparative TLC plate (Fig. 4). Following development with irrigant A, the fluorescent bands were located by long wavelength illumination and photographed. The TLC plate was then scanned to locate the radioactive bands. While the separation of some samples was less than that seen in Fig. 2, it is clear that almost all of the fluorescent bands contained radioactivity. We conclude that most of the fluorophores, especially those in peak 3, were derived from the incorporation of ascorbic acid during the glycation reaction.

3.5. Reversed-phase HPLC profiles of peaks P2 and P3

The fractions isolated from the Bio-Gel P-2 column were subjected to further purification by C-18 reversed phase HPLC. Fig. 5 shows the HPLC profiles for peaks, P2 and P3 from both Indian cataract (Fig. 5A,C) and ascorbic acid modified calf lens pro-

tein Fig. 5B,D). The absorbance profiles at 280 nm and 330 nm, as well as fluorescence at 350/450 nm, were monitored. As shown in Fig. 5A,B, the two main A_{330} and fluorophore peaks, which eluted around 30 min (2-1 and 2-2), were identical for the Indian cataract and the 4-week ascorbic acid modified proteins. A doublet A_{280} -absorbing peak at 28 min was also present in both profiles. Peaks 2-1 and 2-2 both exhibited 350 nm/450 nm fluorescence, but there were several fluorescent peaks that eluted after the main A_{330} peaks in both digests, with the peaks being slightly more prominent in the Indian cataract sample.

Similarly, peak P3 separated into a cluster of five prominent A_{330} peaks as shown in Fig. 5C (3-1 to 3-5), which had very similar counterparts in the profile of peak P3 from the ascorbate-modified lens proteins (Fig. 5D). Two significant A_{330} peaks eluted early in the Indian cataract profile, however, which had no apparent counterpart the ascorbylated sample. The A_{280} peak marked by an asterisk was absent in

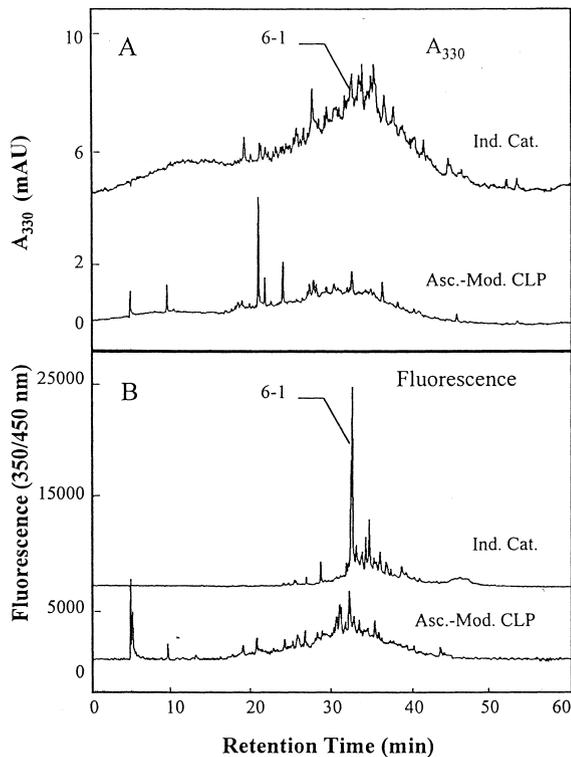


Fig. 7. Reversed-phase HPLC profile for peak P6. Peak P6 from both Indian cataract and ascorbic acid modified calf lens protein were further fractionated by preparative RP-HPLC. The 330 nm absorption (A) and fluorescence at 350/450 nm (B) for both Indian cataract and ascorbic acid-modified calf lens proteins are directly compared in each panel.

peak 3 from the ascorbylated protein digest. For the most part, however, the fluorescence co-eluted with the A_{330} profile. The scale on the fluorescence readings was greatly diminished to make the profile visible below the others. Samples of P2 and P3 from aged normal human lenses gave very similar profiles to those in Fig. 5 (data not shown).

3.6. Reversed-phase HPLC profiles of peaks P4, P5 and 6

The later-eluting peaks were also pooled and subjected to RP-HPLC separation. The A_{330} and fluorescence profiles are directly compared in each panel and are shown for peaks 4 and 5 in Fig. 6 and for peak 6 in Fig. 7. The A_{280} profiles were determined, but not shown because they were dominated by the absorbance of the aromatic amino acids which elute in this area of the P-2 chromatogram. The A_{330} profile in Fig. 6A shows several minor peaks eluting

between 20 and 30 min, which are similar to peaks in the Fig. 6B, but in lesser quantity. The major peak at 33 min was present in both digests. The fluorophores in peak P4 (shown in Fig. 6B) eluted later than the A_{330} peaks, but appear to be almost identical in both digests. Peak P5 (Fig. 6C) showed numerous peaks at 330 nm in the cataract sample, a few of which (5-1, 5-2 and 5-3) may also be present in the ascorbylated protein digest. Peak 5-4, while prominent in the ascorbate-modified CLP digest, was absent in the cataract digest. Fluorescent peaks 5-4 and 5-5 were present in both digests as shown in Fig. 6D. Again, the fluorescence did not correspond exactly to a significant A_{330} peak in Fig. 6C. Peak 6 from the cataract digest contained numerous small A_{330} peaks (Fig. 7). No peak could be clearly ascribed to a companion peak in the ascorbate-modified protein digest. A significant fluorescence peak was present in the cataract digest (6-1), which appears to be similar to a small peak in the digest of the ascorbate-modified proteins. Clearly, there were numerous A_{330} peaks in peaks P-4 and P-5 from the cataract digest that were not present in the ascorbate-glycated digest.

4. Discussion

Aging in the human lens is characterized by the accumulation of protein-bound yellow chromophores and fluorophores, associated with an increase in protein aggregation and protein crosslinking. The significant challenge remains, however, to determine the nature of these protein modifications, the mechanisms that give rise to these modifications, and the relationship between the browning reactions and the extensive protein crosslinking seen in age-onset cataracts. The browning products are capable of absorbing UVA light and producing reactive oxygen species (ROS), which may be the cause of the mixed disulfides and disulfide crosslinks universally seen in age-onset cataracts [31,32] and the low levels of hydroxylated amino acids in brunescant cataracts [33]. Several of these chromophores have been isolated, and their structures determined. These represent two types of compounds, either Trp oxidation products [22,34,35] or advanced glycation endproducts [17–21].

Prior results from our laboratory have argued for

a major role for ascorbic acid oxidation products in AGE formation in vivo. Ascorbic acid, in vitro, glycosylated lens proteins 9-fold, and crosslinked proteins 90-fold more rapidly than either glucose or fructose [26]. Lens proteins incubated with ascorbic acid for 4 weeks exhibited the same absorption and fluorescence spectra as the proteins present in a human lens WI fraction [36]. Ascorbylation also caused the formation of protein-bound sensitizers, which generate the same levels of reactive oxygen species (ROS) in response to UVA light as aged human lens water-insoluble proteins [37,38]. These observations encouraged us to investigate the breadth of the link between ascorbic acid-induced advanced glycation endproducts (AGEs) and the chromophores and fluorophores present in cataract lens proteins by gathering chromatographic information on compounds isolated from both sources. The data presented here provide evidence to support the view that the bulk of the yellow chromophores in human cataracts do arise by ascorbate glycation.

Since AGEs are widely thought to be acid labile, and because acid hydrolysis produces a myriad of yellow fluorescent Trp degradation products, we chose to use the enzymatic hydrolysis method of Luthra et al., [23] to release the modified amino acids from the lens proteins. Several enzymes were added to the procedure to increase the degradation of small peptides. The added enzymes increased the overall proteolysis by only 5–10%, but increased the number of modified amino acids liberated significantly and allowed fractionation of the digests into discrete peaks on the Bio-Gel P2 column. This procedure resulted in 75% release of free amino acids overall, but when corrected for the contribution of the enzymes, an 85% release of amino acids from the digested proteins was obtained. Further, an increase of 8% was seen by acid hydrolysis due to the conversion of Asn and Gln to Asp and Glu. Therefore, a 93% proteolysis to free amino acids was obtained for both the cataract and ascorbylated proteins. Oxidative degradation of constitutive amino acids was kept to a minimum by the presence of ascorbic acid throughout the glycation reactions, and by carrying out the proteolytic digestions under argon and in the dark at all times. Bio-Gel P-2 chromatography was chosen as the initial step to remove undigested peptides, and because it allowed the processing of gram amounts

of digest on a single column. Yellow absorbance was monitored at 330 nm because it was the highest non-Trp absorbance present, and because prior work had shown that UVA light created the greatest damage at this wavelength [13]. Fluorescence was measured at 350 nm excitation and 450 nm emission, as these wavelengths spanned the several fluorophores reported for aged human lens proteins in the literature [1–4] and because these were the fluorescence maxima of both the human WISS fraction and lens proteins ascorbylated in vitro [37].

The fractionation of the lens protein digests by P-2 chromatography shown in Fig. 1 resolved the lens yellow chromophores into six A_{330} -absorbing peaks. The bulk of the absorbance, however, was present in two main peaks eluting either slightly before (peak 2) or coincident with the free amino acid peak (peak 3). Little or no difference was seen between the cataract and ascorbylated proteins, except for a large fluorescent peak (peak 6), which was only present in the cataract protein digest. Side-by-side TLC separation of the fluorophores present in each Bio-Gel P-2 peak, showed a remarkable similarity between the two preparations, except that the cataract proteins had a higher overall fluorescence than the ascorbic acid-modified proteins, while the ascorbylated proteins had a 2-fold higher absorbance at 330 nm. The P-2 profile shown is typical of the five different preparations of cataract water-insoluble (WI) proteins, and the ascorbic acid modified profile is representative of three different preparations. Aged human lens proteins gave similar results to the cataract digest shown. When dehydroascorbic acid was substituted for ascorbate, and the glycation reactions carried out for 2 weeks in the absence of oxygen, a similar profile was seen (data not shown). Further, the P-2 column was eluted under acidic conditions to prevent further reaction of any AGE products, and all peaks were stored in concentrated form under argon in the cold.

The lens proteins glycosylated with [U - ^{14}C]ascorbic acid showed extensive incorporation of radioactivity, whereas previous incubations with [1 - ^{14}C]ascorbate gave little or no incorporation, as carbon 1 is readily lost during oxidative degradation [15]. Recent in vitro evidence argues that the true glycosylating species is L-erythrulose [39], which along with oxalic acid are the only degradation products of ascorbate under the

reducing conditions which exist in the lens. P-2 chromatography of the ascorbylated proteins after extensive dialysis and digestion demonstrated that the ascorbate was incorporated into the protein and co-eluted with the A_{330} absorbing peaks. Furthermore, the fluorophores displayed by TLC were labeled when scanned for radioactivity. These data argue that the yellow chromophores arose by ascorbate incorporation, as opposed to oxidation of amino acids present in the proteins. Controls where ascorbate was omitted showed little or no absorbance peaks.

To allow greater discrimination of the A_{330} -absorbing chromophores in the peaks eluted from the P-2 column, each peak was further separated by RP-HPLC. The Prodigy HPLC profiles in Fig. 5 showed that the major A_{330} peaks present in peak 2 and peak 3 from the Indian cataract eluted at the same retention times as those from the 4-week ascorbic acid modified calf lens proteins. All of these peaks also exhibited absorbance at 280 nm, which is likely due to the AGEs, as calf lens proteins incubated without ascorbate exhibited 3-fold less A_{280} in either peak 2 or 3 from the P-2 column. Peaks P4-P6 also exhibited common major peaks, however, a wide array of minor peaks did not obviously correspond. These data argue that the ascorbic acid glycation may be a significant source of some, but not all, of the yellow chromophores and fluorophores present in aging human lenses and in early brunescant cataracts.

Recently, considerable evidence has been published supporting the binding of filter compounds (e.g., 3-hydroxykynurenine glycoside) to lens proteins as having a major role in the formation of the chromophores and fluorophores present in aged human lenses and cataracts [24,40,41]. While these peaks are surely present in our chromatograms, they were not the major compounds produced by our sample work-up. The propensity of these adducts to dissociate at neutral or slightly basic conditions [41] may mean that they would not have survived our digestion procedure, which includes an incubation at pH 8.5 for 24 h. Also, filter compound adducts, when excited at 380 nm, exhibited emission peaks at roughly 440, 480 and 530 nm [41], but little or no 350/450 fluorescence. Therefore these adducts may not have been detected, or were greatly diminished in our profiles. A major 350/450 fluorescent peak does exist in aged human lenses, and the 330–350 nm range is the ma-

ajor absorbing region shown by Gaillard et al. [42] in sections of aged human lens. The evidence presented here argues that these chromophores and fluorophores likely arise by ascorbic acid glycation. It must be pointed out, however, that we do not know whether other reactive sugars could produce the same display of AGE peaks. The low reactivity of glucose and fructose, however, argue that these sugars are not significant contributors to the AGEs in the lens [26,43].

These results represent only an initial step in the isolation and purification of the yellow chromophores and fluorophores present in either the cataract WISS proteins or the 4-week ascorbic acid modified proteins; however, neither mixture had been successfully fractionated before. Hopefully this work can provide general methods, which can be employed in the future, to isolate individual modified amino acids in sufficient quantities from both sources to allow proof of structural identity. Also, this methodology can be employed to see how these chromophore peaks develop with aging in vivo and during glycation reactions in vitro. This represents the first work showing an extensive chromatographic comparison between AGEs present in aged and pathological tissue with AGEs produced by ascorbic acid glycation in vitro.

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