Identification of 3-Hydroxykynurenine Bound to Proteins in the Human Lens. A Possible Role in Age-Related Nuclear Cataract†

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ABSTRACT: Age-related nuclear (ARN) cataract is a major cause of world blindness. With the onset of ARN cataract, the normally transparent and colorless lens becomes opaque and can take on colors ranging from orange, brown, and even black. The molecular basis for this remarkable transformation is unknown. ARN cataract is also characterized by extensive oxidation, insolubilization, and cross-linking of polypeptides, particularly in the nucleus of the lens. It has been postulated that 3-hydroxykynurenine (3OHKyn) may be involved in these changes. This endogenous tryptophan metabolite is readily oxidized and is involved in the tanning of moth cocoons and the formation of pigments in the eyes of butterflies. 3OHKyn is a component of our primate-specific UV-filter pathway, and the brownish hue of ARN cataract lenses is also unique to humans. Because numerous colored compounds can be produced by autoxidation of 3OHKyn, this process could provide an explanation for the variety of lens colors and other changes seen in ARN cataract. For such a theory to be tenable, it needs to be demonstrated that 3OHKyn is bound to proteins in the human lens. Here, we show that all normal lenses older than 50 have 3OHKyn covalently attached to the nuclear proteins, most likely via cysteine residues. If indeed 3OHKyn is implicated in ARN cataract, a reduction in the levels that are bound in cataract, compared to normal lenses, would be expected. In agreement with this hypothesis, no bound 3OHKyn could be detected in proteins isolated from ARN cataract lenses.

The function of the lens is to transmit, filter, and focus light upon the retina. The high refractive index of the lens is due to the high concentration of structural proteins: α, *β*, and *γ* crystallins (*1*). In addition to transmitting most visible wavelengths, the human lens absorbs UV light in the 300−400 nm region because of the presence of several tryptophan-derived UV-filter compounds (*2*, *3*). These compounds prevent UV-induced photodamage to the retina (*4*) and also aid in visual acuity.

Primate UV filters are kynurenine derivatives, each with a peak absorption centered at about 365 nm. 3-Hydroxykynurenine *O*-*β*-D-glucoside (3OHKynG)[1] is the most abundant in the human lens, followed by 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-*β*-D-glucoside, kynurenine (Kyn), and 3-hydroxykynurenine (3OHKyn) (*2*, *3*, *5*−*7*) (see Figure 1). The content of free UV filters in the lens decreases with age in a linear manner (*8*); however, human lenses become more fluorescent with age because of post-translational modification (PTM) of the structural proteins. Although a number of compounds have been reported as adducts to human lens proteins (*9*−*12*), it is not known which of these is primarily responsible for the increased fluorescence. Human lenses also become increasingly yellow with age; however, it is the unique color of ARN cataract lenses that has puzzled researchers for many years.

In this study, we examined 3OHKyn and its reactivity with proteins. 3OHKyn is a neurotoxic (*13*) *o*-aminophenol that has been implicated in several disease states, for example, Huntington's and Parkinson's disease (*14*−*16*). At neutral pH, kynurenines undergo deamination of the amino acid side chain to produce intermediate compounds (e.g., see Figure 1) that are susceptible to nucleophilic attack via Michael addition. Amino acid residues such as cysteine (Cys), histidine (His), and lysine (Lys) are known to be the most reactive (*17*−*19*). In recent research, it has been found that the spontaneously unstable UV filters, 3OHKynG and Kyn, become covalently attached to proteins in the human lens in an age-dependent manner (*20*, *21*). This is especially true after the lens barrier forms at middle age (*22*−*24*), when UV filters reside for longer time periods within the nuclear region of the lens and are able to deaminate to a greater extent.

3OHKyn differs in one very important respect from the other Kyn UV filters, in that the aromatic portion of 3OHKyn can readily oxidize (*25*, *26*). In model systems, numerous

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[1] Abbreviations: ARN, age-related nuclear; CLP, calf lens protein; Cys, cysteine; ESI, electrospray ionization; Em, emission; Ex, excitation; GSH, glutathione; guanidine HCl, guanidine hydrochloride; His, histidine; HCl, hydrochloric acid; 3OHKyn, 3-hydroxykynurenine; 3OHKynG, 3-hydroxykynurenine *O*-*β*-D-glucoside; Kyn, kynurenine; Lys, lysine; PTM, post-translational modification; RP-HPLC, reversed-phase high-performance liquid chromatography; MS/MS, tandem mass spectrometry; TFA, trifluoroacetic acid.

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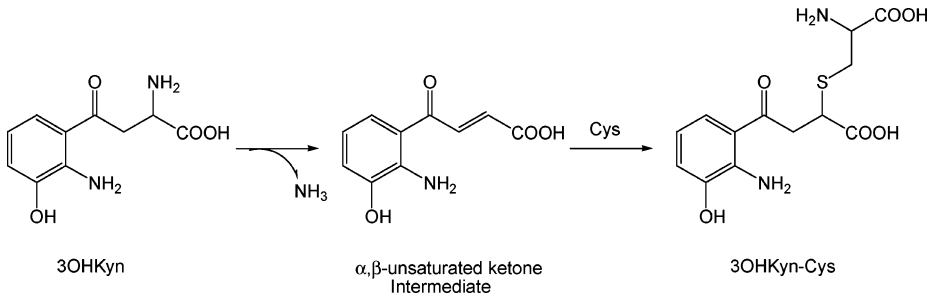

FIGURE 1: Formation of 3OHKyn-Cys adduct via spontaneous deamination of the amino acid side chain of 3OHKyn. His and Lys adducts can be formed in a similar manner.

dimeric and other colored products result from the oxidation of 3OHKyn under physiological conditions (*26*, *27*). If such processes also occur after 3OHKyn has become linked to proteins, colored, insoluble, and cross-linked proteins could result. It has been proposed that this could be a contributing factor in the development of human age-related nuclear (ARN) cataract (*28*−*32*), but proof has been lacking largely because of the problems associated with analyzing the low levels of such a readily oxidized molecule. ARN cataract is characterized by massive oxidation, coupled with extensive coloration, cross-linking, and insolubilization of lens proteins (*33*−*37*).

In the current study, we investigated whether 3OHKyn could bind to proteins under conditions analogous to those in the normal human lens. If so, we could reasonably expect to observe 3OHKyn bound to proteins from older normal human lenses and, further, that these modified proteins may retain the *o*-aminophenol moieties in a state that could be oxidized if conditions within the lens became oxidative, such as in ARN cataract.

As a first step in testing this theory, adducts between 3OHKyn and nucleophilic amino acids were prepared and characterized and the binding of 3OHKyn to proteins was investigated. Using data obtained from stability studies of the synthetic adducts, a novel assay was developed that allowed quantification of the levels of 3OHKyn attached to proteins from both normal and cataract lenses.

EXPERIMENTAL PROCEDURES

Materials. All organic solvents and acids were HPLC-grade (Ajax, Auburn, NSW, Australia). Milli-Q water (purified to 18.2 MΩ/cm^2) was used in the preparation of all solutions. The amino acids (*N*-α-*t*-Boc-L-His, *N*-α-*t*-Boc-L-Lys, and Cys), Hydrochloric acid (HCl), 3OHKyn, thioglycolic acid, phenol, reduced glutathione (GSH), trifluoroacetic acid (TFA), ethylenediaminetetraacetic acid, phenylmethylsulfonyl fluoride, 1,4-dithiothreitol, sodium azide, guanidine hydrochloride (guanidine HCl), and Tris-HCl were obtained from Sigma−Aldrich Chemical Co. (St. Louis, MO).

HPLC. Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on an ICI HPLC system (ICI Instruments, Australia). For analytical scale separation of the 3OHKyn amino acid adducts, a Phenomenex column (Jupiter C18, 300 Å, 5 μm, 4.6 × 250 mm) was used with the following mobile phase conditions: solvent A [aqueous 0.05% (v/v) TFA] for 5 min followed by a linear gradient of 0−50% solvent B [80% (v/v) acetonitrile/H_2O and 0.05% (v/v) TFA] over 20 min, followed by a linear gradient of 50−100% B over 15 min and re-equilibration in the aqueous phase for 15 min. The flow rate was 1 mL/min. Semi-preparative separations were performed using the same conditions as those for the analytical separations, except that a Hypersil (BDS, C18, 5 μm, 10 × 250 mm) column was used at a flow rate of 3 mL/min.

Stability studies and protein analyses were performed on a Shimadzu HPLC system containing a SCL-10A VP system controller, a LC-10AT VP pump, SIL-10AD VP auto-injector with a 50 μL loop, and a SPD-M10AVP diode array detector. The above gradient and the same Phenomenex column were used; however, the flow was 0.5 mL/min, and the concentration of TFA was 0.1%.

Mass Spectrometry. Electrospray ionization (ESI) mass spectra were acquired on a Quadrupole Time-of-Flight Q-TOF2 mass spectrometer (Micromass, Manchester, U.K.) in positive-ion mode. Samples were dissolved in 50% (v/v) aqueous acetonitrile and 0.2% (v/v) formic acid. The mass spectrometer was calibrated with (Glu)-Fibrinopeptide B (0.5 pmol/μL).

Tandem Mass Spectrometry (MS/MS). Tandem mass spectra were acquired using the same conditions as above. The collision energy ranged from 20 to 30 eV.

Synthesis of 3OHKynG. The glucoside of 3OHKyn was synthesized using the method of Manthey et al. (*38*).

Synthesis and Purification of 3OHKyn-Modified Amino Acids (21). 3OHKyn (50 mg) was dissolved in 50 mM Na_2CO_3/$NaHCO_3$ buffer at pH 9.5 (30 mL). The amino acids (*N*-α-*t*-Boc-L-His, *N*-α-*t*-Boc-L-Lys, and Cys) were added to the buffer in 10-fold molar excess. The pH was readjusted to 9.5 with 0.1 M NaOH if required, then the resulting solution was bubbled with argon, sealed, wrapped in foil, and incubated at 37 °C for 48 h. After the pH was adjusted to between 4 and 5 with glacial acetic acid, the resulting mixture was separated by semipreparative or analytical HPLC. The yield, NMR, MS/MS data, and high-resolution mass spectra are given below.

N-α-*tert*-Butoxycarbonyl-L-lysyl-3-hydroxy-DL-kynurenine (3OHKyn-*t*-Boc-Lys): 11.3 mg, 11%. Found: MH^+, 454.2264. Calculated for $C_{21}H_{32}N_3O_8$: MH^+, 454.2189. ESI−MS/MS of *m*/*z* 454 (MH^+): 354 (100%), 247 (7%), 208 (10%), 203 (96%), 152 (24%), 147 (28%), 128 (12%).

δH: 7.54 (1H, d, J = 8.0, H-6), 7.16 (1H, d, J = 7.0, H-4), 6.96 (1H, t, J = 8.0, 8.0, H-5), 4.20 (1H, m, H-9), 4.12 (1H, m, H-15), 3.82 (2H, m, CH_2-8), 3.19 (2H, m, CH_2-11), 1.89 (1H, m, CH_2-14), 1.81 (2H, m, CH_2-12), 1.75 (1H, m, CH_2-14), 1.51 (2H, m, CH_2-13), 1.29 (9H, s, 3×CH_3).

δC: 200.2 (CO-7), 172.8 (CO-10), 146.5 (C-3), 134.1 (C-2), 122.8 (C-6), 120.2 (C-4), 120.1 (C-5), 81.8 (C-18), 57.3

(C-9), 53.7 (C-15), 47.1 (C-11), 38.6 (C-8), 30.1 (C-14), 27.7 (C-19), 25.0 (C-12), 22.3 (C-13).

N-α-*tert*-Butoxycarbonyl-L-histidyl-3-hydroxy-DL-kynurenine (3OHKyn-*t*-Boc-His): 8.6 mg, 9%. Found: MH^+, 463.1771. Calculated for $C_{21}H_{27}N_4O_8$: MH^+, 463.1829. ESI−MS/MS of *m*/*z* 463 (MH^+): 407 (52%), 363 (100%), 317 (15%), 209 (10%), 208 (12%), 156 (52%), 110 (5%).

δH: 8.75 (1H, s, H-11), 7.42 (1H, d, $J = 8.0$, H-6), 7.34 (1H, s, H-13), 7.03 (1H, d, $J = 8.0$, H-4), 6.90 (1H, t, $J = 7.5, 8.0$, H-5), 5.47 (1H, m, H-9), 4.30 (1H, m, H-15), 3.92 (2H, m, CH_2-8), 3.17 (1H, dd, $J = 15.5, 15.5$, CH_2-14), 2.97 (1H, m, CH_2-14), 1.93 (9H, s, $3\times CH_3$).

δC: 199.1 (CO-7), 172.4 (CO-10), 147.0 (C-3), 135.6 (C-11), 135.3 (C-2), 129.7 (C-12), 122.6 (C-6), 122.0 (C-5), 120.7 (C-4), 119.9 (C-13), 119.2 (C-1), 81.8 (C-18), 59.1 (C-9), 52.8 (C-15), 41.9 (C-8), 27.5 (C-19), 26.8 (C-14), 26.8 (C-14).

L-Cysteinyl-3-hydroxykynurenine (3OHKyn-Cys): 12.4 mg, 17%. Found: MH^+, 329.0789. Calculated for $C_{13}H_{17}N_2O_6S$: MH^+, 329.0807. ESI−MS/MS of *m*/*z* 329 (MH^+): 311 (30%), 240 (10%), 208 (60%), 202 (100%), 190 (65%), 162 (80%), 122 (5%), 110 (12%).

δH: 7.69 (1H, d, $J = 7.5$, H-6), 7.43 (1H, t, $J = 8.0$ and 8.5, H-5), 7.32 (1H, d, $J = 8.5$, H-4), 4.35 (1H, m, H-12), 3.97 (1H, m, H-9), 3.77 (1H, m, CH_2-8), 3.66 (1H, d, $J = 4.5$, CH_2-8), 3.43 (1H, d, $J = 4.5$, CH_2-11), 3.33 (1H, d, $J = 5.0$, CH_2-11).

δC: 200.5 (CO-7), 175.9 (CO-10), 170.8 (CO-13), 150.4 (C-3), 128.3 (C-5), 127.3 (C-1), 122.2 (C-6), 121.5 (C-4), 118.6 (C-2), 52.1 (C-12), 41.7 (C-9), 40.9 (C-8), 31.6 (C-11), 31.3 (C-11).

Acid Hydrolysis of 3OHKyn-Modified Amino Acid Adducts (39). Each purified 3OHKyn amino acid adduct (0.5 mg) was hydrolyzed in an evacuated hydrolysis tube with 6 M HCl (1 mL), thioglycolic acid [5% (v/v)], and phenol [1% (w/v)] for 24 h at 110 °C. After hydrolysis, the mixture was freeze-dried, dissolved in 0.1% TFA, and purified by HPLC.

Stability of 3OHKyn Adducts at pH 4. 3OHKyn adducts (0.2 mg) were dissolved in 0.1 M sodium acetate/acetic acid buffer at pH 4 (3 mL), and the resulting solutions were bubbled with either argon or oxygen for 5 min before being sealed, wrapped in foil, and incubated for 48 h at 37 °C. Aliquots were taken every 12 h and examined by HPLC to determine the recovery of the adducts.

Stability of 3OHKyn Adducts at pH 7.2. 3OHKyn adducts (0.2 mg) were dissolved in 0.1 M phosphate buffer at pH 7.2 (3 mL), and chloroform (20 μL) was added to inhibit bacterial growth. The resulting solutions were bubbled with either argon or oxygen for 5 min before being sealed, wrapped in foil, and incubated at 37 °C for 120 h. Aliquots were taken every 24 h and examined by HPLC.

Preparation of Bovine Calf Lens Protein (CLP) (40). Fresh calf eyes were obtained from the local abattoir (Picton, NSW, Australia). The lenses were removed from each eye, and the capsule was removed and then stored at 4 °C until used. A 50 mM Tris-HCl buffer (pH 7.2) containing 5 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1 mM 1,4-dithiothreitol, and 0.04% (w/v) sodium azide was used for extraction. Each lens was homogenized with the buffer (2 mL/lens). Soluble protein remaining after centrifugation (15000*g*, 5 °C, 30 min) was dialyzed against water and freeze-dried.

Incubation of CLP with 3OHKyn at pH 7.2 or 9.5 (21). CLP (50 mg) and 3OHKyn (10 mg) were dissolved in 0.1 M phosphate buffer at pH 7.2 (10 mL) containing chloroform (10 μL) or in 50 mM Na_2CO_3/$NaHCO_3$ buffer at pH 9.5 (10 mL). The solution was bubbled with argon (5 min) to give an oxygen content of 40 mmHg, sealed, wrapped in foil, and incubated at 37 °C for 48 h. At the end of the incubation, the protein was adjusted to a final concentration of 80% ethanol, by the addition of absolute ethanol, and then centrifuged (8000*g*, 10 °C, 20 min). To ensure the removal of all noncovalently bound material, the protein was dissolved in 6 M guanidine HCl (10 mL), dialyzed overnight against 0.1 M sodium acetate/acetic acid buffer at pH 4, and freeze-dried.

Preparation of Human Lens Protein. Normal human aged lenses were obtained from the Sydney Lions Eye Bank. ARN cataract lenses were obtained from K. T. Sheth Eye Hospital, Rajkot, Gujarat, India. All lenses were stored at −80 °C. Nuclei were obtained using a cork borer (5 mm), and the ends were removed (1 mm). The nuclei were homogenized in 500 μL of 6 M guanidine HCl and dialyzed overnight against a 0.1 M sodium acetate/acetic acid buffer at pH 4. The protein was then freeze-dried.

Acid Hydrolysis of Proteins. Proteins modified at pH 7.2 (10 mg), pH 9.5 (10 mg), or human lens protein (5 mg) were hydrolyzed with 6 M HCl (1 mL), thioglycolic acid [5% (v/v)], and phenol [1% (w/v)] as described above. The samples were freeze-dried, dissolved in 0.1% aqueous TFA, and examined by HPLC using the 0.1% TFA buffer system.

Quantification of Protein-Bound 3OHKyn. 3OHKyn was stripped from the proteins by incubation at basic pH. The deaminated 3OHKyn that was released was trapped using excess GSH that both added to the conjugated ketone and prevented autoxidation.

(a) Model studies. The protein modified with 3OHKyn at pH 7.2 (that had previously been dialyzed to remove noncovalently bound material) (50 mg) was dissolved in 6 M guanidine HCl (0.6 mL) containing reduced GSH (500 mg), and 2.4 mL of 50 mM Na_2CO_3/$NaHCO_3$ buffer at pH 9.5 was added. The pH was readjusted to 9.5 with 6 M NaOH, and the resulting solution was bubbled with argon, sealed, wrapped in foil, and incubated at 37 °C for 4 h. After the pH was adjusted to less than 5 with acetic acid, the solution was centrifuged (6000*g*, 4 °C, 60 min) in a Vivaspin concentrator (10 000 MW cut off) to separate the noncovalently bound material (filtrate) from the whole protein. The filtrate was examined by HPLC using the 0.1% TFA buffer system.

(b) Human lenses. Human lens nuclei (22 normal lenses, age range of 17−83, and 20 cataractous lenses, age range of 55−101) were extracted with 100% ethanol and then with 80% ethanol and then centrifuged (8000*g*, 10 °C, 20 min). To ensure the removal of all noncovalently bound material, the insoluble protein was dissolved in 6 M guanidine HCl (2 mL) and dialyzed overnight against 0.1 M sodium acetate/acetic acid buffer at pH 4. The protein was then freeze-dried. The human lens proteins and reduced GSH (100 mg) were dissolved in 6 M guanidine HCl (0.4 mL), and 50 mM Na_2CO_3/$NaHCO_3$ buffer at pH 9.5 (1.1 mL) was added. The pH was readjusted to 9.5 with 6 M NaOH, and the resulting solution was bubbled with argon, sealed, wrapped in foil, and incubated for 4 h at 37 °C. After the pH was adjusted

to less than 5 with acetic acid, the solution was centrifuged (6000*g*, 4 °C, 60 min) in a Vivaspin concentrator (10 000 MW cut off) to separate the noncovalently bound material from the protein. The filtrate was examined by HPLC using the 0.1% TFA buffer system.

RESULTS

Characterization of 3OHKyn Adducts. Three 3OHKyn amino acid adducts were synthesized by incubation of excess *t*-Boc-His, *t*-Boc-Lys, and Cys, with 3OHKyn at pH 9.5. Under these high pH conditions, side chains of Kyn derivatives deaminate more readily than at neutral pH (*41*). The Cys adduct was obtained in the highest yield (17%), followed by Lys (11%) and His (9%) (Figure 1). These compounds were purified by RP-HPLC, and the chromatogram for each adduct showed the presence of other compounds eluting elsewhere, including unreacted 3OHKyn. It is likely that these other compounds represent oxidation products of 3OHKyn because it is known that 3OHKyn readily autoxidizes with the formation of dimeric and other compounds at a neutral pH (*25*, *26*) and that the rate is increased at higher pH (*27*).

NMR studies were undertaken to characterize the adducts. Samples were dissolved in acidified D_2O, to stabilize each adduct, because *o*-aminophenols readily oxidize at neutral pH. The adducts (Figure 2) were not separated into individual diastereoisomers because resolution by HPLC was inadequate. The chemical shifts for the ^1H and ^{13}C NMR spectra are listed in the Experimental Procedures. The chemical shifts for 3OHKyn-*t*-Boc-Lys (Figure 2C) are similar to those reported by Staniszewska et al. (*42*), who synthesized an antigen using 3OHKyn and *N*-α-acetyl Lys, for immunohistochemical studies.

Because there was a limited amount of sample available, the quaternary carbons could not be resolved, and therefore, structural characterization of the 3OHKyn adducts (Figure 2) was further confirmed by mass spectrometry.

ESI mass spectra were acquired for each of the adducts, and the expected molecular ions for each of the adducts (3OHKyn-Cys, *m*/*z* 329; 3OHKyn-*t*-Boc-His, *m*/*z* 463; 3OHKyn-*t*-Boc-Lys, *m*/*z* 454) were present in the spectra (not shown). MS/MS of the molecular ions was also performed to confirm the structures. MS/MS analysis of 3OHKyn-Cys (*m*/*z* 329) yielded fragment ions, 311, 240, 208, 202, 190, 162, 122, and 110 (Figure 3A). Analysis of 3OHKyn-*t*-Boc-His (*m*/*z* 463) resulted in fragment ions, 407, 363, 317, 209, 208, 156, and 110, and 3OHKyn-*t*-Boc-Lys (*m*/*z* 454) resulted in fragment ions, 354, 247, 208, 203, 152, 147, and 128 (spectra not shown). A characteristic fragment present in all adducts was *m*/*z* 208, and this corresponds to 3-hydroxykynurenine yellow (Figure 2D) (*43*), a known intramolecular cyclization product of 3OHKyn at neutral pH (*44*).

UV−vis spectra for the three adducts were found to be essentially identical, with wavelength maxima at 370 and 269 nm. Three-dimensional fluorescence spectra showed that each of the adducts was fluorescent. 3OHKyn-*t*-Boc-His, 3OHKyn-*t*-Boc-Lys, and 3OHKyn-Cys all exhibited maximal fluorescence intensities at excitation (Ex) 370 nm/emission (Em) 495 nm (data not shown).

Stability of Adducts. Because 3OHKyn is known to autoxidize readily at neutral pH with the production of H_2O_2 (*27*) and numerous other products (*25*, *26*), it was of interest to discover if the 3OHKyn amino acid adducts were also unstable under physiological conditions.

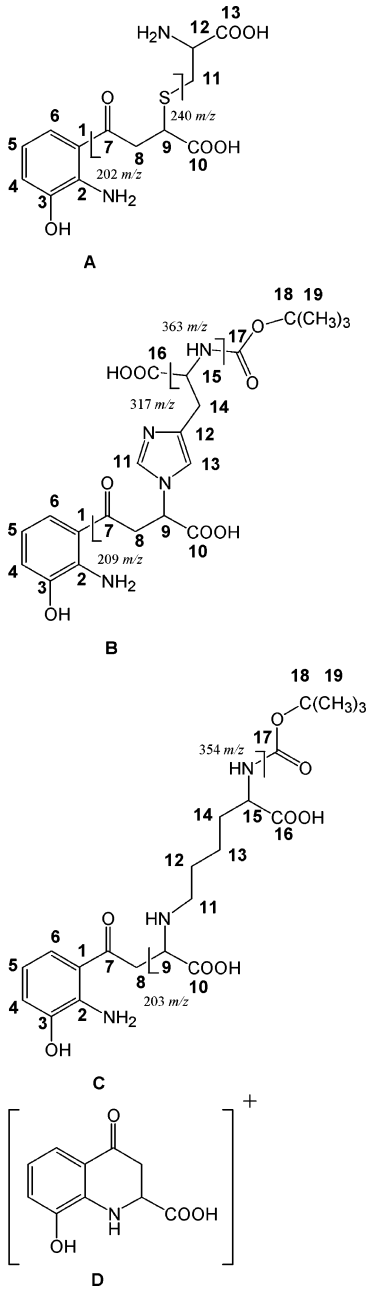

FIGURE 2: Structures of 3OHKyn adducts. (A) 3OHKyn-Cys, (B) 3OHKyn-*t*-Boc-His, (C) 3OHKyn-*t*-Boc-Lys, and (D) 3-hydroxykynurenine yellow, formed by intramolecular condensation (*44*) of the α,*β*-unsaturated ketone (*m*/*z* 208 ion), which was observed in the tandem mass spectra of all three adducts.

Stability experiments were performed at pH 7.2 both in the presence and in the absence of oxygen. The results are shown in Figure 4A. In the absence of oxygen, only 15% of 3OHKyn (part i in Figure 4A) remained after 5 days of incubation, and these results are comparable to those obtained by Taylor et al. (2002), where incubations were done in 25 mM carbonate buffer (pH 7) (*41*). 3OHKyn-Cys and 3OHKyn-*t*-Boc-Lys (parts ii and iv in Figure 4A) adducts were found to be less stable than 3OHKyn, with recoveries of 3 and 7%, respectively, after 24 h. In contrast, under the same conditions, the His adduct (part iii in Figure 4A) was recovered in 51% yield. It is likely that each adduct

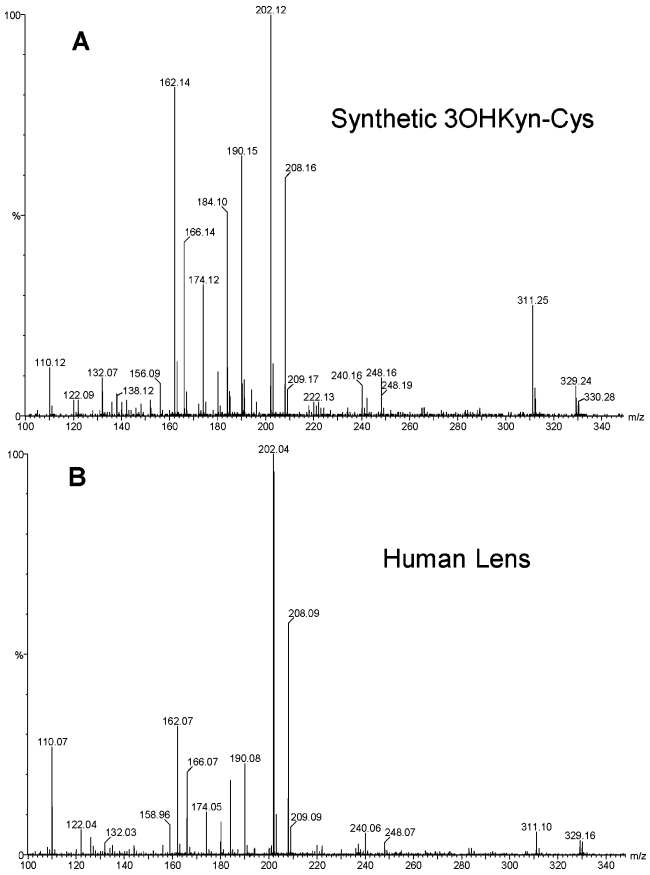

FIGURE 3: Tandem mass spectra of (A) synthetic 3OHKyn-Cys and (B) the HPLC peak isolated from the 6 M HCl digest of an aged human lens. The molecular ion is *m*/*z* 329, and *m*/*z* 311 arises from the loss of water from the molecular ion.

undergoes elimination via an $E1_{cb}$ mechanism, although the reasons for the differences in stability are unclear.

In the presence of oxygen, the adducts were markedly less stable. There was, for example, no 3OHKyn recovered after 96 h at pH 7.2 and no detectable peak for the Cys adduct after only 24 h of incubation (part ii in Figure 4A). No trace of the Lys adduct was observed after 48 h of incubation (part iv in Figure 4A). Again, the His adduct was most stable with a recovery of 11% after 120 h (part iii in Figure 4A).

These data illustrate that all of the 3OHKyn amino acid adducts are unstable under physiological conditions and that this instability is exacerbated by exposure to oxygen. The Cys and Lys adducts were, in fact, more labile than 3OHKyn itself. One objective of the current study was to develop a method for detecting 3OHKyn attached to proteins. Because of their instabilities and susceptibility to oxidation, coupled with the fact that they are likely to be present in low levels, detection of 3OHKyn adducts presents a technical challenge.

On the basis of the studies with 3OHKyn at neutral pH detailed above and previous investigations, which showed that high pH promoted autoxidation (*27*), we investigated the use of acidic pH to stabilize the adducts. If 3OHKyn adducts were found to be stabilized under such acidic conditions, these could be used for their isolation from modified proteins. Stability studies were therefore performed on each of the adducts at pH 4. As described for the incubations at neutral pH, two sets of experiments were performed, one in the absence of oxygen and one in the presence of oxygen. The results are shown in Figure 4B and

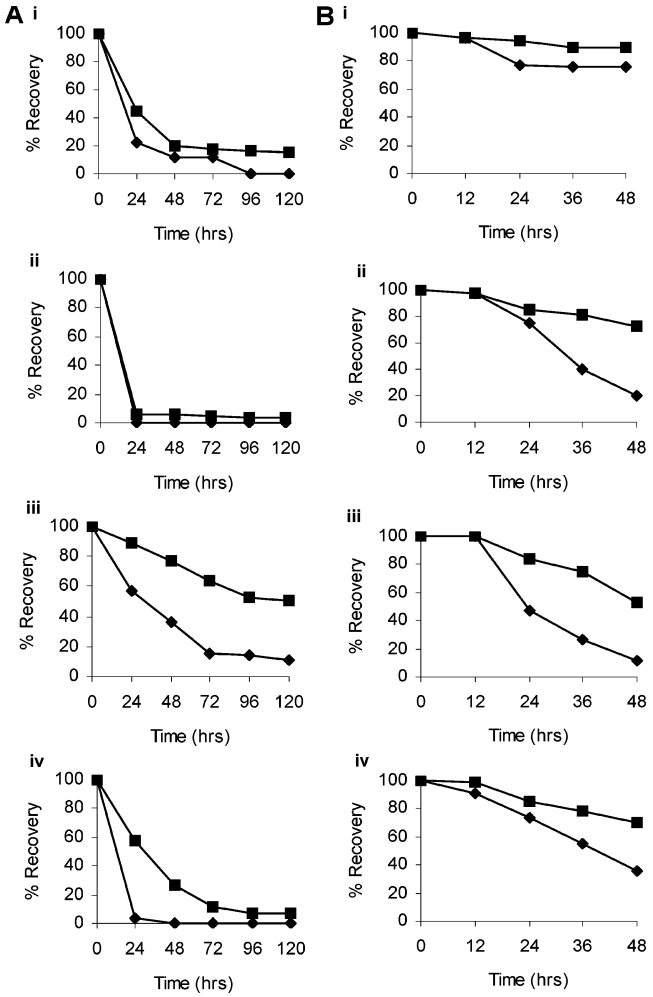

FIGURE 4: (A) Stability of the 3OHKyn amino acid adducts and 3OHKyn at pH 7.2 in the absence (■) and presence (◆) of oxygen. (i) 3OHKyn, (ii) 3OHKyn-Cys, (iii) 3OHKyn-*t*-Boc-His, and (iv) 3OHKyn-*t*-Boc-Lys. (B) Stability of the 3OHKyn amino acid adducts and 3OHKyn at pH 4 in the absence (■) and presence (◆) of oxygen. (i) 3OHKyn, (ii) 3OHKyn-Cys, (iii) 3OHKyn-*t*-Boc-His, and (iv) 3OHKyn-*t*-Boc-Lys.

illustrate a marked effect of pH on adduct stability. In the absence of oxygen, 3OHKyn was recovered in 90% yield (part i in Figure 4B) after 48 h, whereas the Cys and Lys (parts ii and iv in Figure 4B) adducts both showed a 70% recovery, and 53% of the original 3OHKyn-*t*-Boc-His (part iii in Figure 4B) was detected after this time. In the presence of oxygen, the recoveries of all of the compounds were diminished. The recovery of 3OHKyn dropped to 76%, and the Cys, His, and Lys adducts were recovered in yields of 20, 11, and 36%, respectively, after 48 h. On the basis of these data (parts A and B of Figure 4), to maximize recoveries of 3OHKyn-containing amino acids, acidic pH is required during sample isolation. The only exception was 3OHKyn-His, which displayed reasonable stability under both neutral and acidic pH both in the presence and absence of oxygen.

Acid Hydrolysis. The stability of each adduct under conditions used for the hydrolysis of proteins was determined by hydrolyzing with 6 M HCl in the presence of antioxidants (*39*), because low yields (3OHKyn-Cys, 17%; 3OHKyn-His, 20%; and 3OHKyn-Lys, 24%) were recovered initially for each adduct in the absence of such antioxidants. If antioxidants were added, the recoveries were markedly improved,

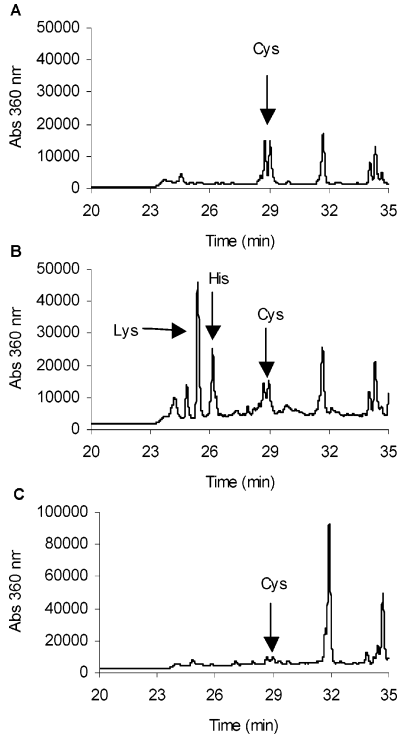

FIGURE 5: HPLC traces of protein samples hydrolyzed with 6 M HCl. The arrows indicate the elution positions of authentic 3OHKyn amino acid adducts. (A) CLP modified with 3OHKyn at pH 7.2 for 48 h, (B) CLP modified with 3OHKyn at pH 9.5 for 48 h, and (C) normal human lens protein from a 76-year-old lens.

3OHKyn-Cys, 87%; 3OHKyn-His, 78%; and 3OHKyn-Lys, 95%. Under the same conditions, the recovery of 3OHKyn was 58%. The HPLC profiles showed the presence of other minor compounds in addition to the major adduct peak, but these minor peaks were not examined further (data not shown).

Protein Modification. CLP was modified with 3OHKyn both at pH 7.2 and 9.5. The higher pH promotes deamination of 3OHKyn and reactivity of the nucleophilic side chains so that higher yields of adducts were expected under these conditions. Modified proteins were hydrolyzed with acid, and components in the hydrolyzates were separated by RP-HPLC. The chromatograms are shown in parts A and B of Figure 5. The HPLC profile of the protein modified at pH 7.2 (Figure 5A) showed a doublet at 28.7 min when monitored at 360 nm. This pair was at the same retention time as synthetic 3OHKyn-Cys, which also eluted as a doublet, corresponding to diastereoisomers. MS/MS of the peaks confirmed that the prominent *m*/*z* 329 ion present, corresponding to the mass of 3OHKyn-Cys, fragmented to give *m*/*z* 208, 202, 190, 162, 122, and 110, which are all characteristic ions of 3OHKyn-Cys. No characteristic ions of the His and Lys adducts could be found at the retention times of the corresponding standards. These results show that, at physiological pH, Cys residues in CLP are major sites of reaction of 3OHKyn.

The HPLC chromatogram of the hydrolyzate of CLP modified by 3OHKyn at pH 9.5 (Figure 5B) also showed a doublet peak eluting at 28.7 min. Using the same conditions as described for the pH 7.2 incubation, MS/MS confirmed that this doublet was 3OHKyn-Cys. Peaks that eluted earlier were also collected and analyzed. The peak at 26.0 min displayed an ion at *m*/*z* 363, which corresponds to the mass of 3OHKyn-His. MS/MS of this molecular ion yielded fragments at *m*/*z* 317, 209, 208, 156, and 110, which are characteristic fragments of authentic 3OHKyn-His. The peak at 25.3 min contained, as the major component, a compound with a molecular ion of *m*/*z* 354, corresponding to the mass of 3OHKyn-Lys. MS/MS analysis of *m*/*z* 354 resulted in major fragments ions, *m*/*z* 208, 203, 152, 147, and 128, all of which are characteristic of 3OHKyn-Lys.

UV−vis and three-dimensional fluorescence spectra were acquired for the CLP modified with 3OHKyn at pH 7.2. This protein exhibited an absorbance peak at 370 nm and fluorescence intensities at Ex 370 nm/Em 490 nm. Lens protein modified at pH 9.5 fluoresced at Ex 370 nm/Em 470 nm. These spectroscopic data are similar to those of the synthetic 3OHKyn amino acid adducts (data not shown).

Human Lens Protein Analysis. Because the *in vitro* study confirmed that 3OHKyn can bind to lens proteins under conditions thought to be similar to those present in the normal human lens and that modified amino acids can be recovered from acid digests of the proteins, we examined proteins isolated from a 76-year-old normal human lens to determine if 3OHKyn−amino acid adducts could be detected. Figure 5C shows the HPLC chromatogram of the lens protein following hydrolysis with 6 M HCl. A small doublet was observed using detection at 360 nm (28.9 min). MS/MS confirmed that the doublet in the sample was indeed 3OHKyn-Cys, because fragmentation of the ion (Figure 3B) at *m*/*z* 329 yielded the same characteristic ions as authentic 3OHKyn-Cys (Figure 3A). Quantitation showed that this peak corresponded to 42.8 mmol of 3OHKyn-Cys/mol of protein. 3OHKyn-Cys was also detected in two other human lens digests at similar levels (59 and 68 years old, 51.8 and 53.4 mmol/mol of protein, respectively, not shown). Peaks corresponding to the elution times of the 3OHKyn-His and Lys standards were also collected; however, these adducts could not be confirmed by mass spectrometry.

Determination of 3OHKyn Bound to Proteins. In previous research, it had been shown that GSH binds covalently to the deamination product that results from the exposure of 3OHKynG to a base. This novel fluorescent diastereoisomeric adduct is found as a UV filter in the human lens (*45*).

Because previous data had shown that exposure to neutral or basic conditions promoted decomposition of the 3OHKyn amino acid adducts, we investigated whether simple exposure of the Cys, His, or Lys adducts or 3OHKyn-modified protein to base may lead to the release of the corresponding deamination product. Basic conditions were used to speed the reaction, but it was recognized that the unsaturated ketone that was released would be highly susceptible to both oxidation and further reactions under such conditions. To prevent oxidation and to rapidly trap the deamination compound, we included excess GSH into the basic solution used to cleave the UV-filter adducts. It was found that the GSH adducts were obtained in high yield and were easily identified by HPLC and mass spectrometry. Time-course experiments showed that maximal yields of the GSH adducts were obtained after incubation for 4 h. Recovery experiments using this methodology with the 3OHKyn adducts of Lys, Cys, and His showed that yields of GSH conjugates of 73, 61, and 0.5% were obtained, respectively, after 4 h of incubation with GSH at pH 9.5. Therefore, this method is

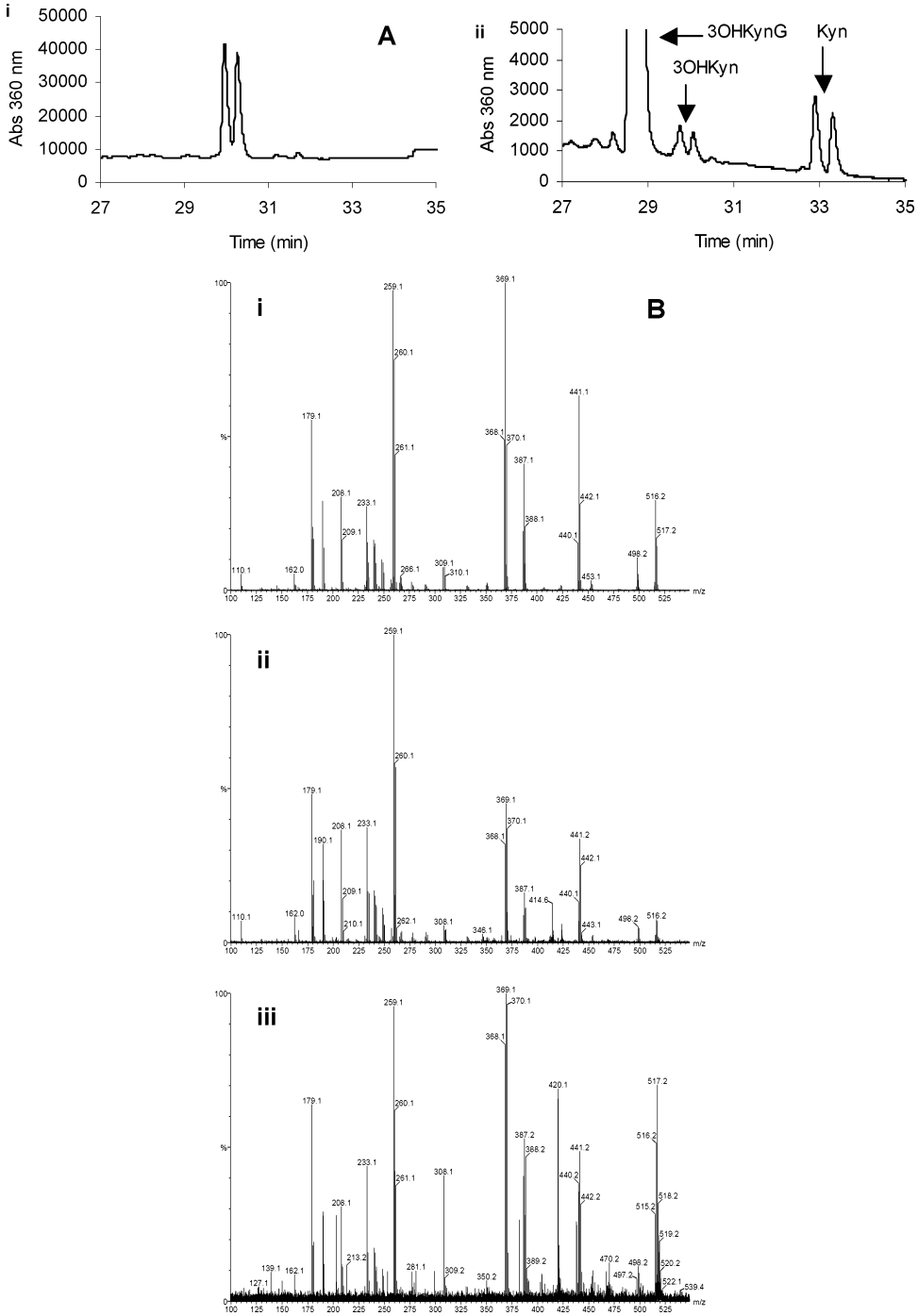

FIGURE 6: UV filters bound to proteins. (A) HPLC traces of the protein-free filtrate following incubation with GSH at pH 9.5 for 4 h. (i) CLP modified with 3OHKyn at pH 7.2 and (ii) human protein from 68-year-old normal lens. (B) Tandem mass spectra of the *m*/*z* 515 ion derived from (i) synthetic 3OHKyn-GSH, (ii) the HPLC doublet peak, which eluted at 30 min in CLP modified with 3OHKyn at pH 7.2 (part i in A), and (iii*)* the HPLC doublet peak, which eluted at 30 min in human protein from a 68-year-old normal lens (part ii in A).

useful for detecting 3OHKyn bound to Lys or Cys residues on proteins but not His residues.

CLP modified with 3OHKyn at pH 7.2 was incubated with excess GSH at pH 9.5 for 4 h because a time course had shown, as for the synthetic adducts, that release was maximal after 4 h. The solution was ultrafiltered to separate the cleaved compounds from the remaining protein. An HPLC chromatogram of the filtrate (part i in Figure 6A) showed a doublet eluting at 30 min. Quantification of this doublet coupled with data from acid hydrolysis, which showed 31.8 mmol of 3OHKyn-Cys/mol of protein, revealed that there was a 67% recovery of 3OHKyn from the modified protein. The ESI spectrum for this peak showed an abundant ion at *m*/*z* 515. MS/MS analysis of this ion (part ii in Figure 6B) confirmed that it corresponded to that of 3OHKyn-GSH, because the spectrum was identical to that of the synthetic compound (part i in Figure 6B). The fragment ion at *m*/*z* 386 corresponds to the loss of glutamate; the ion at *m*/*z* 368 corresponds to the further loss of water; and the ion *m*/*z* 308 corresponds to GSH (*46*). Ions at *m*/*z* 208, 190, 162, and 110 are characteristic fragment ions of 3OHKyn. This methodology was therefore applied to proteins extracted from human lenses. A total of 22 normal human lenses were examined as well as 20 ARN cataractous lenses. Part ii in

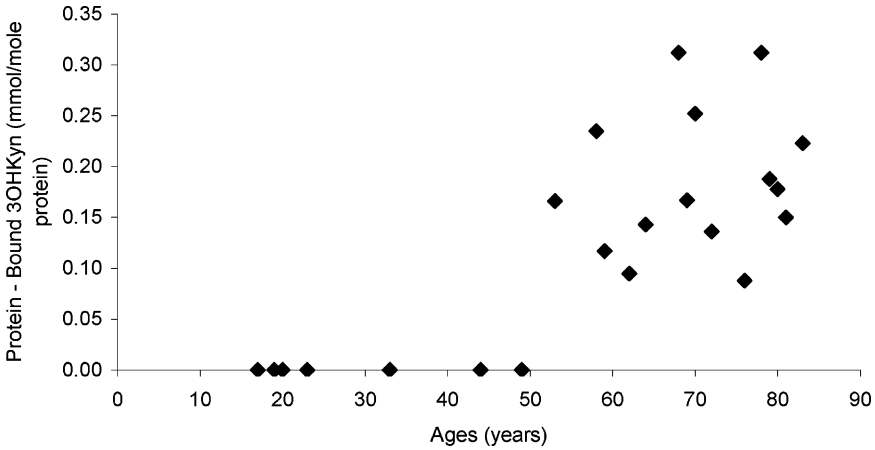

FIGURE 7: Levels of 3OHKyn bound to the nuclear proteins of normal human lenses as a function of age. The values shown have not been corrected for the recovery of 3OHKyn-GSH from 3OHKyn-modified protein.

Figure 6A shows the HPLC chromatogram for the filtrate of a 68-year-old normal lens. This was typical of the HPLC chromatograms of the other older normal lenses examined. The chromatogram showed three doublets eluting at 28.5, 30, and 33 min (part ii in Figure 6A). The large peak at 28.5 min contained a component with a major ion in the ESI spectrum at *m*/*z* 677. MS/MS of this ion (*m*/*z* 515, 386, 368, 208, 190, 162, and 110) confirmed that it was 3OH-KynG-GSH (*45*).

The doublet peak at 33 min was smaller, and the major ion in the ESI spectrum was *m*/*z* 499. MS/MS of this ion (*m*/*z* 370, 352, 308, 192, and 174) identified it as Kyn-GSH because the MS/MS and HPLC elution times were identical to those of Kyn-GSH synthesized by the incubation of Kyn with GSH under basic pH conditions (*46*). The doublet at 30 min eluted at the same time as synthetic 3OHKyn-GSH. A major ion at *m*/*z* 515 was present in the ESI spectrum. MS/MS of this ion (part iii in Figure 6B) showed fragment ions at *m*/*z* 386, 368, 208, 190, 162, and 110, as expected for 3OHKyn-GSH.

Because 3OHKynG was present in much higher concentrations than 3OHKyn, it was important to demonstrate that the 3OHKyn was not derived via the hydrolysis of the glucoside during the incubation period. 3OHKynG was incubated with GSH under the same conditions used for the lens proteins. After 4 h at pH 9.5, a large doublet peak corresponding to 3OHKynG-GSH was observed but there were no detectable peaks in the region where 3OHKyn-GSH elutes. This region was also collected for analysis by mass spectrometry, but no ion at *m*/*z* 515, corresponding to 3OHKyn-GSH, was detected. The absence of hydrolysis is consistent with the stability of glucosides to base.

The amount of 3OHKyn bound to human nuclear proteins as a function of age was determined for 22 normal lenses (Figure 7). No 3OHKyn-GSH could be detected in the lenses 17, 19, 20, 23, 33, 44, or 49 years old. There were however varying amounts of 3OHKyn covalently bound to proteins in lenses aged 53 years and over. This degree of variability is typical of human lens data, and similar distribution patterns were also found in previous studies where the levels of protein-bound 3OHKynG and Kyn were quantified (*20*, *21*). In each of the lenses examined, covalently bound UV filters were detected in decreasing order of abundance, 3OHKynG followed by Kyn and 3OHKyn. The approximate ratio was 150:5:1 (3OHKynG/Kyn/3OHKyn). This reflects the order of abundance of the free UV filters found in human lenses (*8*).

An analysis of 20 human cataract lenses, which included 10 dark [type IV, Pirie classification (*47*)] and 10 light [types II and III, Pirie classification (*47*)], covering the age range from 55 to 101, revealed that 3OHKyn could not be detected in any of the cataract lenses. Compounds eluting at the same HPLC retention time as authentic 3OHKyn-GSH were collected and examined by mass spectrometry to determine if trace levels of 3OHKyn-GSH were present. In some samples, a small molecular ion for 3OHKyn-GSH was detected; however, MS/MS could not conclusively confirm that the product was 3OHKyn-GSH.

DISCUSSION

The aim of the present study was to determine if 3OHKyn may be implicated in the etiology of nuclear cataract, first, by examining normal human lenses for the presence of protein-bound 3OHKyn and, second, by analyzing ARN cataract lenses for any differences from normal human lenses.

ARN cataract is characterized by oxidation, coloration, insolubilization, and cross-linking of proteins in the lens (*33*−*37*). Aging is by far the largest risk factor for cataract (*36*, *48*), and it is likely but not yet clear exactly how the many changes known to take place in the aging lens may predispose it to subsequent ARN cataract. The development of polypeptide coloration and cross-linking makes it probable that PTM of crystallins by small reactive molecules is involved. Candidates include ascorbate (*49*), UV filters (*20*, *21*, *32*), products derived from carbohydrates (*11*, *50*), or lipid decomposition products such as malondialdehyde (*51*).

In this paper, we were able to show that 3OHKyn is bound to human lens proteins in the nucleus and, further, that the amounts bound increase with age. Bound 3OHKyn was not detectable in lenses below the age of 50, but all lenses older than 50 contained measurable levels of this UV filter. In a 68-year-old lens, the amount bound corresponds to approximately 0.312 mmol/mol of protein. 3OHKyn was not detected in the cortex of normal human lenses.

On the basis of acid hydrolysis data of normal human lenses and model experiments, it would appear that most 3OHKyn is attached to Cys residues. Cys was the major site of modification when CLP was incubated with 3OHKyn at

pH 7.2 (Figure 5A). His and Lys residues only became modified significantly when the incubation was performed at pH 9.5, conditions under which the polypeptides are likely to be unfolded.

This is the first structural demonstration that 3OHKyn is bound to human lens proteins, although immunohistochemical evidence was recently published (*42*). As outlined elsewhere (*32*, *52*), this PTM may be deleterious for the lens in several ways. For example, aside from the already noted propensity to oxidize together with the probability of polypeptide cross-linking via nucleophilic addition to the quinonimine, it is also possible that the *o*-aminophenol moiety of 3OHKyn may act as a site for chelation of redox-active metals such as Fe or Cu. On the basis of studies published by Goldstein et al. (*53*), these metals are likely to remain redox-active once bound and could therefore contribute to hydroxyl radical damage if H_2O_2 levels become raised in the lens. Hydroxyl radical oxidation of lens proteins is known to be associated with ARN cataract (*39*).

The finding that 3OHKyn is attached to proteins in the normal lens only after age 50 and that this phenomenon is localized in the nucleus provides additional evidence for the lens barrier. In normal lenses, the onset of the lens barrier at middle age (*22*−*24*) effectively uncouples the metabolically active cortex from the quiescent nucleus and thus increases the likelihood of PTM by reactive molecules. This is because it limits the rate at which GSH enters the nucleus. Any GSSG formed in the nucleus must return to the cortex to be re-reduced. This lessened flux may contribute to a decrease in the concentration of nuclear GSH in older lenses (*8*, *54*).

In addition, because of the decreased general movement of small molecules, including H_2O (*23*), in the nucleus, small molecules that do enter the lens center are then located in this region, on average, for a longer time period than in younger lenses. This latter factor assumes a great importance for molecules that are unstable. Of particular relevance to the lens are UV filters and ascorbate or more correctly dehydroascorbate. In both of these cases, lens proteins can be spared from PTM provided that sufficient GSH is present (*40*, *55*). In the case of UV filters, the deamination products, which form at pH 7 (α,β-unsaturated ketones), react rapidly with the nucleophilic thiol group of GSH (*45*). In this way, proteins in the lens can be spared (*40*), and the GSH adduct that forms can diffuse out of the lens.

It is clear that maintenance of adequate GSH levels in the nucleus of the lens is crucial (*56*−*58*) for minimizing polypeptide modification. In line with these considerations, it has been demonstrated that both 3OHKynG (*20*) and Kyn (*21*) become bound progressively to lens proteins with age. This is particularly noticeable after middle age when nuclear GSH concentrations decrease. The consequences of this for the lens are not known, but crystallins modified by Kyn become susceptible to oxidation by wavelengths of light that penetrate the cornea (*59*). In normal human lenses, the levels of reduced GSH are high, ranging from approximately 10 mM in the outer cortex to 2 mM in the center of the lens (*56*). The major difference between normal and ARN cataract lenses is the environment in the nucleus. On the basis of the lack of reduced GSH in advanced cataract lenses (*60*) and the high levels of oxidized amino acid residues (*35*), it is apparent that the nucleus of cataract lenses is an oxidizing environment.

The binding of 3OHKyn is potentially of far greater significance than the attachment of the other UV filters. This is because of the great sensitivity of 3OHKyn to autoxidation and the fact that the immediate oxidation product, an *o*-quinonimine, is highly reactive (*25*−*27*, *30*, *32*, *61*). Thus, the binding of 3OHKyn to lens proteins after middle age introduces into the older lens proteins sites that are reactive if the general environment of the nucleus becomes less reducing, for example, by a decrease in the concentration of GSH (*8*, *54*). It is of interest that 3OHKyn acts as a pro-oxidant in the lens. For example, if 3OHKyn is introduced into intact bovine lenses (which do not contain UV filters) in culture, there is a marked reduction in the concentration of GSH in these lenses over time of incubation (*62*). It is likely that this is mediated via the production of H_2O_2 that is formed quantitatively during the autoxidation of 3OHKyn (*27*).

Until now, it has proven difficult to demonstrate covalent binding of 3OHKyn to lens proteins. Principally, this has been due to the low levels present, coupled with the known susceptibility to oxidation in the presence of even trace levels of oxygen (*25*−*27*). In an effort to circumvent these issues, we synthesized the three 3OHKyn adducts thought to be present in the lens (*21*). Stability studies revealed that exposure to neutral and basic conditions led to the decomposition of adducts (Figure 4A). Under nonoxidative conditions, both 3OHKyn-Cys and 3OHKyn-*t*-Boc-Lys were less stable than 3OHKyn itself at pH 7.2, whereas 3OHKyn-*t*-Boc-His was significantly more stable. Decomposition of all of these conjugates was accelerated in the presence of oxygen. As such, typical proteolytic techniques involving digestion with trypsin at pH 8 are unlikely to reveal sites of binding of 3OHKyn. In contrast, the 3OHKyn adducts were found to be relatively stable at acidic pH (Figure 4B), particularly in the absence of oxygen.

It was found that the 3OHKyn amino acid adducts could be recovered from acid hydrolyzates in good yields provided that antioxidants were included. When hydrolysis of aged human lens proteins with HCl was undertaken, 3OHKyn-Cys was observed by HPLC (Figure 5C). However, this experiment cannot distinguish whether the 3OHKyn-Cys was derived from 3OHKyn attached to Cys residues of proteins or whether it was in fact 3OHKynG-Cys, because the glucoside bond, if present, will also be hydrolyzed by acid. Previously, studies using tryptic digestion of proteins from older human lenses at pH 6 revealed that 3OHKynG is attached to several sites on the crystallins. These include three Cys residues in γS crystallin and one in βB1 crystallin (*63*).

If 3OHKyn-His, 3OHKyn-Lys, or the corresponding glucoside adducts were present in human lens proteins, the levels were below the limits of detection. These data together with the results of incubation of lens proteins with 3OHKyn suggest that Cys may be an important site for the modification of lens crystallins. In addition, the results of stability studies of the adducts at pH 7.2 (Figure 4A) suggest that the binding of 3OHKyn to crystallins in the lens may be a dynamic process, with binding and release both taking place. If sufficient GSH is present in the nucleus, as in a young normal lens, the reactive deamination product that is released from the protein would most likely react with GSH and much of it may then diffuse into the lens cortex and out of the

lens. In the absence of excess GSH, such as in an older or nuclear cataract lens, the deaminated 3OHKyn may oxidize and bind to the proteins irreversibly (*30*−*32*). In this way, it is probable that the nuclear proteins would become colored as well as cross-linked. Oxidation of the *o*-aminophenol would also generate H_2O_2 that could oxidize other residues.

If indeed oxidation of bound UV filters was responsible for the coloration and other features associated with ARN cataract, one would expect that the levels of 3OHKyn attached to the protein may be decreased in cataract lenses. This was found to be the case, and 3OHKyn was not detected in the proteins in human cataract lenses. The levels of the other two UV filters were diminished compared to age-matched normal lenses. This result of decreased UV-filter attachment in cataract agrees with that published for Kyn using acid hydrolysis (*64*), but the reason for this is not yet clear.

Our results suggest that 3OHKyn may play a role in the substantial changes that occur in the lens with the onset of ARN cataract. Although we have shown here that 3OHKyn is indeed bound to nuclear proteins, it is likely that other compounds known to be attached to normal aged lens proteins (*9*−*12*) may also play a role in the coloration of the cataract lens. To prove conclusively that 3OHKyn is involved in the biochemical processes associated with ARN cataract, it will be necessary to isolate and characterize oxidation products that incorporate the 3OHKyn moiety from such cataract lenses. Because of the complexity of pathways for oxidation of 3OHKyn (*25*−*27*, *32*) and the lability of some intermediates, coupled with the low levels and the multitude of protein nucleophilic groups, the isolation and characterization of oxidized 3OHKyn adducts from cataract proteins will represent a considerable technical achievement.

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REFERENCES

1. Berman, E. R. (1991) *Biochemistry of the Eye*, Plenum Press, New York.
2. van Heyningen, R. (1971) Fluorescent glucoside in the human lens, *Nature 230*, 393−394.
3. van Heyningen, R. (1973) The glucoside of 3-hydroxykynurenine and other fluorescent compounds in the human lens, *Ciba Found. Symp. 19*, 151−171.
4. Zigman, S., and Paxhia, T. (1988) The nature and properties of squirrel lens yellow pigment, *Exp. Eye Res. 47*, 819−824.
5. Wood, A. M., and Truscott, R. J. W. (1993) UV filters in human lenses: Tryptophan catabolism, *Exp. Eye Res. 56*, 317−325.
6. Truscott, R. J. W., Wood, A. M., Carver, J. A., Sheil, M. M., Stutchbury, G. M., Zhu, J., and Kilby, G. W. (1994) A new UV-filter compound in human lenses, *FEBS Lett. 348*, 173−176.
7. Wood, A. M., and Truscott, R. J. W. (1994) Ultraviolet filter compounds in human lenses: 3-Hydroxykynurenine glucoside formation, *Vis. Res. 34*, 1369−1374.
8. Bova, L. M., Sweeney, M. H. J., Jamie, J. F., and Truscott, R. J. W. (2001) Major changes in human ocular UV protection with age, *Invest. Ophthalmol. Vis. Sci. 42*, 200−205.
9. Nagaraj, R. H., Sell, D. R., Prabhakaram, M., Ortwerth, B. J., and Monnier, V. M. (1991) High correlation between pentosidine protein crosslinks and pigmentation implicates ascorbate oxidation in human lens senescence and cataractogenesis, *Proc. Natl. Acad. Sci. U.S.A. 88*, 10257−10261.
10. Tessier, F., Obrenovich, M., and Monnier, V. M. (1999) Structure and mechanism of formation of human lens fluorophore LM-1 relationship to vesperlysine a and the advanced maillard reaction in aging, diabetes, and cataractogenesis, *J. Biol. Chem. 274*, 20796−20804.
11. Argirov, O. K., Lin, B., and Ortwerth, B. J. (2004) 2-Ammonio-6-(3-oxidopyridinium-1-yl)hexanoate (OP-lysine) is a newly identified advanced glycation end product in catarctous and aged human lenses, *J. Biol. Chem. 279*, 6487−6495.
12. Cheng, R., Feng, Q., Argirov, O. K., and Ortwerth, B. J. (2004) Structure elucidation of a novel yellow chromophore from human lens proteins, *J. Biol. Chem. 279*, 45441−45449.
13. Chiarugi, A., Meli, E., and Moroni, F. (2001) Similarities and differences in the neuronal death processes activated by 3OH-kynurenine and quinolinic acid, *J. Neurochem. 77*, 1310−1318.
14. Reynolds, G. P., and Pearson, S. J. (1989) Increased brain 3-hydroxykynurenine in Huntington's disease, *Lancet 2*, 979−980.
15. Pearson, S. J., and Reynolds, G. P. (1992) Increased brain concentrations of a neurotoxin, 3-hydroxykynurenine, in Huntington's disease, *Neurosci. Lett. 144*, 199−201.
16. Ogawa, T., Matson, W. R., Beal, M. F., Myers, R. H., Bird, E. D., Milbury, P., and Saso, S. (1992) Kynurenine pathway abnormalities in Parkinson's disease, *Neurology 42*, 1702−1706.
17. Garner, B., Shaw, D. C., Lindner, R. A., Carver, J. A., and Truscott, R. J. W. (2000) Non-oxidative modification of lens crystallins by kynurenine: A novel post-translational protein modification with possible relevance to ageing and cataract, *Biochim. Biophys. Acta 1476*, 265−278.
18. Aquilina, J. A., and Truscott, R. J. W. (2000) Cysteine is the initial site of modification of α-crystallin by kynurenine, *Biochem. Biophys. Res. Comm. 276*, 216−223.
19. Aquilina, J. A., and Truscott, R. J. W. (2001) Kynurenine binds to the peptide binding region of the chaperone αB-crystallin, *Biochem. Biophys. Res. Comm. 285*, 1107−1113.
20. Hood, B. D., Garner, B., and Truscott, R. J. W. (1999) Human lens coloration and aging evidence for crystallin modification by the major ultraviolet filter, 3-hydroxykynurenine *O*-β-D-glucoside, *J. Biol. Chem. 274*, 32547−32550.
21. Vazquez, S., Aquilina, J. A., Jamie, J. F., Sheil, M. M., and Truscott, R. J. W. (2002) Novel protein modification by kynurenine in human lenses, *J. Biol. Chem. 277*, 4867−4873.
22. Sweeney, M. H. J., and Truscott, R. J. W. (1998) An impediment to glutathione diffusion in older normal human lenses: A possible precondition for nuclear cataract, *Exp. Eye Res. 67*, 587−595.
23. Moffat, B. A., Landman, K. A., Truscott, R. J. W., Sweeney, M. H. J., and Pope, J. M. (1999) Age-related changes in the kinetics of water transport in normal human lenses, *Exp. Eye Res. 69*, 663−669.
24. Truscott, R. J. W. (2000) Age-related nuclear cataract: A lens transport problem, *Ophthalmic Res. 32*, 185−194.
25. Butenandt, A., Schiedt, U., and Biekert, E. (1954) Uber ommochrome. 3. Mitteilung: Synthese des xanthommatins, *Liebigs Ann. 588*, 106−116.
26. Butenandt, A., and Schafer, W. (1962) *Ommochromes. In Chemistry of Natural and Synthetic Colouring Matter*, Academic Press, New York.
27. Vazquez, S., Garner, B., Sheil, M. M., and Truscott, R. J. W. (2000) Characterisation of the major autoxidation products of 3-hydroxykynurenine under physiological conditions, *Free Radical Res. 32*, 11−23.
28. Tomoda, A., Yoneyama, Y., Yamaguchi, T., Shirao, E., and Kawasaki, K. (1990) Mechanism of coloration of human lenses induced by near-ultraviolet-photo-oxidized 3-hydroxykynurenine, *Ophthal. Res. 22*, 152−159.
29. Ellozy, A. R., Wang, R. H., and Dillon, J. (1994) Photolysis of intact young human, baboon, and rhesus monkey lenses, *Photochem. Photobiol. 59*, 474−478.
30. Stutchbury, G. M., and Truscott, R. J. W. (1993) The modification of proteins by 3-hydroxykynurenine, *Exp. Eye Res. 57*, 149−155.
31. Aquilina, J. A., Carver, J. A., and Truscott, R. J. W. (1997) Oxidation products of 3-hydroxykynurenine bind to lens proteins: Relevance for nuclear cataract, *Exp. Eye Res. 64*, 727−735.
32. Aquilina, J. A., Carver, J. A., and Truscott, R. J. W. (1999) Elucidation of a novel polypeptide cross-link involving 3-hydroxykynurenine, *Biochemistry 38*, 11455−11464.

33. Dilley, K. J., and Pirie, A. (1974) Changes to the proteins of the human lens nucleus in cataract, *Exp. Eye Res. 19*, 59−72.
34. Truscott, R. J. W., and Augusteyn, R. C. (1977) Changes in human lens proteins during nuclear cataract formation, *Exp. Eye Res. 24*, 159−170.
35. Truscott, R. J. W., and Augusteyn, R. C. (1977) Oxidative changes in human lens proteins during senile nuclear cataract formation, *Biochim. Biophys. Acta 492*, 43−52.
36. Harding, J. J. (1991) *Cataract Biochemistry, Epidemiology and Pharmacology*, Chapman and Hall, London, U.K.
37. Spector, A. (1984) Oxidation and cataract, *Ciba Found. Symp. 106*, 48−64.
38. Manthey, M. K., Jamie, J. F., and Truscott, R. J. W. (1999) Synthesis of human ultraviolet filter compounds: *O*-β-D-Glucopyranosides of 3-hydroxykynurenine and 2-amino-3-hydroxy-γ-oxobenzenebutanoic acid, *J. Org. Chem. 64*, 3930−3933.
39. Fu, S., Dean, R., Southan, M., and Truscott, R. J. W. (1998) The hydroxyl radical in lens nuclear cataractogenesis, *J. Biol. Chem. 273*, 28603−28609.
40. Taylor, L. M., Aquilina, J. A., Jamie, J. F., and Truscott, R. J. W. (2002) Glutathione and NADH, but not ascorbate, protect the lens proteins from modification by UV filters, *Exp. Eye Res. 74*, 503−511.
41. Taylor, L. M., Aquilina, J. A., Jamie, J. F., and Truscott, R. J. W. (2002) UV filter instability: Consequences for the human lens, *Exp. Eye Res. 75*, 165−175.
42. Staniszewska, M. M., and Nagaraj, R. H. (2005) 3-Hydroxykynurenine-mediated modification of human lens proteins structure determination of a major modification using a monoclonal antibody, *J. Biol. Chem. 280*, 22154−22164.
43. Vazquez, S. (2001) Human lens protein modification: The role of kynurenine and 3-hydroxykynurenine, Ph.D. Thesis, University of Wollongong, Wollongong, New South Wales, Australia.
44. Tokuyama, T., Senoh, S., Sakan, T., Brown, K. S. J., and Witkop, B. (1967) The photoreduction of kynurenic acid to kynurenine yellow and the occurrence of 3-hydroxy-L-kynurenine in butterflies, *J. Am. Chem. Soc. 89*, 1017−1021.
45. Garner, B., Vazquez, S., Griffith, R., Lindner, R. A., Carver, J. A., and Truscott, R. J. W. (1999) Identification of glutathionyl-3-hydroxykynurenine glucoside as a novel fluorophore associated with aging of the human lens, *J. Biol. Chem. 274*, 20847−20854.
46. Taylor, L. M. (2002) Human lens UV filters, Ph.D. Thesis, University of Wollongong, Wollongong, New South Wales, Australia.
47. Pirie, A. (1968) Color and solubility of the proteins of human cataracts, *Invest. Ophthalmol. 7*, 634−650.
48. Taylor, H. R. (2003) Eye care for the future the Weisenfeld lecture, *Invest. Ophthalmol. Vis. Sci. 44*, 1413−1414.
49. Ortwerth, B. J., Feather, M. S., and Olesen, P. R. (1988) The precipitation and cross-linking of lens crystallins by ascorbic acid, *Exp. Eye Res. 47*, 155−168.
50. Ahmed, N., Thornalley, P. J., Dawczynski, J., Franke, S., Strobel, J., Stein, G., and Haik, G. M. (2003) Methylglyoxal-derived hydroimidazolone advanced glycation end-products of human lens proteins, *Invest. Ophthalmol. Vis. Sci. 44*, 5287−5292.
51. Bhuyan, K. C., and Bhuyan, D. K. (1984) Molecular mechanism of cataractogenesis: 3. Toxic metabolites of oxygen as initiators of lipid peroxidation and cataract, *Curr. Eye Res. 3*, 67−81.
52. Truscott, R. J. W. (2003) Human cataract: The mechanisms responsible; light and butterfly eyes, *Int. J. Biochem. Cell Biol. 35*, 1500−1504.
53. Goldstein, L. E., Leopold, M. C., Huang, X., Atwood, C. S., Saunders, A. J., Hartshorn, M., Lim, J. T., Faget, K. Y., Muffat, J. A., Scarpa, R. C., Chylack, L. T. J., Bowden, E. F., Tanzi, R. E., and Bush, A. I. (2000) 3-Hydroxykynurenine and 3-hydroxyanthranilic acid generate hydrogen peroxide and promote α-crystallin cross-linking by metal ion reduction, *Biochemistry 39*, 7266−7275.
54. Dickerson, J. E., Jr., and Lou, M. F. (1997) Free cysteine levels in normal human lenses, *Exp. Eye Res. 65*, 451−454.
55. Ortwerth, B. J., and Olesen, P. R. (1988) Glutathione inhibits the glycation and crosslinking of lens proteins by ascorbic acid, *Exp. Eye Res. 47*, 737−750.
56. Reddy, V. N., and Giblin, F. J. (1984) Metabolism and function of glutathione in the lens, *Ciba Found. Symp. 106*, 65−87.
57. Giblin, F. J. (2000) Glutathione: A vital lens antioxidant, *J. Ocular Pharmacol. Ther. 16*, 121−135.
58. Truscott, R. J. W. (2005) Age-related nuclear cataract−Oxidation is the key, *Exp. Eye Res. 80*, 709−725.
59. Parker, N. R., Jamie, J. F., Davies, M. J., and Truscott, R. J. W. (2004) Protein-bound kynurenine is a photosensitizer of oxidative damage, *Free Radical Biol. Med. 37*, 1479−1489.
60. Truscott, R. J. W., and Augusteyn, R. C. (1977) The state of sulphydryl groups in normal and cataractous human lenses, *Exp. Eye Res. 25*, 139−148.
61. Aquilina, J. A., Carver, J. A., and Truscott, R. J. W. (2000) Polypeptide modification and cross-linking by oxidized 3-hydroxykynurenine, *Biochemistry 39*, 16176−16184.
62. Berry, Y., and Truscott, R. J. W. (2001) The presence of a human UV filter within the lens represents an oxidative stress, *Exp. Eye Res. 72*, 411−421.
63. Aquilina, J. A., and Truscott, R. J. W. (2002) Identifying sites of attachment of UV filters to proteins in older human lenses, *Biochim. Biophys. Acta 1596*, 6−15.
64. Vazquez, S., Parker, N. R., Sheil, M. M., and Truscott, R. J. W. (2004) Protein-bound kynurenine decreases with the progression of age-related nuclear cataract, *Invest. Ophthalmol. Vis. Sci. 45*, 879−883.

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