HUMAN UV FILTERS, UV LIGHT AND OXIDATIVE DAMAGE IN AGE RELATED NUCLEAR CATARACT

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ABSTRACT

Purpose

The lenses of humans contain a group of small protective molecules that are known as UV filters. The main UV filters present in human lenses are 3-hydroxykynurenine *O*-β-D-glucoside (3OHKG), kynurenine (Kyn), 3-hydroxykynurenine (3OHK) and 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid-*O*-β-D-glucoside (AHBG). These UV filters protect the lens from UV damage and it is believed that there may be other novel related species present in the lens that act analogously. The UV filters are also implicated in the progressive modification of lens proteins that occur with aging, and the development of age related nuclear cataract in older humans.

The first aim of this thesis was to detect, quantify and elucidate the structures of novel 3-hydroxykynurenine glucoside-derived UV filter metabolites present in the human lens. The second aim of this thesis was to investigate bovine lens proteins modified through covalent binding with UV filters and determine if these modified UV filter metabolites could be photosensitisers of oxidative damage. The final aim was to describe the detection and identification of amino acid residues involved in the covalent binding of human lens UV filters and metabolites to bovine lens proteins.

Methods

Three novel UV filter compounds 4-(2-amino-3-hydroxyphenyl)-2-hydroxy-4-oxobutanoic acid *O*-β-D-glucoside (3OHKG-W), 3-hydroxykynurenine *O*-β-D-glucoside yellow (3OHKG-Y) and 2-amino-3-hydroxyacetophenone *O*-β-D-glucoside (AHAG), along with Kyn, 3OHK, AHBG and 3OHKG, were detected and quantified by LC-MS/MS in 24 human lenses of different ages, of which 22 were normal and two had age related nuclear cataract. The structures of these compounds were confirmed through total synthesis and spectral analysis.

The synthesised analogues (3OHKG-Y, 3OHKG-W, AHAG), together with a previously identified analogue (3OHKG-D) and the major UV filters (3OHKG, Kyn, 3OHK), were incubated with bovine lens proteins (BLP) at pH 7.4, to mimic physiological conditions, and at pH 9.5 (which accelerates deamination and encourages binding to protein residues). The modified lens proteins were exposed

to UV light under aerobic and anaerobic conditions and in the presence and absence of free UV filters for varying periods of time. The UV light irradiated BLP were then separated through SDS-PAGE to observe protein binding, aggregation and any other observable changes.

Finally, the amino acid adducts produced from this incubation were identified by spectral analysis and identities confirmed by total synthesis. Their concentrations in the modified proteins were then quantified following enzymatic hydrolysis of the modified lens proteins with Pronase.

Results

As reported in previous studies, 3OHKG concentrations decreased with age in the lens nuclei, whereas the levels of three novel species, 3OHKG-Y, 3OHKG-W and AHAG increased, though to different extents. The deaminated metabolite of 3OHKG (3OHKG-D) was also detected, though in very low concentrations, and only in some of the lenses. In contrast, the concentrations of all the UV filters measured present in the cortex of the lens remained constant with age.

Upon irradiation of the modified BLP, different changes were observed between bound UV filters having both an *o*-aminophenol and α,β-unsaturated carbonyl moieties (3OHK) and those with only an α,β-unsaturated carbonyl moiety (3OHKG, Kyn, 3OHKG-D). Metabolites with only an *o*-aminophenol (AHB) or without an α,β-unsaturated carbonyl moiety (3OHKG-Y, 3OHKG-W or AHAG) no did not experience any visible changes. Evidence of cross-linking and non-disulphide binding was observed in the BLP modified with Kyn, 3OHK, 3OHKG and 3OHKG-D. Irradiation (of at least 120 min) resulted in a significant loss of soluble protein, suggesting aggregation of modified BLP, with the greatest extent of aggregation detected with samples modified at pH 9.5.

There was evidence of UV filter binding to BLP under both physiological and basic conditions. The major lens UV filter, 3OHKG, was found to react with lens proteins at cysteine and histidine residues, with the greatest level of binding on Cys at pH 9.5. Low levels of binding were detected at the Lys residues at pH 9.5. These were also observed at pH 7.4, however, only at close to the detection limit. Comparative studies with Kyn and 3OHK similarly resulted in lens proteins modified at Cys residues. The extent of modification was found to be significantly higher at pH 9.5 in all cases. LC-MS/MS analysis of tryptic digests showed modifications at both α- and β-, but not γ-crystallins. By contrast, UV filter

compounds that cannot form α,β-unsaturated carbonyl compounds (*i.e.* cannot deaminate) did not produce covalent modification of lens proteins *via* side-chain interaction. 3OHKG, Kyn and 3OHK modified lens proteins were found to be coloured and fluorescent, resembling those from aged and age related nuclear cataractous lenses. There was also strong evidence that the structure of the protein in itself inhibits binding to internally (protected) Cys residues.

Conclusion

Three novel 3OHKG derived metabolites have been detected in extracts from normal and cataractous human lenses. In the subsequent binding studies of these metabolites (and other UV filter compounds), it was confirmed that binding can only occur between BLP and a UV filter with α,β-unsaturated carbonyl moieties. Covalent binding is a prerequisite to photo-oxidative damage occurring upon irradiation with UV light. No evidence of binding or protein damage was observed in samples treated with AHB, 3OHKG-Y, 3OHKG-W or AHAG upon UV irradiation compared to irradiated untreated BLP. Even though AHB could covalently bind to proteins *via* auto-oxidation of the *o*-aminophenol, this was not observed in this study even though AHB displayed evidence of non-covalent binding (at pH 9.5 only). Protein modification/treatment was also examined with the UV filter metabolites 3OHKG-W and AHAG, however no modified amino acid residues were observed. There was a very low level of modification with 3OHKG-Y, of similar proportions in both pHs, suggesting some level of spontaneous binding. This generally suggests that, other than 3OHKG-D, 3OHKG derived metabolites are relatively stable and unlikely to react with lens proteins.

THESIS DECLARATION

This thesis contains no material that has been accepted for the award of any higher degree or diploma at any University or Institution, and to the best of my knowledge contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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This thesis is dedicated to

my son

Christian Severin Gad

May the Lord keep you safe and guide you along the right path.

PUBLICATIONS AND CONFERENCE PRESENTATIONS

NSW Royal Australian Chemical Institute (RACI) Organic One Day Symposium, Sydney (NSW, Australia) 2 December 2009. Nicholas Gad, Jasminka Mizdrak, David Pattison, Roger Truscott, Michael Davies, Joanne Jamie - Novel UV Filter Compounds in the Human Lens

Royal Australian Chemical Institute (RACI) Natural Products Chemistry Group Annual One Day Symposium Sydney (NSW, Australia) 1 October 2010. Nicholas Gad, Jasminka Mizdrak, David Pattison, Roger Truscott, Michael Davies, Joanne Jamie - New 3-Hydroxykynurenine-Glucoside Derivative UV Filters Found in Human Lenses of Varying Age

Society for Free Radical Biology and Medicine (SFRBM) 17th Annual Meeting, Orlando (Florida, USA) 17-21 November 2010. Nicholas Gad, Jasminka Mizdrak, David Pattison, Roger Truscott, Michael Davies, Joanne Jamie – Role of Novel UV Filters in Human Lens Protein Damage – Link to Age Related Nuclear Cataract

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LIST OF ABBREVIATIONS

The following abbreviations are used throughout the text:

ABG	2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl bromide
AcOH	Acetic acid
AGE	Advanced glycation end
Arg	Arginine
Asp	Aspartic acid
AHA	4-(2-Aminophenyl)-4-oxobutanoic acid
AHAG	2-Amino-3-hydroxyacetophenone *O*-β-D-glucoside
AHB	4-(2-Amino-3-hydroxyphenyl)-4-oxobutanoic acid
AHBG	4-(2-Amino-3-hydroxyphenyl)-4-oxobutanoic acid-*O*-β-D-glucoside
AHBDG	4-(2-Amino-3-hydroxyphenyl)-4-oxobutanoic acid-*O*-diglucoside
ARN	Age related nuclear
BLP	Bovine lens proteins
BOC	Butyloxycarbonyl
CD_3OD	Deuterated methanol
CH_3CN	Acetonitrile
Cys	Cysteine
Cys-3OHKG	Cysteinyl-3-hydroxykynurenine-*O*-β-D-glucoside
di-Tyr	Di-tyrosine
DOPA	3,4-Dihydroxyphenylalanine
D_2O	Deuterium oxide
ESI	Electrospray ionisation mass spectrometry
Em	Emission
Ex	Excitation
Gly	Glycine
GSH	Glutathione
GSH-3OHKG	Glutathionyl-3-hydroxykynurenine-*O*-β-D-glucoside
GSH-Kyn	Glutathionyl-kynurenine
GSH-3OHK	Glutathionyl-3-hydroxykynurenine
GSSG	Glutathione disulphide
HCl	Hydrochloric acid

His	Histidine
H_2O_2	Hydrogen peroxide
Kyn	Kynurenine
LC-MS	Liquid chromatography-mass spectrometry (ESI)
Lys	Lysine
MeOH	Methanol
Met	Methionine
MS/MS	Tandem mass spectrometry
m/z	Mass to charge ratio
NAD(P)H	Reduced β-nicotinamide dinucleotide (phosphate)
NMR	Nuclear magnetic resonance
NaOH	Sodium hydroxide
$^1O_2$	Singlet molecular oxygen in its $^1\Delta_g$ state
$O_2^{\cdot-}$	Superoxide radical anion
3OHK	3-Hydroxykynurenine
3OHKG	3 Hydroxykynurenine-*O*-β-D-glucoside
3OHKG-D	4-(2-amino-3-hydroxyphenyl)-4-oxobut-2-enoic acid *O*-β-D-glucoside
3OHKG-W	4-(2-amino-3-hydroxyphenyl)-2-hydroxy-4-oxobutanoic acid *O*-β-D-glucoside
3OHK-Y	3-Hydroxykunurenine yellow
3OHKG-Y	3-Hydroxykunurenine-*O*-β-D-glucoside yellow
RP-HPLC	Reversed phase high performance liquid chromatography
RP-UPLC	Reversed phase ultra performance liquid chromatography
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
Trp	Tryptophan
Tyr	Tyrosine
UV	Ultraviolet
UVA	400-320 nm UV light
UVB	320-280 nm UV light
UVC	280-100 nm UV light
UV/Vis	Ultraviolet-visible (spectroscopy)

CHAPTER 1

INTRODUCTION TO HUMAN LENS UV FILTERS

The lenses of humans and closely related primates contain a group of small protective molecules that are known as UV filters. These molecules are synthesised in the lens through the kynurenine pathway, with the main UV filter present in humans being 3-hydroxykynurenine *O*-β-D-glucoside. Four major UV filters and three UV filter analogues have been identified in previous studies, and three more analogues were identified and detected in this study. With age, the production of UV filters in the lens decreases, and it has been shown that *in vivo* these UV filters can bind to lens proteins. It is believed that this progressive modification of lens proteins with aging and their subsequent oxidation are the main cause of the development of age related nuclear cataract in older humans.

1.1 Introduction

Cataract is a major cause of visual impairment in humans and is defined generally as any opacity of the lens in the eye [1]. There are a number of forms of cataract, with most early onset cataracts having a genetic or disease-based origin [2]. The most common form, and the focus of this study, is age related nuclear (ARN) cataract, which occurs through the gradual discolouration and opacity of the lens as a person ages [3]. It is well known that both UVA (320-400 nm) and UVB (295-320 nm) radiation are damaging to the eye and play a role in the development of ARN cataract [4-7]. The only treatment upon the development of any form of cataract is surgery, though UV protective eyewear may reduce or delay the onset of ARN cataract in a person [8]. In 2002, of the estimated 37 million world cases of blindness, 47.8% were believed to be due to cataract, followed by 12.3% caused by glaucoma and 8.7% by age related macular degeneration [9]. Visual impairments, including blindness, are not equally distributed in the world's populations. The greatest prevalence is in the developing world, particularly in Asia and Africa, and the greatest correlating risk factor is the age of a person [9]. As visual impairments are largely confined to people 50 years of age and over, and as life expectancy of the developing world increases, over time cataract related blindness is also expected to greatly increase. Hence, it is of great

importance that the mechanisms by which ARN cataract develops are understood, and that effective means of delaying or even preventing its onset are discovered and implemented.

1.2 Human Lens

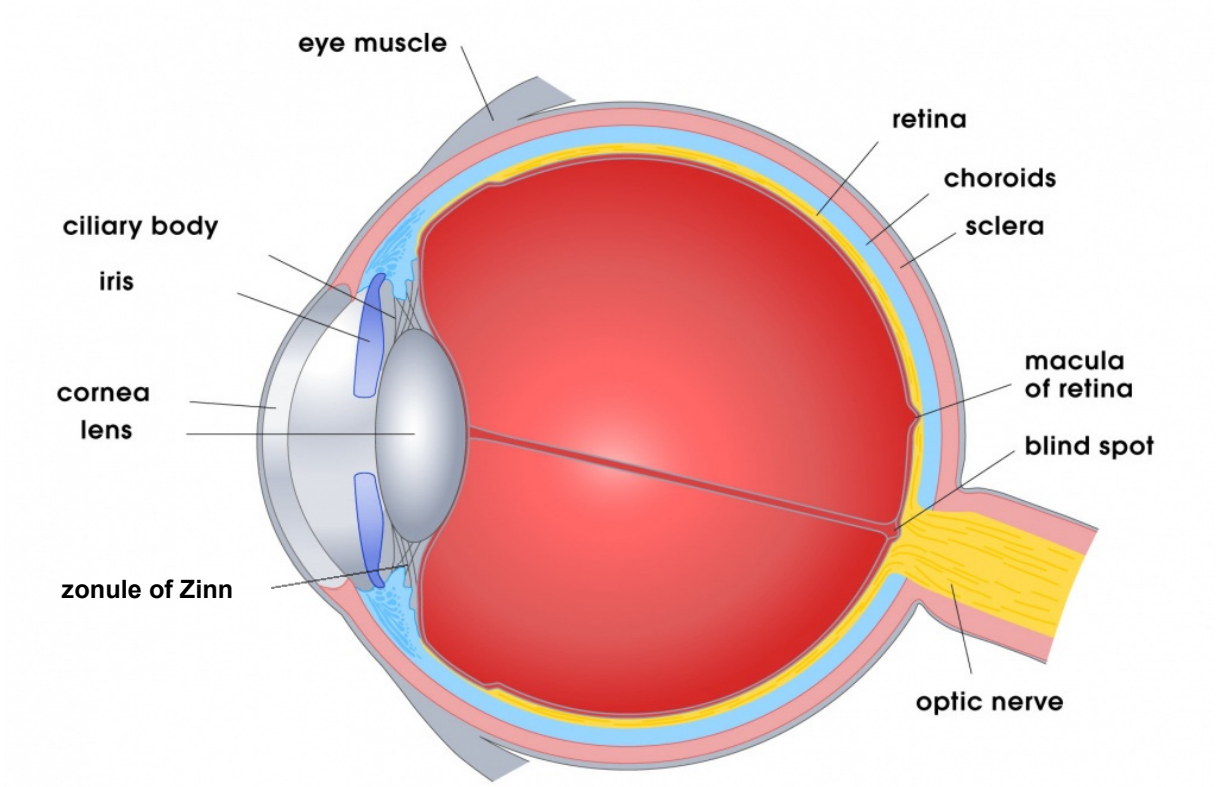


Figure 1.1: The human eye (cross-section) [10].

The lens is a transparent protein based structure present inside the eye that is necessary for clear discernible vision. As a biconvex and flexible structure, the lens focuses light entering the eye onto the retina by expanding or contracting [11]. The lens has a relatively simple structure, with the main sections being the lens capsule, the lens epithelium and the lens itself (the main protein structure). The lens capsule is a highly elastic collagen based membrane that encloses the lens. The ciliary muscles of the eye are connected to the lens capsule by connective tissue called the zonule of Zinn. It is through the stretching and relaxation of the lens capsule that the lens changes shape to focus incoming light [11]. The lens is also an avascular organ, with all its nutrients and oxygen coming from the aqueous and vitreous humour surrounding it. The regulation of the flow of nutrients and ions into the lens is performed by the lens epithelium, which is

present in the front portion of the lens, between the lens capsule and the lens itself [12].

1.2.1 Lens composition

The lens consists of 60% water, 35% proteins and 5% free amino acids and small molecules, including UV filters. 90% of all lens proteins are crystallins, which are divided into three main types (α, β, and γ crystallins) [13, 14]. Due to the high concentration of crystallin proteins and the hexagonal arrangement of its anucleate fibre cells (which keeps extracellular space to a minimum), a healthy lens is transparent to light in the 400-800 nm region, and light passes through the lens without scattering [15]. The epithelial cells of the lens also maintain tight control of electrolytes, so as to ensure the lens remains constantly hydrated [16]. It is believed that as a person ages, this highly effective structure begins to break down due to oxidative stresses, resulting in gradually increasing levels of visual impairment [17, 18]. Unlike in most other parts of the body, proteins in the lens do not turn over and thus serve a person throughout their whole life [19]. The lens does not contain either DNA or RNA, and though the outer parts of the lens can undergo some level of repair, the nucleus cannot [20]. Despite the lack of protein turnover in the nucleus, the lens continuously grows during a person's life. At birth, a person is born with approximately 1.6 million fibre cells. This doubles to 3 million cells by age 20, and continues to grow to around 3.5 million fibre cells by 80 years of age [15]. Likewise, the human lens grows throughout all stages of a person's life, with men generally having larger lenses than women [21]. This difference in size may also be a factor in why women over 50 years of age develop cataract more often than men [21].

1.2.2 Antioxidants in the lens

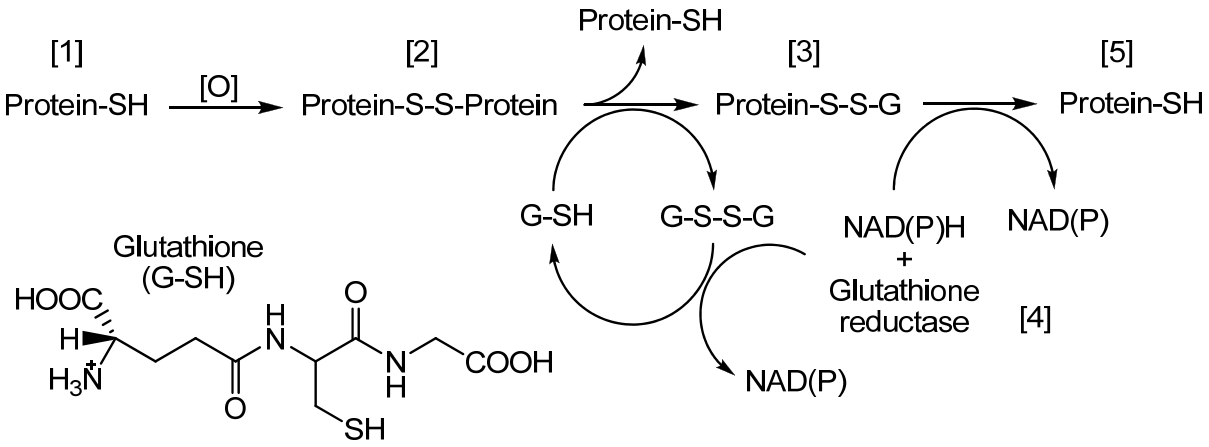
The eye has both enzymatic and non-enzymatic means of protecting the lens from oxidative damage. As in the rest of the body, the lens is protected from oxygen radicals by the enzymes superoxide dismutase and catalase. In addition, the lens has multiple non-enzymatic compounds present to help reduce oxidative damage to the lens. The two most noteworthy ones are glutathione (with its associated enzymes; glutathione peroxidase and glutathione reductase) and ascorbic acid. Both glutathione and ascorbic acid, however, are also linked to the formation of cataract in the lens. Glutathione interacts with the protective UV filters in the

lens, while ascorbic acid (under certain conditions) can cause cataract-like damage in the lens [22]. Some of the other minor protective compounds include α-tocopherol, β-carotene, uric acid, lutein and zeaxanthin. The latter two (lutein and zeaxanthin) are xanthophylls. Their hydroxyl functional groups permit them, and their structural isomers, to cross both the blood-ocular and blood-brain barriers, and they have both been linked to reduced incidence of ocular opacity [23]. Other carotenoids (β-carotene and lycopene) contain only carbon and hydrogen, and hence do not cross the blood-ocular or blood-brain barriers [24]. In general, the activity of all the protective compounds and mechanisms in the lens is greatest in the epithelial layers. Hence, the cortex is more protected from oxidative damage than the nucleus, which is consistent with ARN cataract beginning in the nucleus [25, 26].

1.2.2.1 Glutathione

Glutathione is a linear peptide of three amino acids: glutamic acid, cysteine (Cys) and glycine. In the human lens, its concentration can have a large range with levels of 1 to 6 mM being observed in normal human lenses [27]. Glutathione is arguably the most important of the self-generated antioxidants in all mammals, and its protective capacity is particularly important in the human lens [28, 29]. The strong reducing power of the thiol group in the cysteine portion of the peptide protects the lens (and most other cells in the body) from peroxides, reduces disulphides and regulates the reactivity of other biologically significant compounds (like ascorbate) that are required for the normal function of the lens [30-32].

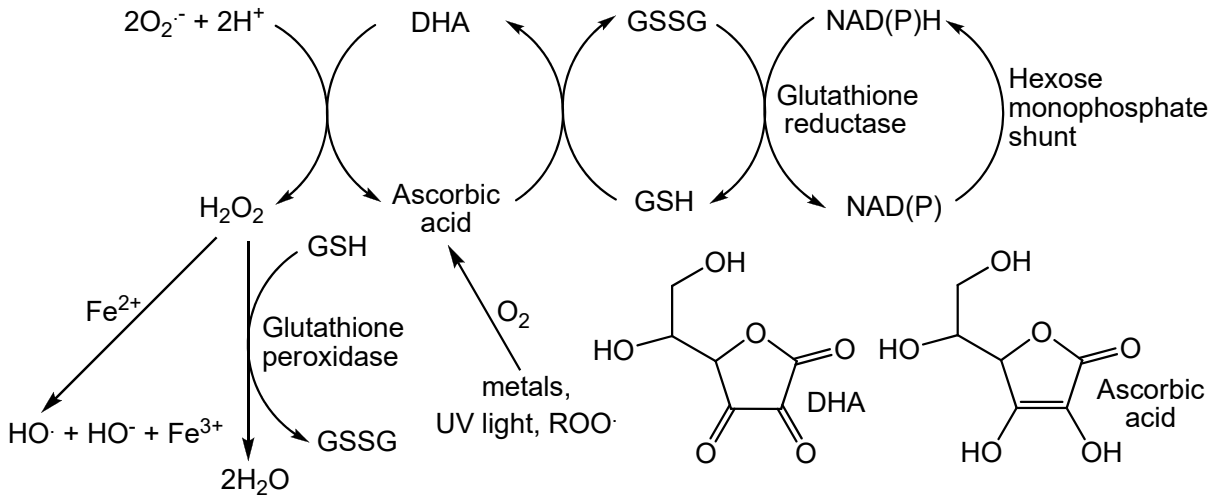
In most of these protective mechanisms, upon oxidation, glutathione forms a disulphide with another glutathione molecule making glutathione disulphide. This is a favoured reaction due to the relatively high concentration of glutathione in the lens. The disulphide is then reduced by glutathione reductase, hence bringing the two glutathiones back into their protective state [27].



Scheme 1.1: Protective cycle of glutathione and glutathione structure [33, 34] Lens proteins with a cysteine residue [1] are oxidised, forming a cystine bond between two cysteine residues on the protein [2]. GSH reduces one of the cysteines [3] and binds to the other, leaving glutathione disulphide (GSSG) as a by-product. Glutathione reductase with NAD(P)H, then recovers the 3 GSH molecules [4], restoring the original cysteine residue on the protein [5].

1.2.2.2 Ascorbic acid

Similar to glutathione, ascorbic acid is also present in relatively high concentrations in the normal human lens, though the observed range (1-2 mM) is much narrower [35]. Ascorbic acid can react with singlet oxygen [$^1O_2$] and many other oxygen free radicals, including the superoxide radical [$O_2^{\cdot-}$], hydroxyl radical [$HO^{\cdot}$], alkoxyl radical [$RO^{\cdot}$] and the peroxyl radical [$ROO^{\cdot}$] [36]. Studies have also shown that if the levels of glutathione are depleted in the lens (as they would be in an older lens), vitamin C (ascorbic acid) and E (tocopherols/tocotrienols) can restore some of the lens epithelial cell's resistance to hydrogen peroxide [37]. This ability of ascorbic acid to indirectly scavenge peroxides, along with glutathione, is of particular significance as there is a correlation between the level of hydroxyl radical formation in the lens and the severity of nuclear cataract [38].



Scheme 1.2: Protective cycle of ascorbic acid and how it interacts with GSH, superoxide radicals and other potential sources of protein damage (metals, UV light and protein peroxides) [39].

The ascorbate oxidation pathway is itself partly regulated by glutathione, with the intermediate dehydroascorbic acid being reduced by glutathione so as to regenerate ascorbic acid in the lens [40, 41]. Likewise, under specific conditions (that are possible in the lens) ascorbic acid can act as an oxidant. Ascorbic acid can form peroxides when in the presence of even trace amounts of iron or copper, and by reacting with reduced metals may then itself generate hydroxyl radicals in the lens [22, 42].

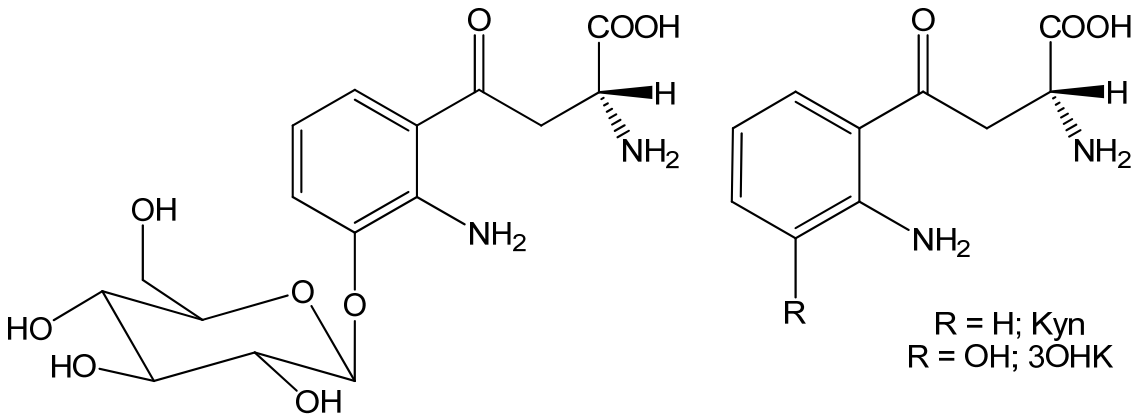
1.3 UV Filters

The human lens contains a class of protective, normally free in solution, small molecules collectively known as UV (ultraviolet) filters. These molecules absorb light in the 300-400 nm range (predominantly in the UVA spectrum), and protect the lens and retina from UV damage [43]. The majority of all UV radiation entering the eye in that range is absorbed by the UV filters, hence preventing damage from occurring to the retina [44, 45]. These compounds are constantly synthesised through the Kynurenine pathway in the lens epithelium [46], with production of UV filters slowly diminishing as a person ages [17]. Some of the first identification of [47] and quantification of UV filters in the human lens were performed by Van Heyningen in the early 1970s [48], who identified three of the four most common UV filters. The second most abundant UV filter was not identified until 20 years later by Truscott *et al.* in 1994 [49]. It has been proposed, and further evidence is provided in this thesis, that these UV filter compounds contribute to the protein

oxidation, cross-linking, and lens discolouration observed in age related nuclear (ARN) cataractous lenses [50].

1.3.1 UV filters in the human lens

In order of abundance, the main UV filters in human lenses are: 3-hydroxykynurenine-*O*-β-D-glucoside (3OHKG), 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid-*O*-β-D-glucoside (AHBG), kynurenine (Kyn) and 3-hydroxykynurenine (3OHK) [26]. The main UV filters differentiate themselves from other UV filters (and UV filter like molecules) in that they are present in lenses of all ages and generally decrease in concentration as a person ages [26]. Two other significant UV filters include glutathionyl-3-hydroxykunurenine-*O*-β-D-glucoside (GSH-3OHKG) and its derivative 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid-*O*-diglucoside (AHBDG) [51]. Though GSH-3OHKG has been found in concentrations higher than AHBG, Kyn and 3OHK, it has predominantly been observed in older, damaged and cataract lenses [26]. The other minor UV filter metabolites, as has been found in this study, generally increase in concentration as a person ages. 3OHKG, Kyn and 3OHK are all unstable under physiological conditions and can undergo non-enzymatic deamination at physiological pH forming α,β-unsaturated carbonyl compounds, which are highly reactive compounds [52]. These compounds can then react with nucleophiles, including lens proteins [50]. It is believed that upon binding to the lens proteins, these same protective UV filters become photosensitisers. Upon further oxidation they have been shown to cause damage to the lens crystallin proteins, essentially damaging their specialised quaternary structure through cross-linkage, truncation and dehydration of lens proteins [50, 53-56]. In understanding the role that UV filters have at the molecular level in the development of ARN cataract, it may become possible for preventative and/or therapeutic treatments to be developed so as to minimise the onset of this disease in older people.



Scheme 1.3: Major UV filters in the human lens: 3OHKG, Kyn and 3OHK.

1.3.2 Colouration, fluorescence and opacity of human lenses

At birth, young human lenses are colourless. As that person ages, the lens undergo a gradual increase in colouration due to the synthesis of 3OHKG and 3OHK, towards a pale yellow colour [57]. The level of light absorption by the human lens increases with increased adsorption in the 400–500 nm (blue to green) range [58]. Likewise, an accumulation of pigmentation takes place with age, and from this change in pigmentation cataracts are categorised and described in the literature. An early scale for this colouration was suggested by Pirie, with a gradation of the level of opacity from 1 to 5 (pale yellow through to dark brown discolouration) [59]. This classification system has been refined, and the modern equivalent is the Lens Opacity Classification System III (LOCS 3) [60]. It is known that UV filters bind to lens proteins (predominantly colourless crystallins) and discolour them. It is believed that that cataract discolouration occurs because of this semi-permanent binding and the resulting protein damage [50, 61] and protein glycation [62, 63]. Along with the colouration of the lens, the fluorescence of the human lens also increases with age, with two main emissions being reported in the blue (maxima 365 nm / emission 420-440 nm) and green (maxima 435 nm / emission 500-520 nm) regions. Over time, the optical density of the lens also increases, leading to increased scattering of light as it enters the lens (as opposed to true absorption of the light) [64]. Cumulatively, all these changes to the lens first affect the perception of colour by the individual, followed by a gradual blurring of the vision and ultimately ending with blindness.

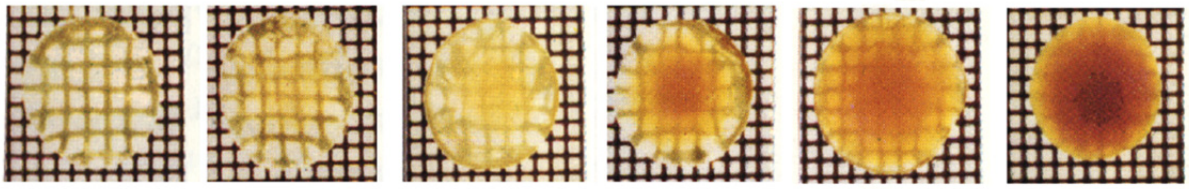


Figure 1.2: Normal lens and cataract human lenses.
Normal lens, followed by Pirie/LOCS 3 cataract type I, II, III, IV and V (left to right) [33].

1.4 Natural changes in the lens with age

The proteins in the lens nucleus are formed mainly whilst in utero. As there is little or no protein turnover throughout a person's life, these proteins gradually become modified and essentially damaged over the lifetime of an individual [43]. There are both internal and external factors affecting this modification, and many of the currently known internal factors occur naturally (*e.g.* oxidative stress) and are largely unavoidably. Hence, it would be of immense value to identify what the external factors are, and by what mechanism they interact, as they are potentially avoidable and controllable (*e.g.* protection from UV radiation).

1.4.1 Formation of a lens barrier

The evidence is that a significant factor in the rapid aging of the lens from approximately 50 years of age is due to the decreased transport of nutrients and antioxidants (specifically GSH) into the lens nucleus from the metabolically active outer cortex and lens epithelium cells [17]. The fibre cells of the nucleus rely on the transport of water soluble nutrients, such as GSH, from the metabolically active outer cortex and epithelium into the lens nucleus [65]. The human lens does not have a vascular system, and has little extracellular water (approximately 5% of the total lenticular water), so diffusion through the gap junctions is the major mode of transportation into the lens. From around middle-age, however, a barrier appears to the diffusion of these small molecular nutrients into the lens nucleus [66, 67]. The approximate location of this lens barrier is believed to be at the junction of the nuclear and cortical region, though at this time further study is required to determine the molecular mechanism by which it functions [66, 67]. As a result of this barrier, unstable UV filters, ascorbic acid, NAD(P)H and GSH are retained for a longer period within the nuclear region as their efflux from the nucleus is impeded. This increased concentration of unstable UV filters (and

inhibition of antioxidant turn-over) may be part of the reason for the greater extent of decomposition and reactivity of those compounds whilst in the nucleus (in contrast to the cortex) [68] and it is speculated that hence there is a diminished chance of any damage being repaired. Likewise due to this inhibition, the concentration of the available GSH antioxidant decreases over time in the nucleus, so its response to oxidative stress also reduces [67]. As a consequence of the decreasing levels of free GSH, deaminated UV filters and ascorbic acid are believed to react with and permanently modify the long lived proteins in the human lens [32, 69, 70]. These modifications (*i.e.* damage) slowly accumulate, altering the tertiary protein structure and function, and thus are believed to be a precursor to age related nuclear cataract [71, 72].

1.4.2 The antioxidant glutathione in the lens

GSH is present in the lens nucleus at approximate concentrations of 4.5 mM in the young lens, and this concentration decreases to approximately 1 mM by the time an individual reaches their 90s [73]. The concentration in the cortex of the lens also decreases, but to a lesser degree (from approximately 6 mM to 3 mM) over the same time period [26, 74]. It is believed that this greater decrease in the nucleus is as a result of barrier formation between the nucleus and cortex as a person ages [27, 74-76]. Concurrently, the activity of γ-glutamylcysteinyl synthetase in the human lens also decreases with age to low levels, and it is reasonable to assume that it is at least in part responsible for the decline of GSH content with age in the lens [77-80]. Another cause for part of the decline in GSH levels may be due to the formation of GSH-3OHKG, GSH-Kyn and GSH-3OHK, through the Michael addition of GSH to the respective deaminated Kyn-backbone UV filters [81, 82]. GSH-3OHKG levels have been found to increase significantly in human lenses after approximately 50 years of age, from small/negligible quantities until its concentrations are of a similar order to the most abundant UV filter 3OHKG [26, 81]. The concentrations of GSH-Kyn and GSH-3OHK also increase with age, though to a lesser extent [81]. Interestingly, the activity of glutathione reductase (which reduces glutathione disulphide) in the lens, remains relatively constant throughout a person's lifetime, giving further evidence to the closed nature of the lens [79]. Hence, as both the concentration of and activity levels of the antioxidant GSH decreases the most in the nuclear region of the lens, it becomes readily

apparent that the proteins in that region should be more susceptible to oxidative damage and post-translational modifications (in contrast to the cortex of the lens).

1.4.3 The modification of proteins in the lens

It is important to consider the types and extent of post-translational modification in the human lens. Broadly, the various modifications fit into the categories of neutral/subtractive modifications (examples being truncation or racemisation), which in general seem to have a lesser effect on the functionality of lens crystallins; and additive modifications (examples being glycosylation, phosphorylation, methylation, acetylation, carbamylation and UV filter modification), which add potentially unstable molecules to the polypeptide, and are generally believed to have a much greater effect on the functionality of the lens crystallins. Modification is likely to influence the conformation, aggregation state and solubility of the lens crystallins. The greater the level of alteration to the crystallin's polypeptide (and particularly to the functional groups of its amino acids), then the greater likelihood of colouration of the lens proteins, aggregation and loss of solubility, increased light scattering and the eventual loss of its transparency. It is believed that gradual changes to proteins of the lens nucleus are what lead to the development of ARN cataract in humans.

1.4.3.1 Neutral and subtractive modifications of crystallins

The main neutral and subtractive modifications observed with lens crystallins include truncation, racemisation and deamidation. The net effect of deamidation is a 1 Da addition, and over time this can affect the functions of a protein. The deamidation of glutamine and asparagine leads to the formation of a polar carboxylate anion and this change in charge may result in different ionic interactions in the protein (hence causing a change in protein conformation) [83]. The level of deamidation is linked to the age of a protein and is one of the major age related post-translational modifications found in the human lens [84]. Though it is more abundant in the water insoluble proteins of older lenses, it is not the only change observed in older lenses [3, 85]. Truncation of lens crystallins, at both the N- and C-termini of the α-, β- and γ-crystallins, increases as a person ages [86-88]. This truncation of lens crystallins is not unique to humans, as it has also been observed in bovine crystallins [89-91]. Though βB1- and αA-crystallins with truncated N- and C- termini display different protein aggregation and chaperone

activity than their native proteins, their substantive functional properties remain largely unchanged, specifically transparency, water solubility and reactivity [91, 92]. The last major type of neutral/subtractive modification is racemisation of the L-amino acids in lens proteins to their D-amino acid equivalents. Racemisation is a very slow that occurs in proteins, with for example L-aspartic acid having been observed to undergo racemisation at a rate of 0.14% per year in both normal and cataractous lenses [93, 94]. Hence, racemisation is a good marker for the aging process [95]. Racemisation in the lens is believed to occur spontaneously through the removal of the α-carbon proton, therefore forming a planar carbanion [96]. The main factors that affect its occurrence are the position of the amino acid in the peptide chain, the functional group on the amino acid and the position of the amino acid in the protein's tertiary structure [97]. The racemisation of tyrosine (Tyr) should be particularly noted in regards to lens proteins as Tyr residues affect the hydrophobic interactivity within proteins [98].

1.4.3.2 Simple additive modifications of crystallins

As opposed to neutral/subtractive modifications, additive modifications can have a much greater impact on the shape and functionality of a protein. A number of the simpler additive modifications, including carbamylation, acetylation, phosphorylation and methylation occur naturally or spontaneously in the lens and have not been observed to cause significant damage.

Carbamylation has been observed on more than 50% of the available N-termini on γB-, γC- and γD-crystallins of adult lenses. In addition, a further 10-12% of these carbamylated crystallins also have acetylation [99]. In very young lenses (11 days old), carbamylation and acetylation have been observed on approximately 2-3% of N-termini on γB-, γC- and γD-crystallins. The presence of these modifications in such young lenses suggests that they may not have such a significant effect on protein function, transparency and solubility, particularly as the γ-crystallins make up only a small percentage of the overall crystallins present in the eye [99]. On the C-terminal there is a particular acetylation of importance that occurs at Lys-70 on the αA-crystallin, the significance being that it occurs on of the major crystallins in the human lens, and its location may affect the chaperone function of the crystallin [100].

Two (apparently) less functionally significant modifications include phosphorylation, which occurs on the serine, threonine and tyrosine residues of α-

and β-crystallins at approximate levels of 5% [88, 101], and methylation, which occurs on exposed cysteine residues at levels of 50% on γD-crystallins and 12% in γC- and γB-crystallins [99]. Generally, the extent of methylation and phosphorylation is similar throughout the nucleus and the cortex of the lens, with a slightly greater level of methylation being present in the nucleus of the γS-crystallin [102]. It has been suggested that methylation, as well as glutathionylation with GSH, may be of benefit to crystallins by inhibiting the formation of disulphide bonds within the protein itself, or with other proteins [76, 103].

1.4.3.3 Glycation of lens proteins

The spontaneous modification of lens crystallins through glycation and by modification with lens UV filters appear to have a much greater impact (compared to the above modifications) on the functional properties of lens crystallins, and leads to damage through colouration, increased fluorescence and cross-linkage of the affected crystallins [104-109].

Glycation occurs *via* a Maillard reaction between free amino groups on the crystallins (lysine and arginine) with reducing carbohydrates (like glucose), or dicarbonyl molecules (like glyoxal and methylglyoxal), forming a ketoamine adduct through an Amadori rearrangement [110, 111]. These ketoamine adducts are relatively stable, however they may undergo further oxidation forming compounds known as advanced glycation end (AGE) products [112], which accumulate over time in long lived proteins (such as lens crystallins) and upon sufficient accumulation result in colouration, fluorescence and cross-linking of the protein [104-109]. These glycations are generally spontaneous, however diabetes mellitus is a risk factor for protein glycation in the lens, with diabetics having approximately twice as much glucitol-lysine present when compared to control samples [113].

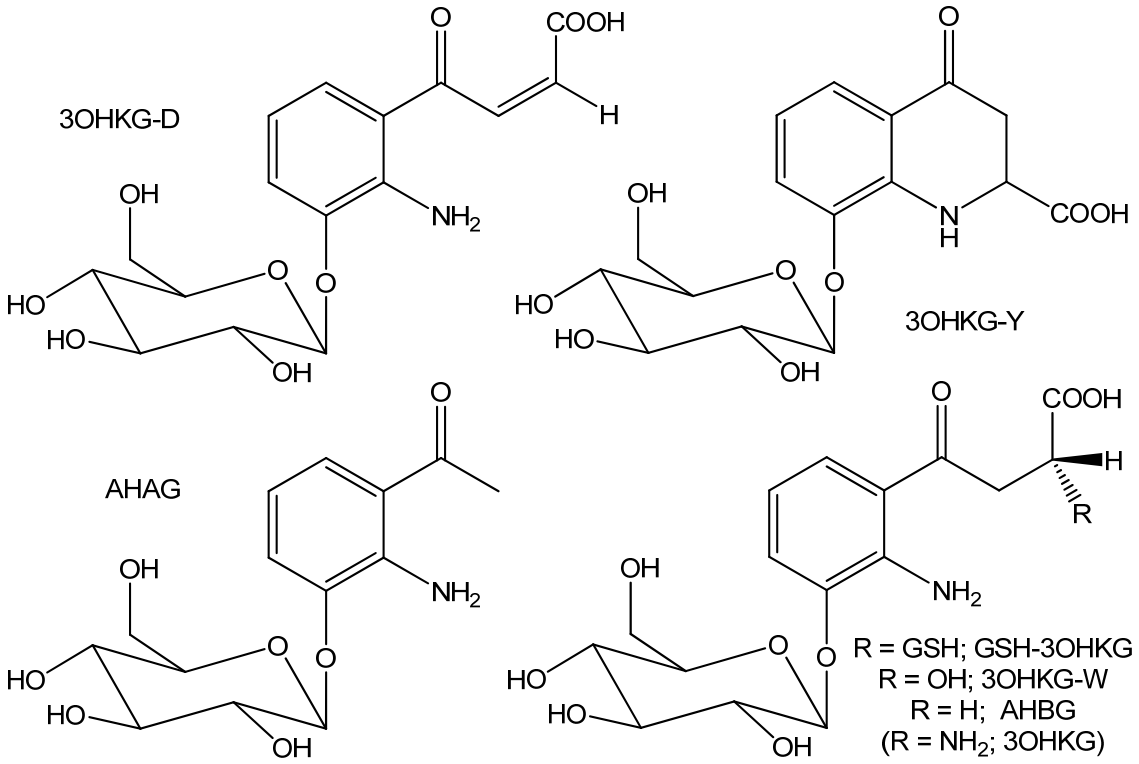
1.4.3.4 Effects of ascorbic acid on the aging lens

Though ascorbic acid is an essential non-enzymatic antioxidant in the lens, as the lens ages and various cell control mechanisms are weakened, the various oxidation products of ascorbic acid can quickly react *via* glycation with lens proteins leading to similar types of damage as caused by carbohydrate glycation (colouration and fluorescence) [114]. When these reactions are replicated *in vitro*, the modified proteins have similar appearance and similar absorbance/fluorescence profiles to those found in older and cataractous human

lenses [62, 70, 115, 116]. Subsequently, upon UV irradiation of these ascorbic acid products it has been observed that further aggregation, copper chelation and the production of reactive oxygen radicals (superoxide and singlet oxygen) occurred in the modified proteins [117-122]. As ascorbic acid is more reactive and in higher concentrations than glucose (1-2 mM, compared to less than 1 mM for glucose), post-translational modification by ascorbic acid may be much more significant than with glucose. Likewise in the presence of air, ascorbic acids glycates lens proteins approximately nine times faster, and forms cross-linkages 90 times faster than either glucose or fructose can [63, 110].

1.4.3.5 Effects of UV filter modification on lens crystallins

It is believed that a major yet possibly preventable source of lens protein damage arises from the protective UV filters found in the human lens. Similar compounds are found in the lenses of primates and other longer lived animals. All the major UV filters are synthesised from tryptophan (Trp), and with the exceptions of AHBG and GSH-3OHKG, all can deaminate under physiological conditions forming unstable compounds that can bind to lens proteins and can then act as photosensitisers [50, 52]. The instability of 3OHKG, 3OHK and Kyn in the human lens is well documented, with metabolites and derivative UV filters having been observed from each of these UV filters [49, 68, 81]. Most derivative compounds occur as a result of the binding of the UV filter (to itself or to another molecule) upon deamination. A summary of the main metabolites and derivatives is presented in Scheme 1.4 [49, 68, 81]:



Scheme 1.4: UV filter metabolites in the human lens: 3OHKG-D, 3OHKG-Y, AHAG, GSH-3OHKG, 3OHKG-W and AHBG. The latter three are all derivatives of 3OHKG, *via* the unstable metabolite 3OHKG-D.

In previous protein binding studies, it has been established that Kyn, 3OHK and 3OHKG can bind to cysteine residues in the lens crystallins and that these adducts are photosensitisers of oxidative damage [18, 50]. Upon the illumination of lens proteins (modified with either Kyn, 3OHK or 3OHKG) with UV light in the 300-400 nm range in the presence of air, all three UV filters caused a significant increase in the formation of peroxides [18, 50]. Though it has been long hypothesised that 3OHKG has a part in the photo-oxidation of lens proteins in a similar manner to Kyn and 3OHK (particularly as it is present in much higher quantities than either 3OHK or Kyn in the human lens) [123], experimentation has been difficult as 3OHKG is not available commercially. 3OHKG (along with Kyn and 3OHK) favour binding to the Cys residues in lens crystallins, with binding being identified on the βB1- and γS-crystallins (NOTE: referred to as βS-crystallin in newer literature) [72]. The main crystallins in the eye αA- and αB-crystallins, only have one and zero Cys residues (respectively), whilst βB1-crystallin has two Cys residues and γS-crystallin has seven Cys residues, hence it is not surprising that binding of 3OHKG was only found on these two lower concentration crystallin types. Nevertheless, 3OHK (which is a smaller and less hydrophilic molecule)

appears to favour binding to the one Cys residue in the αA-crystallin [124]. Studies have also shown that 3OHKG is able to bind to free cysteine molecules present in the lens (albeit in picomolar quantities), forming a highly reactive UV filter derived metabolite cysteine-L-3-hydroxykynureine *O*-β-D-glucoside (Cys-3OHKG) [125].

1.5 Age Related Nuclear Cataract

Age related nuclear (ARN) cataract (the most common form of cataract) is a major cause of human blindness, and besides macular degeneration is the leading cause of blindness [126-128]. The genetic make-up of an individual is a factor, but not the only one and not necessarily the most important factor with a person's health and environmental and geographic locations (*i.e.* living closer to the equator where there is more sun exposure) having a much greater affect [2, 129]. Other cataract types (apart from nuclear) include cortical, sub-capsular and congenital cataract. Most are disease related secondary cataracts, with diabetes (cortical, sub-capsular cataract) or herpes group virus infection (congenital cataract) in an immunosuppressed mother being common causes. Steroids and phototoxic drugs are also factors in sub-capsular cataract.

In contrast to the other types of cataract, ARN cataract is believed to be a result of the human lens deteriorating with age and being unable to repair itself. An estimated 75% of people develop cataract in Australia by the time they reach their 80s or above [130]. If any particular individual should live long enough, then they will eventually develop ARN cataract. As a condition that predominantly affects the elderly, and with the increasing average age of the world's populations, cataract treatment will become an even greater burden on the health budgets of both the developed and developing world. In intermediate or mild cases of cataract, the vision of the individual can be improved with corrective lenses, however this improvement is only temporary as the cataract gradually becomes more severe over time. In advanced stages, when the individual is partially or completely blind, the only treatment is surgical removal of the lens and its replacement with an artificial lens. Thus the benefits of understanding the mechanisms involved in the development of ARN cataract have enormous potential, and are necessary if a less invasive treatment is to be developed. Even if the onset of ARN cataract were delayed for 5-10 years (through a greater understanding of the underlying causes and then educating people on effective

preventative behaviour) the benefits of this research would have an immense impact on the health and living standards of people world-wide.

When healthy, the normal human lens is almost colourless, is transparent, and all the crystallins forming the lens are completely in solution. As a cataract in the nucleus develops with age, the lens becomes opaque from the centre outwards, and results in regressively worsening vision with distortions in the perception of colours and eventually leads to blindness. The other symptoms of ARN cataract include blurred vision, light sensitivity, reduced night vision, seeing halos and the need for frequent changes in corrective lens prescriptions.

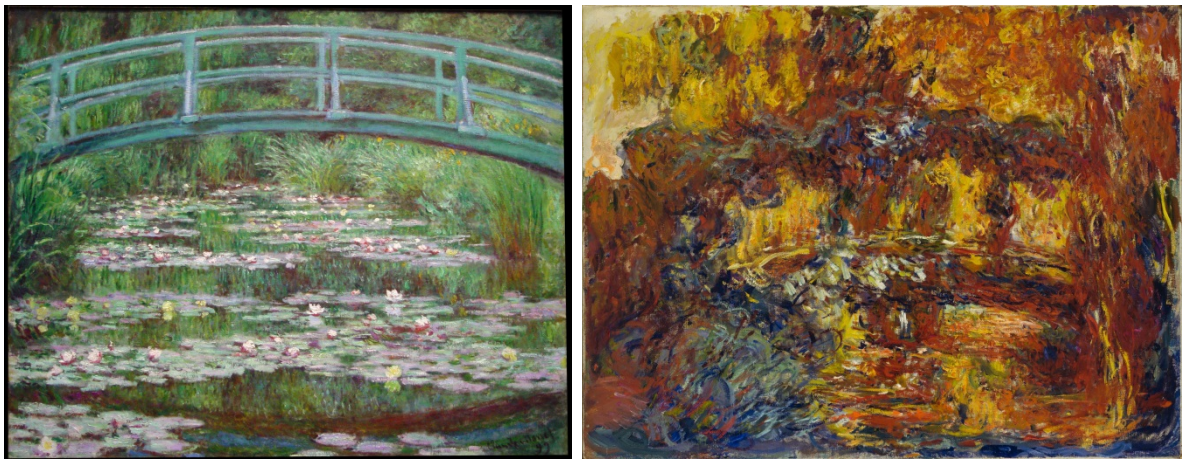


Figure 1.3: "The Japanese Footbridge" painted by Claude Monet, the one on the left in 1899 [131], and the one on the right in 1922 [132].

A beautiful illustration of the effects that cataract has on a person's vision, particularly their perception of light, is provided by the Claude Monet's two paintings of (and both titled) "The Japanese Footbridge", the first being painted in 1899 whilst the lenses in his eyes were still healthy, and the second painted around 1922 after he had developed cataract (**Figure 1.3**).

1.5.1 Changes in the lens with age

As mentioned earlier, ARN cataract is the culmination of multiple forms of damage experienced by the lens as a result of aging, and is essentially the natural point reached by an old lens. The most obvious and easily quantifiable changes that occur in the human lens with aging (or the slow and gradual development of ARN cataract) are the progressive increases in colouration from yellow to brown,

fluorescence and precipitation of the normally clear hydrophilic crystallins proteins in the lens, beginning in the centre of the lens (the nucleus) [59, 133-136]. Interestingly, the level of colouration in the lens strongly correlates with the level of protein damage on the microscopic level, namely the oxidation and cross-linkage of the lens crystallins [137]. As the level of colouration and opacity directly relates to the level of blindness, it is the main factor in classification of the cataract in the Pirie / LOCS 3 cataract scales [59, 60].

1.5.1.1 Oxidation

Oxidation of lens proteins is a major feature of ARN cataract and is believed to be the main source of all the subsequent negative changes in the lens [17]. As the lens ages it is less able to protect itself from oxidative damage due to wear and tear, however it is also possible that the lens experiences an increase in the formation of specific oxidants like superoxide and singlet oxygen [33]. At first, oxidation appears to occur more rapidly in the lens nucleus (due to a number of factors) and then slowly spreads out from the nucleus and eventually into the cortex by the continued addition of molecules to an ever increasingly insoluble network of damaged (adversely modified) proteins [33].

1.5.1.2 Decreases in protective compounds

Numerous changes have been detected in the aging lens, and likewise in the development of ARN cataract. Some of the most significant changes include a marked decrease in glutathione concentration (with decreases up to 80% in ARN cataract) [138, 139] . At the same time, the levels of glutathione disulphide and GSH-3OHKG (the glutathione adduct of the main UV filter 3OHKG) increase in the lens [29, 51, 140]. The other major non-enzymatic antioxidant ascorbic acid also decreases in concentration, though to a lesser extent, and as it can also be a pro-oxidant, the reduced glutathione cannot regulate it as effectively [141]. It is also noteworthy that the other major protective compounds that decrease with age (and particularly in ARN cataract) are the UV filters, with the most abundant UV filter 3OHKG decreasing by approximately 12% for each decade in an individual's age [26, 67]. Glutathione (along with NADH) is also responsible for protecting the lens crystallins from modification by the unstable UV filters, so its protective function in the lens is much more significant [69]. There is evidence that these decreases may be as a result of the formation of the lens barrier between the nucleus and

the cortex, as the decreases in the cortex compared to the nucleus are much lower with age [67]. It is clear that glutathione plays a crucial role in protecting lens crystallins from oxidative damage, hence it is undeniable that its decrease with age will have adverse effects on the health of the lens [142].

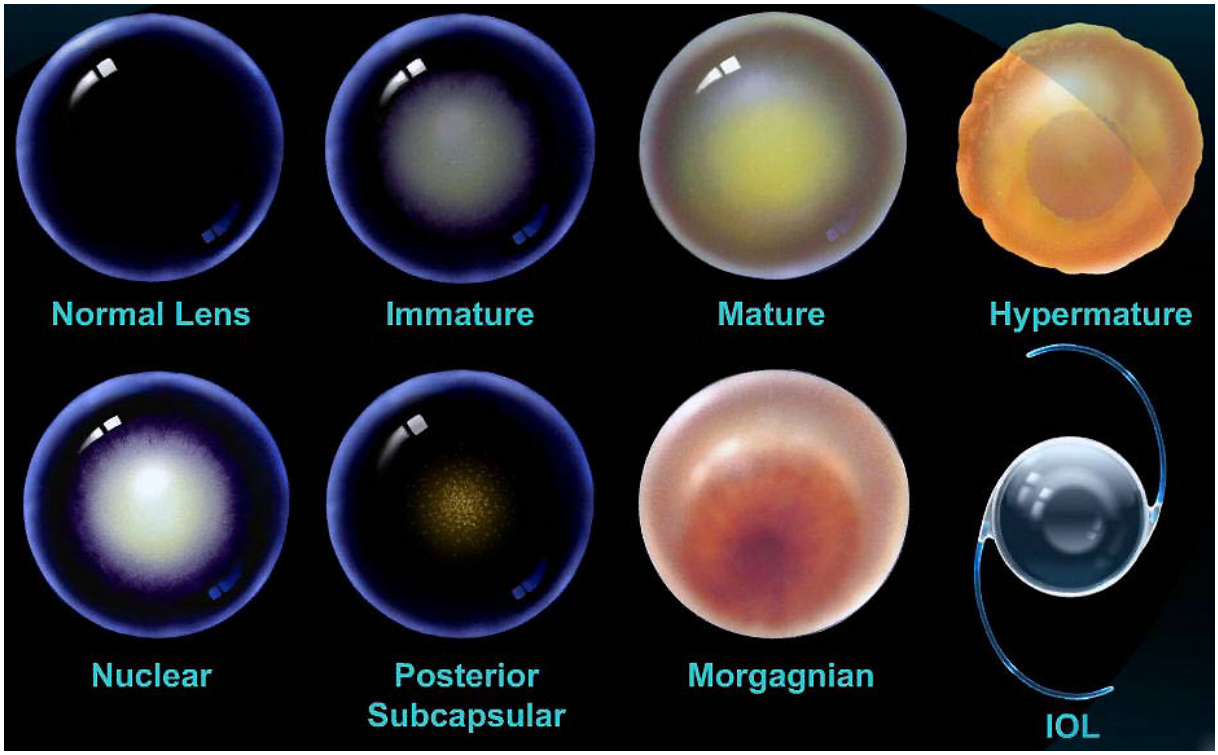


Figure 1.4: Different types of cataract. In extremely mature cataracts (Morgagnian cataract), the cortical proteins dissolve hence only leaving a small section of the nucleus (which sinks to the base of the lens cavity). The last image is of an intraocular lens implant (IOL) [143].

1.5.1.3 Oxidation of sulfhydryl groups

As referred to previously, the sulfhydryl group on cysteine is one of the most reactive functional groups in a protein, and more than half of them are oxidised in advanced ARN cataract [137, 143]. Methionine (Met) residues are also oxidised, though only to around 50%. This may be partly explained by the fact the Met is hydrophobic, and usually found towards the centre of a folded protein [144], however the lens crystallins are naturally truncated over time, and upon breaking down into smaller fragments in older age, more Met residues become exposed [53]. Along with glutathione, cysteine residues can also react (forming a disulphide bond) with free cysteine molecules (which are present in minute amounts in the lens) and with other proteins or peptides containing cysteine [34, 142, 145].

1.5.1.4 Aggregation

As the lens ages and develops ARN cataract, the amount of protein aggregation and colouration increases in the nucleus forming larger and less water soluble proteins [146, 147]. Other parts of the protein, namely the N-terminal of α-crystallins, are also involved in the colouration of the lens proteins through the formation of cross-linkages. It is believed that this cross-linking is as a result of the α-crystallin's chaperone function, which encourages binding to the denaturated crystallins in older lenses [148]. As these crystallin aggregates increase to sizes greater than 50 MDa, the lens loses its transparency and likewise develops a yellow colour [149]. These large protein aggregates make up approximately 50% of the coloured proteins in lenses with advanced cataract, and the hardening in these aggregates is so great that they resist the effects of strong denaturants like 6 M guanidine hydrochloride and 8 M urea and are essentially insoluble [135, 137]. As is the case with most changes involved in ARN cataract, the aggregation occurs primarily in the nucleus and then extends out into the cortex, hence the greatest effect on vision and colour perception is in the centre of the lens. Though the process has not been fully characterised, it is believed that oxidised Trp and advanced glycation end (AGE) products are involved in the formation of these yellow chromophores [44, 108, 150-152]. The cross-links due to AGE products do not involve disulphide bonds, and mainly involve arginine (Arg) and lysine (Lys) residues in the affected protein [109, 111]. The level of AGE product cross-linking is greatest in the water insoluble protein fraction of cataractous lenses [153-155].

1.5.1.5 UV filter binding

The four main UV filters (Kyn, 3OHK, 3OHKG and AHBG) are all unstable, however 3OHK is particularly unstable as it is an *ortho*-aminophenol which can readily auto-oxidise when in an oxidative environment, regardless of the pH of that environment [71]. ARN cataract, with its reduced levels of antioxidants (glutathione, ascorbic acid etc.) provides such an environment. 3OHK can undergo a complex reaction process, forming a reactive quinone imine intermediate. This quinone imine can react with itself (to form the metabolite 3OHK yellow), but can also react with functional groups on crystallins, thus creating a redox reactive substrate on the protein [156, 157]. Multiple oxidation products formed from 3OHK have been detected with absorbance maxima in the

mid 400-500 nm range [158-160]. Most of these oxidation products (for example, dihydroxanthommatin, 4,6-dihydroxyquinolinequinone carboxylic acid**,** benzoxazole and benzimidazole) have strong red-brown colours, with the most common product formed, xanthommatin, having been found to be the main cause of the brown pigmentation in ARN cataract lenses [161-165]. 3OHK is known to form yellow/coloured protein products when incubated with lens products under both oxidative and even physiologically neutral conditions, and these products have similar appearance and properties to the modified protein products in ARN cataract lenses [71, 124, 166]. The 3OHK azole oxidative products cross-link the peptides Gly-Lys, Gly-Gly and Thr-Lys-Pro-Arg [158, 167].

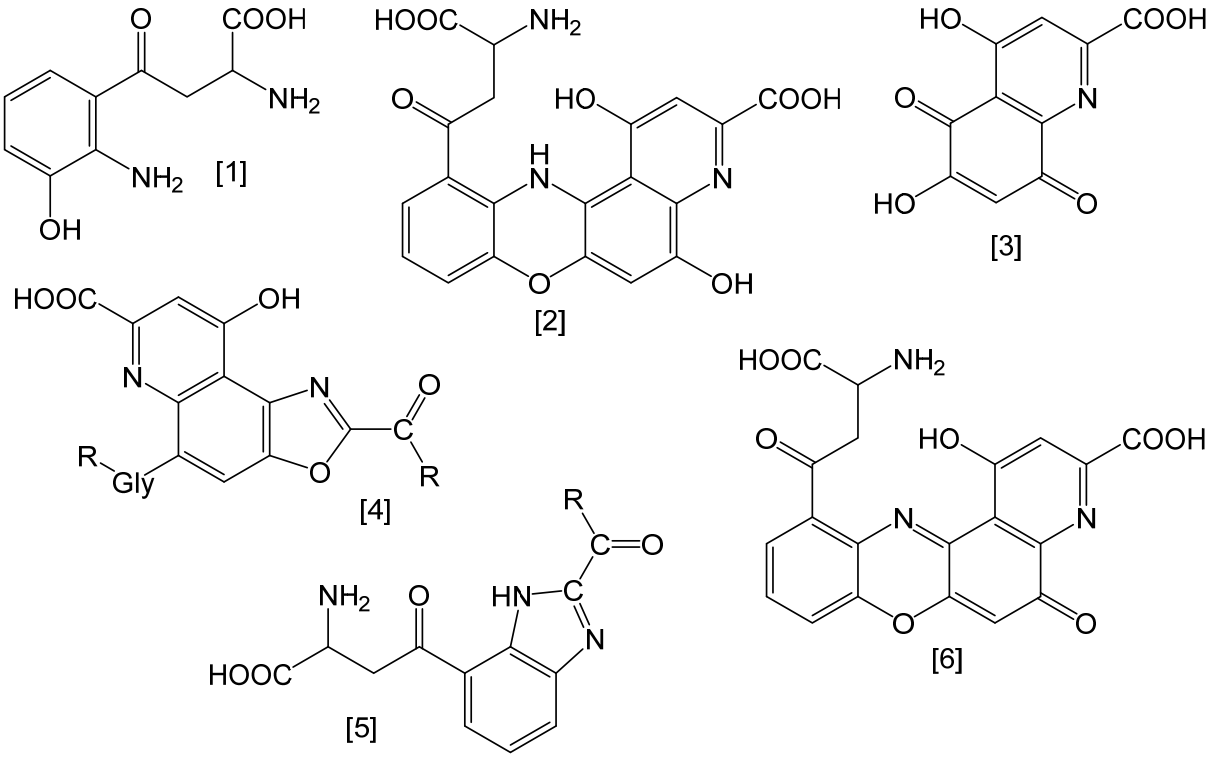


Figure 1.5: Oxidation products of 3OHK

[1] 3OHK, [2] dihydroxanthommatin, [3] 4,6-dihydroxyquinolinequinone carboxylic acid, [4] R=H benzoxazole, R=CH_3 methylbenzoxazole, [5] R=H benzimidazole, R=CH_3 methylbenzimidazole and [6] xanthommatin [159, 160, 165].

Unlike 3OHK, Kyn when bound to lens proteins produces a pale white product that visually is not very distinguishable from unmodified crystallins. The metabolite 3OHKG-D (or 3OHKG under higher pH conditions) both produce a more strongly coloured yellow product, resembling the modified proteins found in ARN cataract [50]. Though not required for cross-linking, reduced metal irons

enhance the level of cross-linking and are believed to lead to greater aggregation and precipitations of crystallins [167, 168].

1.5.2 Other factors in age related nuclear cataract development

There are a number of means by which the lens proteins may become oxidised [169]. These factors include both internal (or the failure of internal) lens processes, as well as external factors like deficiencies in nutrition, ionising radiation, UV light and other external pollutants to which the eye may be exposed [170-172]. Reactive oxygen species like superoxide, hydrogen peroxide and singlet oxygen are known to be significant in the modification of the lens proteins and ARN cataract [50], however the sources and exact mechanisms by which these oxidants are created are not well understood (due to their interrelated formation) [173].

1.5.2.1 UV light

UV light has been long postulated as a major factor in the development of ARN cataract, with links being suggested for nearly 90 years [174, 175]. Correlations have also been observed between the occurrence of ARN cataract and the geological placement of the individual, with greater incidences being recorded by individuals that have greater exposure to the sun [176-179]. UV light, which spans an invisible light range from 10 nm through to the almost visible 400 nm, is divided into four broad categories: UVA (400 – 320 nm), UVB (320-280 nm), UVC (280-100 nm) and extreme UV (100-10 nm). The sun produces UV light of all frequencies, however almost all UVC and extreme UV light is absorbed by the oxygen in the atmosphere, forming ozone in the process, whilst UVB is mostly absorbed by ozone. The majority of the UV light experienced by the surface of the earth is therefore in the 400-300 nm region, essentially UVA (or sometimes also called Near UV light) [16, 180]. Even if UV light below 300 nm were not absorbed by the earth's atmosphere, the cornea absorbs wavelengths below 300 nm, thus those wavelengths do not affect the lens or the retina [44, 181]. UV light in the 300-400 nm region can pass through the cornea into the lens, hence the role of the UV filters is to absorb that damaging light, protecting the retina from photochemical damage, and concurrently improving contrast sensitivity and reducing colour aberrations [180, 182]. UVA light is known to damage other cells in the body through the production of reactive photo-products. The shorter wavelength UVB, however, can directly damage DNA, proteins and other compounds in the body [183-185].

Though different to ARN cataract, cortical and sub-capsular cataracts have also been linked to UV light exposure, with sun exposure being a well-known risk factor [186-188].

1.5.2.2 Direct UV damage

For UV light above 300 nm that enters the eye, almost all of it is absorbed by UV filters, Trp (in proteins) and other free aromatic groups present in the lens solution. As direct damage to the lens proteins is unlikely (as the wavelengths of UV light are too long to be absorbed), the only photo-oxidative damage that UV light can induce is through an indirect process involving photosensitisers and the production of oxidants [122].

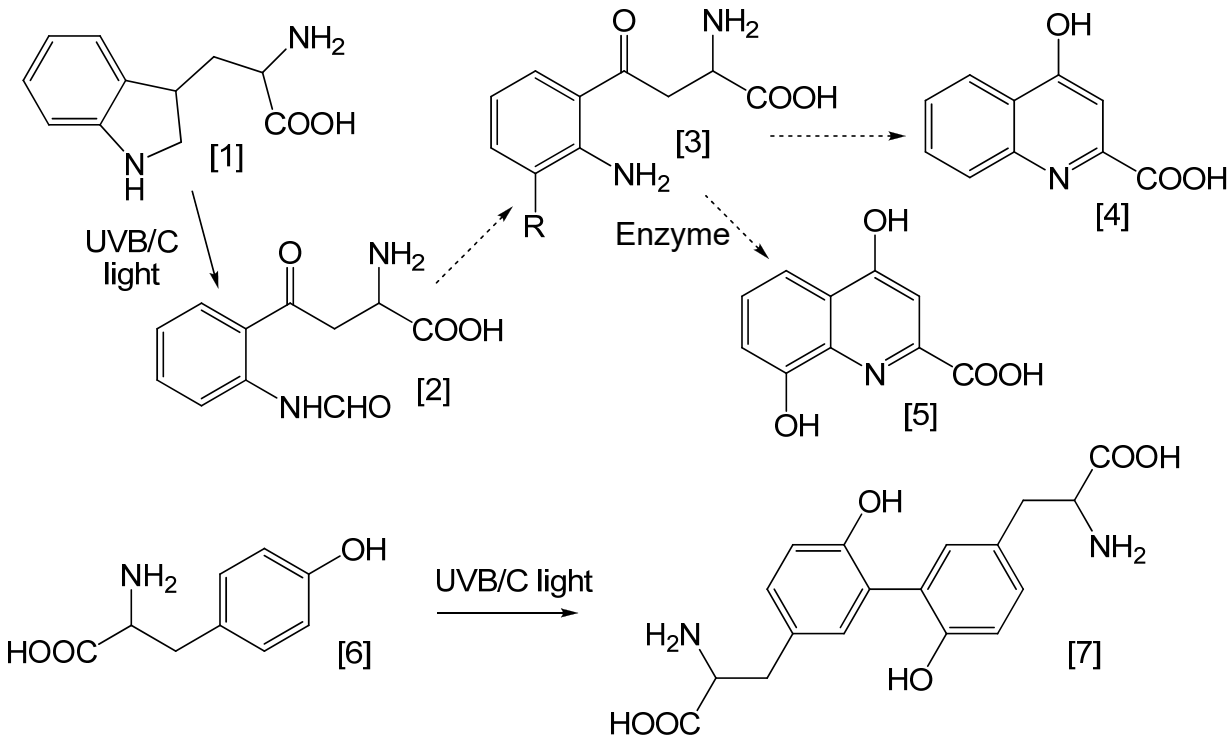


Figure 1.6: UVB/C direct irradiation products of Trp and Tyr residues [1] Trp, [2] *N*-formyl-L-kynurenine, [3] R=H Kyn, R=OH 3OHK, [4] kynurenic acid, [5] xanthurenic acid, [6] Tyr, and [7] di-Tyr [18, 189-195].

If UVB and UVC light were to directly hit the lens (without absorption by the cornea), they would be mainly absorbed by Trp and Tyr [195], producing *N*-formyl-L-kynurenine, kynurenic acid, di-Tyr and other similar by products, all of which are known to be present in the older nucleus and to increase with ARN cataract [18, 189-194]. Xanthurenic acid or its glucoside, two other compound linked

to ARN cataract, can then form enzymatically from 3OHK or 3OHKG respectively [193, 196]. *N*-formyl-L-kynurenine, kynurenic acid and xanthurenic acid are also likely to be involved in the generation of singlet oxygen, which would induce further damage to the lens [197-199]. However, damage *via* this direct route is unlikely as most UVB light (and lower wavelengths) is absorbed by the cornea. As the wavelengths required for direct damage to the lens cannot reach the lens, it is believed that oxidation by-products and lens damage found in ARN cataract must be occurring *via* a different mechanism, likely through photosensation [18, 50, 200].

1.5.2.3 Photosensitisers

A photosensitiser is a chromophore that can absorb light of a particular wavelength, producing an excited state of the chromophore. It's energy is then transferred to another species or compound of which molecular oxygen is a likely target, producing singlet oxygen, which is biologically very damaging [201, 202]. The Kyn based UV filters (Kyn, 3OHK and 3OHKG) are poor sensitisers of oxidative damage when in solution, however their photosensitising potential is believed to be much greater when they are bound to lens proteins [18, 50, 203]. It has been demonstrated that upon the illumination of lens proteins bound to 3OHKG, 3OHK and Kyn derivatives, increased colouration and fluorescence of the lens proteins occurs. When repeated in the presence of a free radical scavenger, the level of colouration and fluorescence was greatly reduced [50, 123, 204]. It is believed that formation of these highly fluorescent products is due to damage through covalent binding of the Kyn based UV filters to the lens amino acids. This binding of the free UV filters to the lens proteins can also help explain the gradual decrease of UV filters in the human lens with age [26, 204].

In a study by Parker *et al.*, when Kyn bound to lens proteins is illuminated with UV light above 305 nm, both hydrogen peroxide and protein peroxides (in lower concentrations) were detected [18, 205]. Hydrogen peroxide can oxidise Cys and Met, as well as (at a much slower rate) Trp and Tyr amino acid residues in lens crystallins, leading to lens opacity, increased formation of disulphide bonds and protein insolubility, all in a similar pattern to that observed in human ARN cataract [140, 206-208]. A number of other types of damage were also observed, with protein-bound Tyr residues being also oxidised to di-tyrosine (di-Tyr) and 3,4-dihydroxyphenylalanine (DOPA) when exposed to UV light. Like the previous changes to the lens protein, this modification of Tyr and phenylalanine residues in

proteins has been previously observed to increase with age, and to be most severe in cases of ARN cataract [209]. UVA sensitiser activity is mainly present in the water insoluble fraction of the human lens, with assays producing results equivalent to lenses damaged by longer term UV light exposure [210].

1.6 Aims of thesis

The aims of this thesis were:

- To determine the presence and structure of novel human lens Kyn-based UV filter metabolites;
- To determine the extent and location of UV filter binding in a human lens protein analogue (using bovine lens proteins); and
- To observe the effects of UV light on lens proteins when bound with unstable UV filters and in the presence/absence of oxygen.

CHAPTER 2

IDENTIFICATION OF 3-HYDROXYKYNURENINE *O*-B-D-GLUCOSIDE DERIVED NOVEL UV FILTERS

Purpose

3-Hydroxykynurenine *O*-β-D-glucoside (3OHKG) protects the lens from UV damage, and novel related species may act analogously. The aim of this study was therefore to detect, quantify and elucidate the structures of novel 3-hydroxykynurenine glucoside-derived UV filter metabolites present in the human lens.

Methods

Compounds were detected and quantified by LC-MS/MS in 24 human lenses of different ages, of which 22 were normal and two had cataract. Structures of these were confirmed through total synthesis and spectral analysis.

Results

3OHKG concentrations decreased with age in the lens nuclei, whereas the levels of three novel species: 4-(2-amino-3-hydroxyphenyl)-2-hydroxy-4-oxobutanoic acid *O*-β-D-glucoside (3OHKG-W), 3-hydroxykynurenine *O*-β-D-glucoside yellow (3OHKG-Y) and 2-amino-3-hydroxyacetophenone *O*-β-D-glucoside (AHAG) increased, though to different extents. In contrast, the concentrations present in the cortex of the lenses remained constant with age.

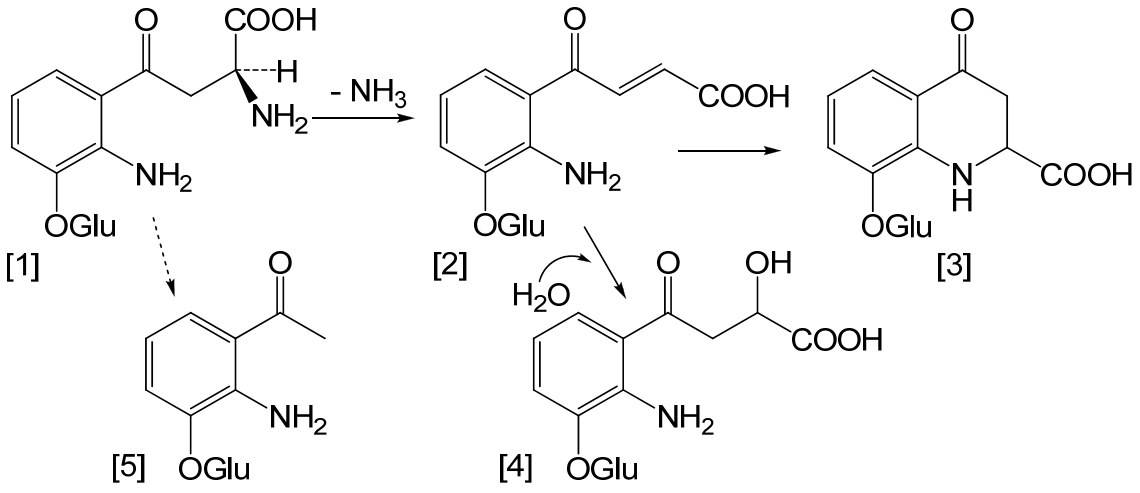
Conclusion

Three novel 3OHKG derived metabolites were detected in extracts from human lenses.

2.1 Introduction

Kynurenine (Kyn) based compounds are derived from the amino acid L-tryptophan (Trp) and have strong optical absorbance bands in the 300-400 nm wavelength range, also categorised as the ultraviolet A light region. Kyn and these related molecules are present in the human lens and protect it and the retina from UV-induced damage. The main mechanism by which they perform this function is by acting as physical quenchers of excited state species generated by incident radiation [47]. These compounds are collectively known as UV filters and occur

predominantly in primates [43]. They include (in order of abundance): 3-hydroxykynurenine *O*-β-D-glucoside (3OHKG, 2000-3600 pmol/mg), 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-β-D-glucoside (AHBG, 400-700 pmol/mg), Kyn (180-380 pmol/mg) and 3-hydroxykynurenine (3OHK, <100 pmol/mg) [46, 81]. 3OHKG, 3OHK and Kyn can deaminate at physiological pH values to form reactive α,β-unsaturated carbonyls [52]. The deaminated form of 3OHKG (3OHKG-D) appears to be a precursor of already known UV filters including the glutathione and cysteine adducts of 3OHKG and the reduced compound AHBG [82, 125]. The deaminated forms of Kyn and 3OHK have likewise been reported to form glutathione adducts and reduced compounds that are present in the human lens, albeit at pmol/mg protein levels [81]. Kyn and 3OHK are also able to undergo ring closure reactions, forming the known compounds kynurenine yellow and 3-hydroxykynurenine yellow [211], so it is hypothesised that the main UV filter 3OHKG may undergo the same type of reaction and ring close in a similar manner. It has been proposed that these UV filter compounds may contribute to the protein oxidation, cross-linking, and lens discolouration observed in age related nuclear (ARN) cataractous lenses [50]. Hence, the identification of other possibly reactive metabolites is important to create a better model of, and to further understand, ARN cataract.



Scheme 2.1: Proposed mode of formation of metabolites [4] 3OHKG-W, [3] 3OHKG-Y, [5] AHAG and [2] 3OHKG-D from [1] 3OHKG.

In this chapter the identification and quantification of three novel UV filters, found in human lenses varying from 18 to 84 years of age, are described; 4-(2-amino-3-hydroxyphenyl)-2-hydroxy-4-oxobutanoic acid *O*-β-D-glucoside

(3OHKG-W), 3-hydroxykynurenine *O*-β-D-glucoside yellow (3OHKG-Y), and 2-amino-3-hydroxyacetophenone-*O*-β-D-glucoside (AHAG). It is proposed that 3OHKG-W and 3OHKG-Y are derived from 3OHKG in the lens *via* the intermediate 3OHKG-D. AHAG is believed to be produced through the reverse-Aldol cleavage of 3OHKG, which though not a favoured reaction, can potentially occur spontaneously under mild basic conditions.

2.2 Results

Twenty four human lenses in an age range from 18 to 84 years were obtained from the Sydney Eye Bank. Twenty two of these were normal lenses (four of those twenty two were the second lens of the same donor) and two had developed ARN cataract. A larger number of intact cataract lenses could not be investigated in this study primarily due to improvements in techniques that result in cataract lenses being rarely removed whole, despite the overall increasing numbers of cataract surgery. Each whole lens was separated into its nucleus and cortex and the UV filters extracted as described previously using ethanol [212].

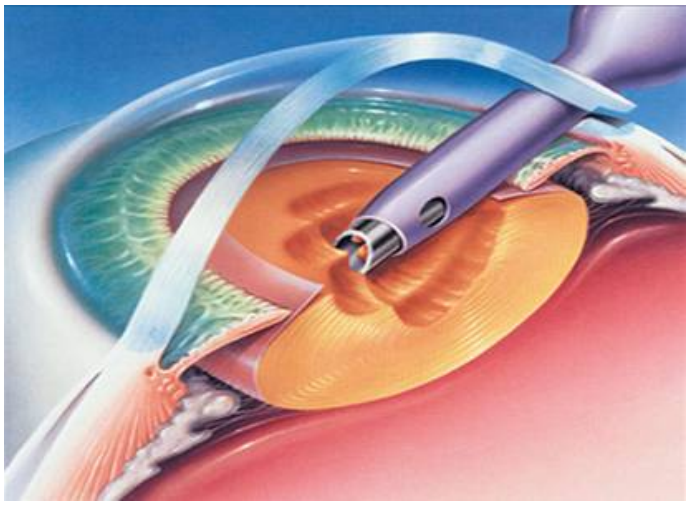


Figure 2.1: Modern cataract surgery - the cataract lens is broken up with ultrasonic vibrations and then sucked out with the same probe [213].

2.2.1 Mass spectrometric (LC-MS/MS) analysis of lens extracts

The lens extracts were centrifuged to remove high-molecular-mass compounds, lyophilised and then resuspended in acetonitrile/water before analysis. Materials present in the extracts were separated and analysed by reversed phase LC-MS/MS (ESI+) with data for the nuclear and cortex regions of the normal and cataractous lenses examined separately (**Figure 2.2 and Table 2.1**). ESI analysis

in positive mode confirmed the presence of 3OHKG *m/z* 387.1 (peak 1; Figure 2.1(a)), along with protonated molecular ions *m/z* 388.1 (peak 2; Figure 2.1(b)), *m/z* 314.1 (peak 3; Figure 2.1(c)), *m/z* 370.1 (peak 4; Figure 2.1(d)), *m/z* 314.1 (peak 5; Figure 2.1(c)), *m/z* 372.1 (peak 6; not shown) and *m/z* 370.1 (peak 7; Figure 2.1(d)). These peaks also showed fragment ions consistent with the loss of a glucose residue (162.0 Da).

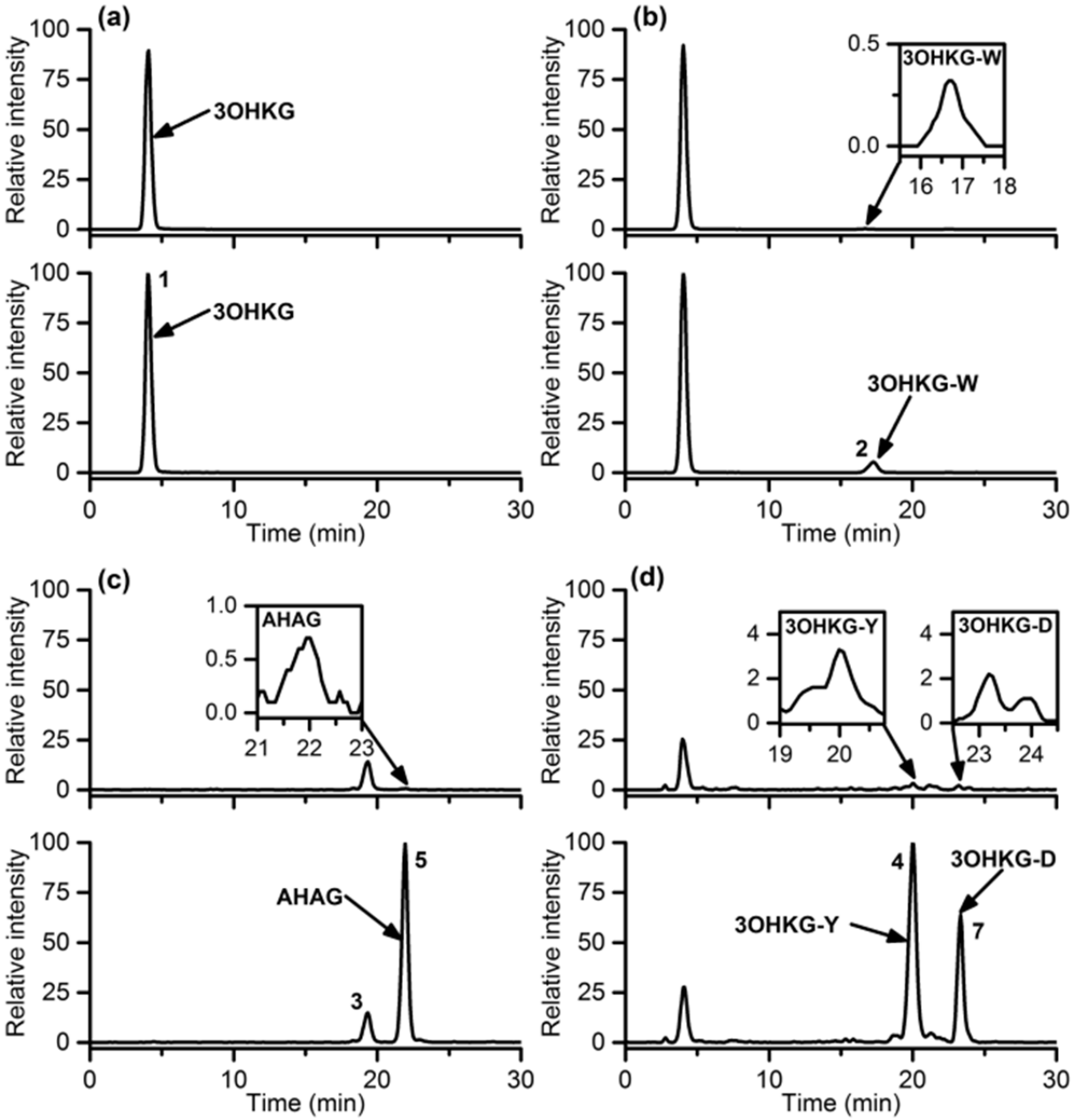


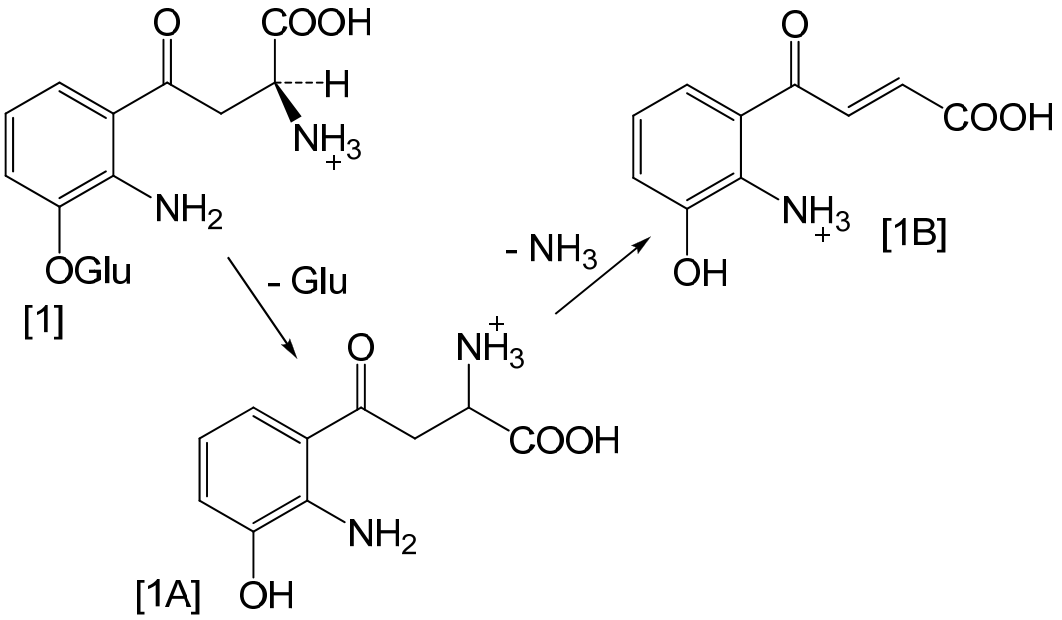
Figure 2.2: LC-MS chromatograms of novel UV filters (a) 3OHKG *m/z* 387.1 (b) 3OHKG-W *m/z* 388.1 (c) AHAG *m/z* 314.1 (d) 3OHKG-Y, 3OHKG-D, both *m/z* 370.1, in lens samples that are unspiked (above) or spiked with synthetic standards (below). Peak numbers correlate with text and Table 2.1. Insets show expanded peaks of interest in unspiked samples.

Table 2.1: UV filter LC-MS retention times, *m/z* M+H$^+$ and *m/z* M+H$^+$ of fragment ion following loss of glucose.

Peak	Expected Compound	Retention (min)	*m/z* M+H$^+$	*m/z* M+H$^+$ - glucoside
1	3OHKG	4.1	387.1	225.1
2	3OHKG-W	16.7	388.1	226.0
3	Unknown 3	19.3	314.1	152.3
4	3OHKG-Y	20.1	370.1	208.0
5	AHAG	21.9	314.1	152.1
6	AHBG	22.6	372.1	210.0
7	3OHKG-D	23.2	370.1	208.0

2.2.1.1 Peak 1 (3OHKG)

Loss of the glucose moiety from 3OHKG (*m/z* 387.1) produced a fragment ion with *m/z* 225.1 and a stronger *m/z* 208.0 fragment ion due to a further loss of ammonia (17.0 Da). The main fragment ion observed at *m/z* 370.1 corresponds to the mass of 3OHKG-Y and 3OHKG-D, which are formed by the loss of the amine group (17.0 Da) from 3OHKG. Two minor fragments with *m/z* 314.1 (AHAG) and *m/z* 388.1 (3OHKG-W) were also observed.

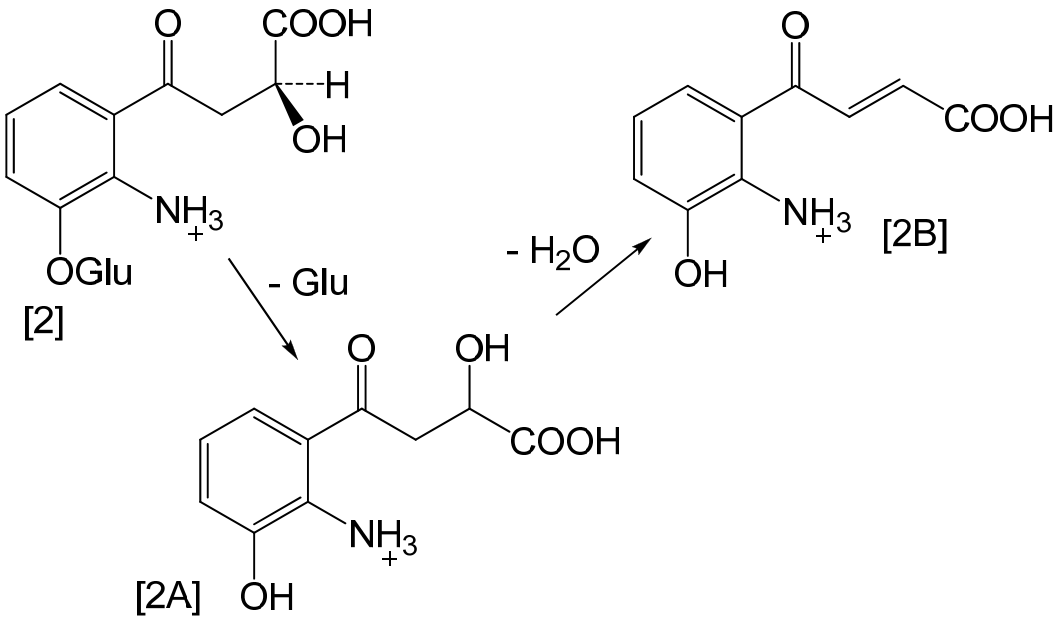


Scheme 2.2: LC-MS Fragmentation of 3OHKG

Fragmentation of protonated [1] 3OHKG to [1A] 3OHK (*m/z* 225.1) through loss of [Glu] glucoside, with further fragmentation to [1B] deaminated 3OHK (*m/z* 208.0). Positive charge may not necessarily be localised to the amine.

2.2.1.2 Peak 2 (3OHKG-W)

Peak 2 exhibited a protonated molecular ion of 1 Da greater mass than 3OHKG (*m/z* 388.1), which is consistent with the α-hydroxyl analogue of 3OHKG (3OHKG-W). A *m/z* 226.0 fragment ion eventuates upon loss of the glucoside, with a weaker intensity *m/z* 208.0 fragment ion due to a further loss of the α-hydroxyl group.

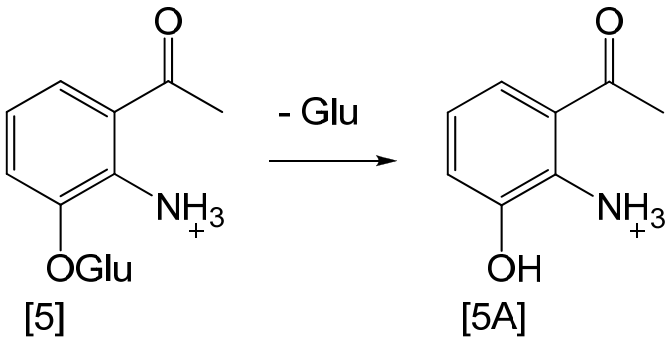


Scheme 2.3: LC-MS fragmentation of 3OHKG-W

Fragmentation of protonated [2] 3OHKG-W to [2A] 3OHK water adduct (*m/z* 226.0) through loss of [Glu] glucoside, with further fragmentation to [2B] deaminated 3OHK (*m/z* 208.0). Positive charge may not necessarily be localised to the amine.

2.2.1.3 Peaks 3 and 5 (Unknown 3 and AHAG)

Two peaks with *m/z* 314.1 (peaks 3 and 5) were observed, each having a mass consistent with the cleavage of the 3OHKG side-chain at carbon 3 *via* a reverse Aldol reaction. Both peaks also displayed a fragment ion with *m/z* 152.3. Spiking of the samples with authentic AHAG confirmed that AHAG co-eluted at peak 5. The species corresponding to peak 3 (Unknown 3) gave a stronger signal for a *m/z* 152.3 fragment ion than AHAG, but displayed no UV activity at 360 nm, though this is probably due to very low concentration. Thus, peak 3 is believed to correspond to either a contaminant or another small molecule present in the human lens that is not a UV filter.

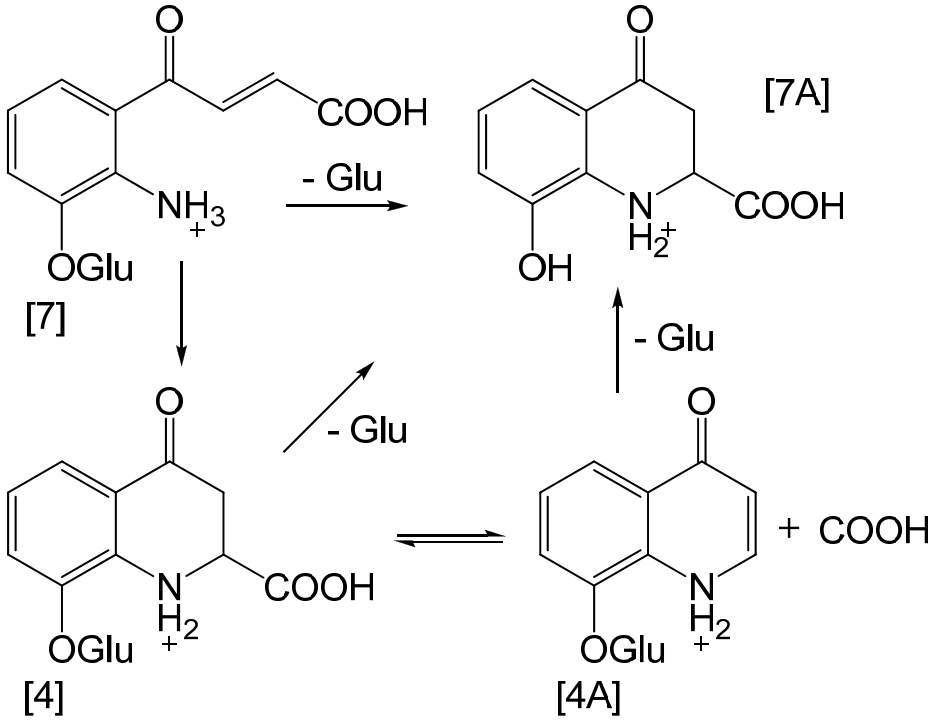


Scheme 2.4: LC-MS fragmentation of AHAG

Fragmentation of [5] AHAG to [5A] AHAA (*m/z* 152.1) through loss of [Glu] glucoside. Positive charge may not necessarily be localised to the amine.

2.2.1.4 Peaks 4 and 7 (3OHKG-Y and 3OHKG-D)

The observation of two species with *m/z* 370.1 (peaks 4 and 7) was consistent with the presence of the deaminated species 3OHKG-D and 3OHKG-Y. 3OHKG-D (assigned to peak 7 through spiking experiments) can isomerise through a ring-closing Michael addition, resulting in peak 4 *m/z* 370.1 (3OHKG-Y).



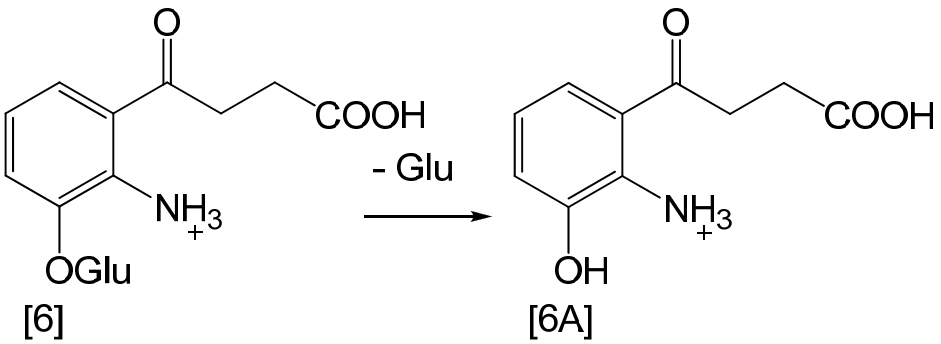
Scheme 2.5: LC-MS Fragmentation of 3OHKG-Y and 3OHKG-D

The unstable [7] 3OHKG-D can undergo Michael addition to form [4] 3OHKG-Y, which can undergo thermal loss of formic acid to form [4A] decarboxlylated 3OHKG-Y (*m/z* 324.1). All [4, 4A, 7] fragment to form [7A] 3OHK yellow (*m/z* 208.0) through loss of [Glu] glucoside (which is of equivalent mass to deaminated 3OHK). Positive charge may not necessarily be localised to the amine.

Both compounds gave the same two fragment ions with *m/z* 208.0 and *m/z* 370.1. 3OHKG-Y was differentiated from 3OHKG-D as it co-eluted with a compound having *m/z* 324.1, consistent with loss of formic acid (46.0 Da) from 3OHKG-Y in the heated source of the mass spectrometer. In contrast, 3OHKG-D does not readily lose its carboxylic acid and hence does not exhibit a co-eluting species corresponding to this thermal decomposition.

2.2.1.5 Peak 6 (AHBG)

Peak 6 (not shown) had a mass (*m/z* 372.1) and UV profile consistent with the structure of the known UV filter AHBG, with a fragment ion of *m/z* 210.0 due to the loss of the glucoside.



Scheme 2.6: LC-MS fragmentation of AHBG
Fragmentation of [6] AHBG to [6A] AHB (*m/z* 210.0) through loss of [Glu] glucoside. Positive charge may not necessarily be localised to the amine.

2.2.2 Total synthesis of novel UV filters

As the quantities of the lens UV filters were too low for unambiguous structure elucidation by NMR, the proposed compounds 3OHKG-W, 3OHKG-Y and AHAG were synthesised for comparison with the lens-derived compounds (**Scheme 2.1**). Kyn and 3OHK are both available commercially, however all the other UV filters and metabolites had to be synthesised, mainly *via* 3OHKG. The synthesis of 3OHKG was first reported as an intermediate product towards the synthesis of the related compound xanthurenic acid 8-*O*-β-D-glucoside by Real and Ferré in 1990 [214]. A similar crude and fast method was later developed by Heckathorn *et al.* in 2001 [215]. Unfortunately, neither method was suitable for large scale synthesis as both required the very expensive 3OHK as a starting compound and both produced 3OHKG in unreported yields (hence assumed to be low yields). Both methods were acceptable for the synthesis of 3OHKG as a standard, but not

for structural elucidation or protein binding as larger quantities of the UV filters are needed. Hence, 3OHKG was synthesised from the inexpensive starting compound 3-hydroxyacetophenone using a method developed by Manthey *et al.* [216] and subsequently improved by Mizdrak *et al.* [81, 125]. The synthesis proceeded as expected in moderate yield (as described in the experimental). The purified 3OHKG was then refluxed under argon under basic conditions to encourage deamination using a modified method of Tokuyama *et al.* [217]. This resulted in the formation of 3OHKG-Y, 3OHKG-W and 3OHKG-D, which were isolated by reversed phase HPLC. AHAG was synthesised using a method reported by Kumar and Saha [218]. The structures of the synthesised compounds were confirmed by NMR, HRMS and UV/Vis spectroscopy and were consistent with the above LC-MS structural assignments. All synthesised compounds (except for AHAG) eluted as diastereomers, with NMR spectra indicating approximate 1:1 ratios of each. Although UV/Vis spectra could not be obtained for these compounds from the lens samples, due to their low concentrations, such data was obtained for the synthetic 3OHKG-W, 3OHKG-Y and AHAG, with each of these having significant absorption maxima in the region 300-420 nm. The presence of 3OHKG-D in cataract human lenses has been reported previously [219].

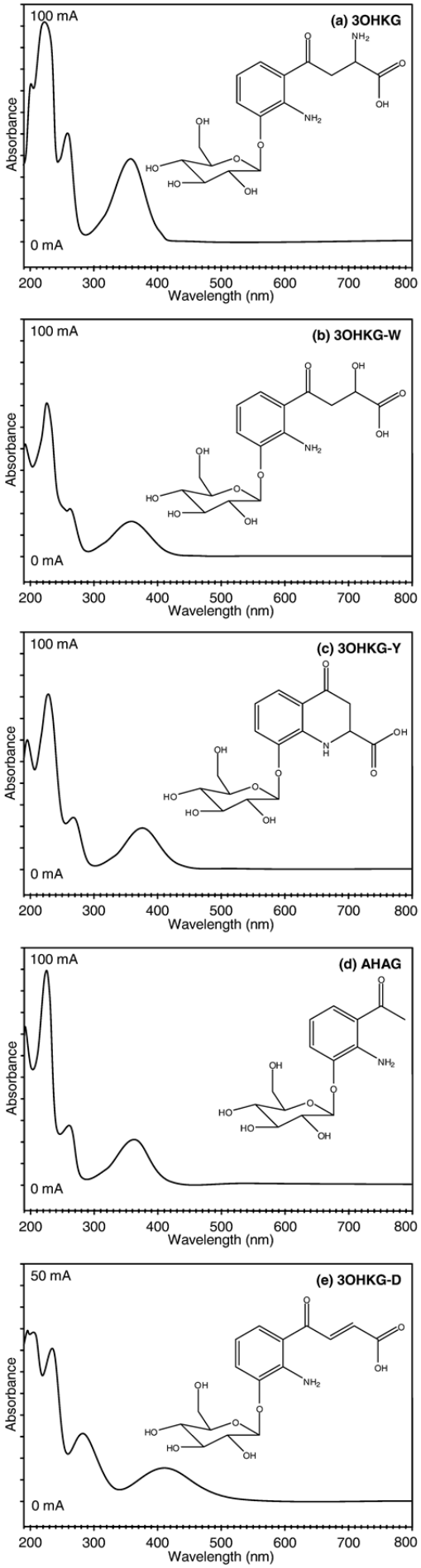


Figure 2.3: UV/Vis profiles of synthesised standards

2.2.3 Quantification of UV filters in human lenses

The three new UV filters (3OHKG-Y, 3OHKG-W and AHAG) and two known UV filters (3OHKG and 3OHKG-D) were quantified using LC-MS/MS detection. The compounds were detected by selected reaction monitoring (SRM) of the loss of the glucoside ($[M+H]^+ \rightarrow [M\text{-glucoside}+H]^+$) and their peak intensities were compared to standard curves generated from the synthetic samples. An extraction efficiency of 60% was used for all concentration data calculations (except in figure 2.4).

2.2.3.1 Normal lenses

All of the new UV filters were found in normal lenses, with 3OHKG-Y, 3OHKG-W and AHAG being present at concentrations in the pmol/mg of dry protein range (**Table 2.2**). The concentrations of these materials were correlated with the age of the lens using Fisher's P=0.05, 2 tailed test (**Figure 2.4**). In the normal lens nucleus, 3OHKG decreased in concentration with the age of the lens in a statistically significant manner (**Table 2.2**). The concentration of 3OHKG approximately halved in the nucleus of lenses aged from 18 to 84 years of age, consistent with previous reports [26, 81]. The other UV filters examined increased significantly in concentration with lens age, with the greatest increase occurring with AHAG and 3OHKG-Y. 3OHKG-W showed a trend towards an increase with age, but this was not statistically significant. 3OHKG-D was detected in low concentrations in some, but not all lenses, with no statistically significant changes in 3OHKG-D concentration being detected. In the normal lens cortex samples, the concentrations of most of the tested UV filters did not change in a statistically significant manner. 3OHKG was an exception that showed a decrease with age, though the levels of this material varied markedly, particularly in the young lens samples.

2.2.3.2 Cataract lenses

Two cataract lenses were also examined. These cataract lenses were found to have similar concentrations of the various UV filters in both the nucleus and the cortex. Low levels of 3OHKG were detected in the lens nuclei with these values being 32% of the average 3OHKG concentration of normal lenses (2.01 nmol/mg normal lens protein vs. 0.64 nmol/mg cataract lens protein), whilst the 3OHKG concentrations detected in cataract cortex were 28% of those detected in normal

lens cortex (1.63 nmol/mg normal lens protein vs. 0.46 nmol/mg cataract lens protein). 3OHKG-Y and AHAG concentrations in cataract lenses were similar to those of normal lenses having the same age. For both cataract lenses, the concentration of 3OHKG-W was close to the detection limit and therefore could not be quantified. 3OHKG-D was only observed at a low level in one of the cataract lenses.

Table 2.2: Concentrations of novel and known UV filter compounds in human lens nucleus and cortex extracts, and correlation of data with age.

NUCLEUS				
UV Filter	**Concentration Range**	**Pearson**	**P (2 tail)**	**Significance**
3OHKG	0.09 – 4.16 nmol/mg	-0.5960	0.0021	Yes
3OHKG-W	0.36 – 5.60 pmol/mg	0.2059	0.3705	No
3OHKG-Y	0.31 – 4.08 pmol/mg	0.5336	0.0072	Yes
AHAG	0.28 – 3.41 pmol/mg	0.5997	0.0025	Yes
3OHKG-D	0.06 – 0.71 pmol/mg	0.0806	0.7505	No
CORTEX				
UV Filter	**Concentration Range**	**Pearson**	**P (2 tail)**	**Significance**
3OHKG	0.05 – 5.61 nmol/mg	-0.4867	0.0159	Yes
3OHKG-W	0.23 – 1.63 pmol/mg	-0.3233	0.1778	No
3OHKG-Y	0.18 – 1.81 pmol/mg	-0.2843	0.1758	No
AHAG	0.08 – 1.14 pmol/mg	-0.0886	0.6965	No
3OHKG-D	0.04 – 0.13 pmol/mg	-0.6777	0.0654	No

2.2.4 Extraction efficiency

As the levels of the UV filters determined above would be expected to depend on the efficient extraction of these UV filters/metabolites from the lens samples, the extraction efficiency was examined. This was assessed by two independent methods using bovine lens tissue extracted with either ethanol (*Method 1*) or 5% KOH in 80% ethanol/water (*Method 2*) (**see Experimental section**).

2.2.4.1 Method 1

Extraction efficiencies for four of the UV filters were determined; data for 3OHKG-D was not obtained as this proved to be too unstable for reliable quantification.

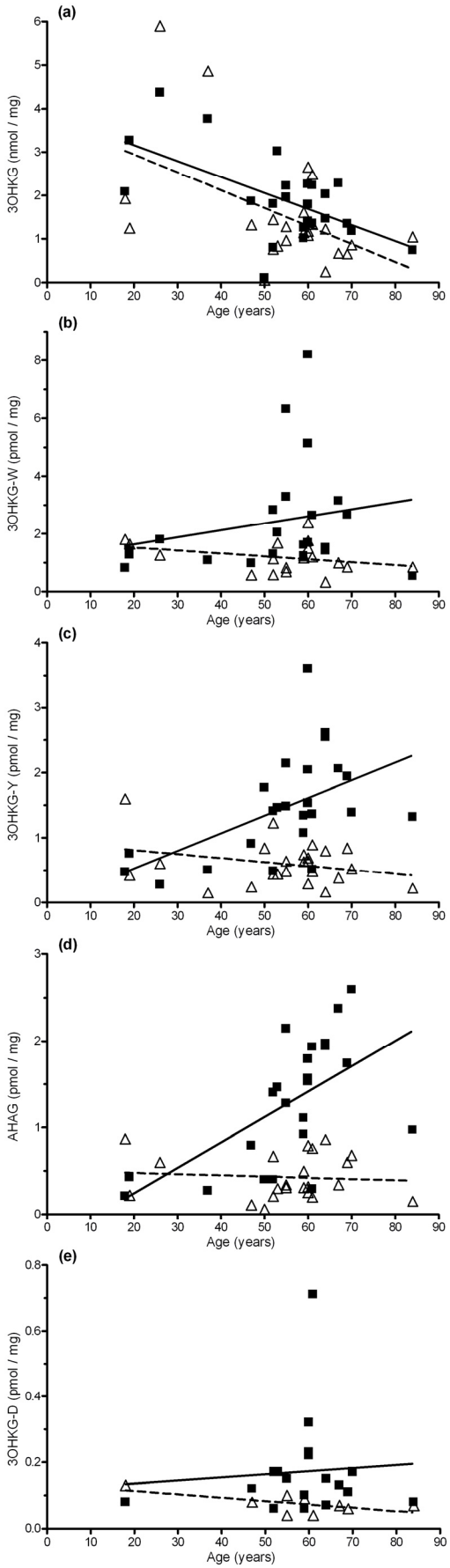


Figure 2.4: Correlation plots of the concentration of novel UV filters in the nucleus (■, solid line) and cortex (Δ, dashed line) of the human lenses with age of the donor.

Reversed phase LC-MS data for 3OHKG did not show any evidence for breakdown and conversion to other UV filters (such as AHAG), and an extraction efficiency of 57 ± 4% (mean ± standard deviation) was determined. For the other species, the recovery efficiencies were: AHAG, 79 ± 15%; 3OHKG-Y, 68 ± 14%; 3OHKG-W, 41 ± 12%. These data indicate that the detection of these species in the lens samples is not a result of artefacts arising from the extraction method. All data presented in Figure 2.4 (except for 3OHKG-D) has been corrected for the corresponding average extraction efficiency.

2.2.4.2 Method 2

Under the basic conditions of this extraction method, the 3OHKG concentration decreased and that of AHAG increased, over time. After 48 h, ~10% of the starting 3OHKG was still detectable by reversed phase LC-MS, with AHAG the only other detectable product. These data indicate that 3OHKG can be converted to AHAG under basic conditions, but that this is a slow process. The absence of other products is consistent with the detection of the other UV filters in the lens samples being a true reflection of their presence, and not mere artefacts of the extraction process.

2.3 Discussion

Three novel UV filters, 3OHKG-W, 3OHKG-Y and AHAG, were identified and quantified in human lenses varying in age from 18 to 84 years. The synthetic compounds had high-resolution masses and fragmentation patterns consistent with the structures postulated for the materials identified in the lens extracts. The UV/Vis spectra determined for the synthetic compounds are also consistent with these species acting as UV filters and are in accord with reported literature [219], strongly suggesting that they may have similar activity to the more abundant UV filters in the human lens.

In the nucleus all three novel UV filters increased in quantity with age with AHAG showing the greatest increase, a 6.5-fold increase between ages ~20 to ~68 (0.42 to 2.71 pmol/mg lens protein). 3OHKG-Y showed a 3.3-fold increase over the same age period (0.69 to 2.26 pmol/mg lens protein) and 3OHKG-W increased 2.8-fold (0.71 to 1.98 pmol/mg lens protein). The only compound that decreased in concentration over the same age period was 3OHKG (2.54 to 1.72 nmol/mg protein). This decrease in 3OHKG concentration levels is consistent with

other reports and is of a similar magnitude [25]. 3OHKG-Y and 3OHKG-W are postulated to arise from a common intermediate, 3OHKG-D ([2] in Scheme 2.7). Both 3OHKG-Y and 3OHKG-W were found to increase in concentration with age, though 3OHKG-W was found at a higher average concentration in young lenses. In contrast, 3OHKG-Y was present at higher levels in old lenses (>57 years of age). These differences with age were not observed in the lens cortex, where the concentrations of 3OHKG-W, 3OHKG-Y and AHAG either stayed the same or decreased moderately with age. Overall UV filters concentrations in the cortex were, in general, 2-3 fold lower than those found in the nucleus, and these levels did not vary dramatically with age, with the concentrations remaining at approximately similar levels to those detected in young lenses. The cortex is usually obscured by the iris; hence most light (including UV light) passes through the centre of the lens (and the nucleus) rather than the outer cortex. This fact may explain, at least in part, why more UV filter compounds are retained in the nucleus, however they also degrade more quickly in the nucleus over a person's life, hence that is the location where ARN cataract formation is most likely to begin.

The third quantified UV filter, AHAG, is postulated to arise from a reverse Aldol reaction of 3OHKG *via* cleavage of the amino acid side-chain between the second and third carbon atoms [3]. AHAG has been reported previously as a UV filter present in cataract lenses [220], however the detection of this species may be due, at least in part, to the basic extraction conditions used (5% KOH in 80% ethanol), which accelerates side-chain cleavage of 3OHKG. Thus the extraction method used previously [220] results in diminishing levels of 3OHKG over time, with almost complete conversion to AHAG after 48 h. The current study provides data consistent with the presence of AHAG in normal and cataract lenses, as the extraction method employed here did not give rise to any detectable artefactual generation of AHAG from 3OHKG. In contrast to the other novel UV filters reported here, AHAG appears to be relatively stable, nevertheless, the concentrations detected in the lens were low, with this likely to be due to the deamination of 3OHKG being a more favoured reaction than the reverse Aldol reaction that yields AHAG.

3OHKG-D was observed in only some of the normal lens samples, both in the nucleus and cortex, and only in very low quantities in one of the cataract lenses tested. As no significant changes in the concentration of 3OHKG-D over

time were detected in the normal lenses, no conclusion could be drawn on its production throughout the lifetime of a person. Nevertheless this does not negate its gradual formation over time and is consistent with the observation that 3OHKG-D is highly reactive, hence precluding any form of longer term accumulation as observed with the other UV filters.

2.4 Conclusion

Three novel UV filters, 3OHKG-Y, 3OHKG-W and AHAG, have been identified and quantified in 24 human lenses. These UV filters were present at pmol/mg lens protein concentrations, and at lower levels than the major UV filter 3OHKG (which is present at nmol/mg lens protein) [25]. Although these novel UV filters are present at low concentrations, the data obtained were consistent with their presence in both normal and cataractous lenses. The concentrations of these compounds may reflect their reactivity when compared to other species, with this potentially resulting in additional products and/or binding to lens proteins. In the nucleus, 3OHKG-Y and AHAG increased in concentration with age, and high levels were detected in cataract lenses, though additional lens analyses are required to confirm the latter observation. These novel UV filters, with the exception of 3OHKG-D, which has an additional longer wavelength absorption band, have similar UV absorbance profiles to the abundant UV filter compound 3OHKG and other known UV filters [219], consistent with these species contributing to protection against UV-induced damage to the lens and retina.

2.5 Experimental

2.5.1 Materials and apparatus

Twenty four human lenses aged from 18 to 84 years, were obtained from the Sydney Eye Bank after ethical approval (University of Sydney Human Research Ethics Committee # 7292). The nucleus and cortex of each lens was extracted separately, first with absolute ethanol and then 80% ethanol, as described previously [212]. All chemicals used were of analytical reagent grade or higher. Non-HPLC grade organic solvents were distilled prior to use. Trifluoroacetic acid (TFA, >99%) and formic acid (>98%), were obtained from Sigma-Aldrich. 3OHKG was synthesised as described previously [216]. Milli-Q water was used to prepare all solutions. TLC plates used were reversed phase silica gel 60 RP-18 F_{254} plates

(Merck, Germany) with a mobile phase of *n*-butanol/acetic acid/water (12:3:5, v/v/v). Plates were sprayed with ninhydrin and visualised using 254 and 365 nm UV light.

2.5.2 Nuclear magnetic resonance (NMR) spectroscopy

^{1}H, ^{13}C, COSY, HSQC (^{1}H–^{13}C heteronuclear single quantum correlation) and HMBC (^{1}H–^{13}C heteronuclear multiple bond correlation) data were obtained using a Bruker Avance 400 spectrometer (^{1}H, 400 MHz; ^{13}C, 100 MHz) at 25 °C. Compounds were dissolved in D_2O or CD_3OD, with undeuterated residual compounds used as a reference. Resonances are quoted in ppm and coupling constants (*J*) are given in Hz.

2.5.3 Mass spectroscopy (LC-MS/MS)

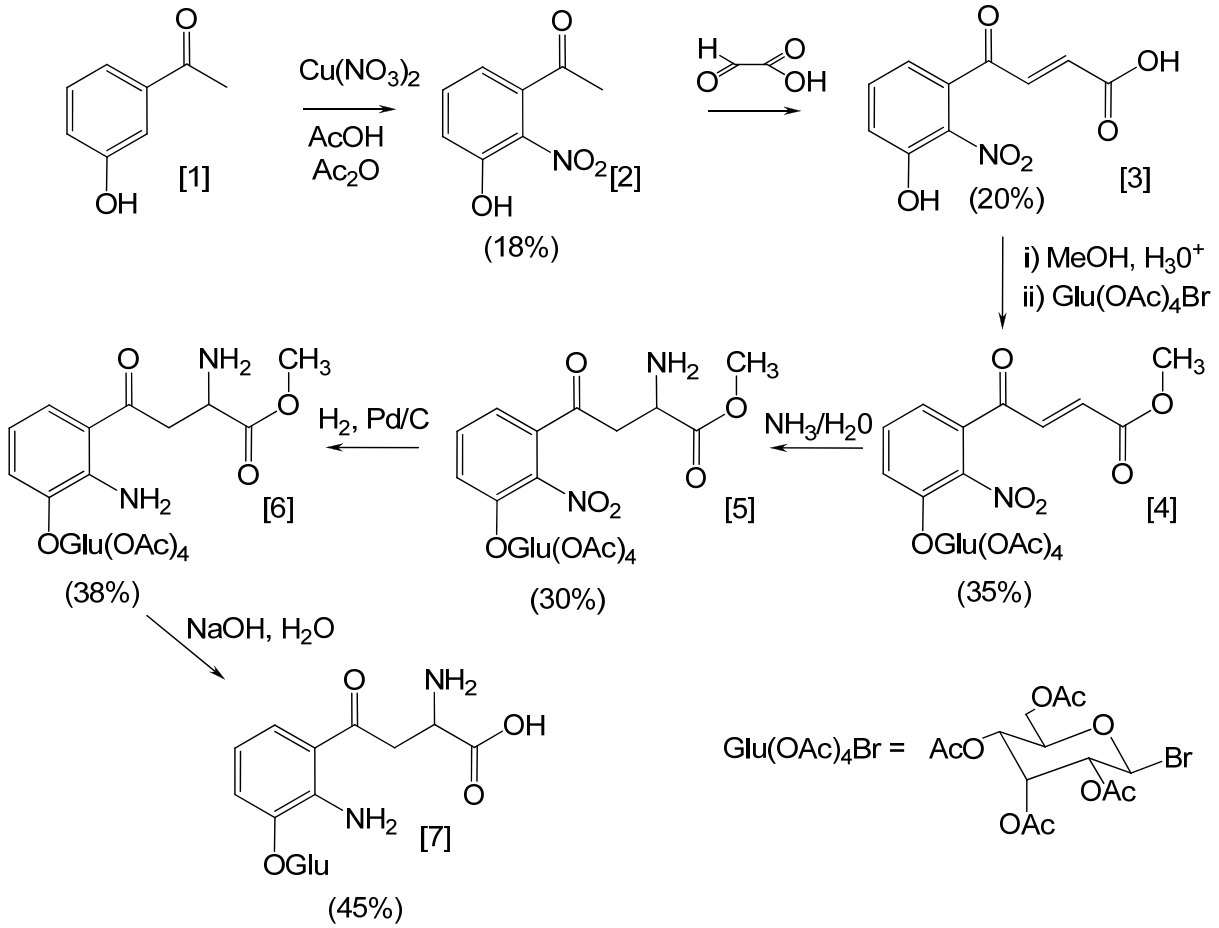
Lens extracts were centrifuged through a 10 kDa cut-off filter to remove high-molecular-mass compounds, lyophilised and then resuspended in 50 µL 1% (v/v) CH_3CN/water. Samples were separated using a Thermo Finnigan Surveyor HPLC system with a Phenomenex Synergi 4µ Fusion-RP 80 column (150 mm x 2.0 mm x 80 Å; 30 °C) coupled to a Thermo Finnigan LCQ Deca XP Max ion trap with electrospray ionisation in positive ion mode. Samples were eluted at a flow rate of 0.2 mL/min using a gradient system with 100% buffer A (5 min), before a linear increase to 40% buffer B over 40 min, then increasing to 95% B over 2 min, washing with 95% B for 1 min, before returning to 100% A over 2 min and re-equilibration with 100% A (buffer A: 0.05% (v/v) formic acid in water; buffer B: 100% CH_3CN). Eluted compounds were detected by UV absorbance at 360 nm, and MS selected reaction monitoring (SRM) of expected ion fragments (**Table 2.1**). Mass spectrometer conditions were: electrospray needle voltage, 4.5 kV, sheath gas flow rate 42, sweep gas flow rate 24 and a capillary temperature of 275 °C. The sheath and sweep gases were nitrogen, while the collision gas was helium. High resolution MS data were obtained on a Thermo LTQ FT Ultra Hybrid Mass Spectrometer (A/Prof Mark Raftery, University of NSW, Sydney, Australia).

2.5.4 UV/Vis absorbance spectroscopy

UV/Vis spectra were recorded using a Shimadzu SPD-M10A VP Photodiode array detector.

2.5.5 Synthesis of 3OHKG

3OHKG was synthesised from 3-hydroxyacetophenone using a method developed by Manthey *et al.* [216] and modified by Mizdrak *et al.* [81, 125].

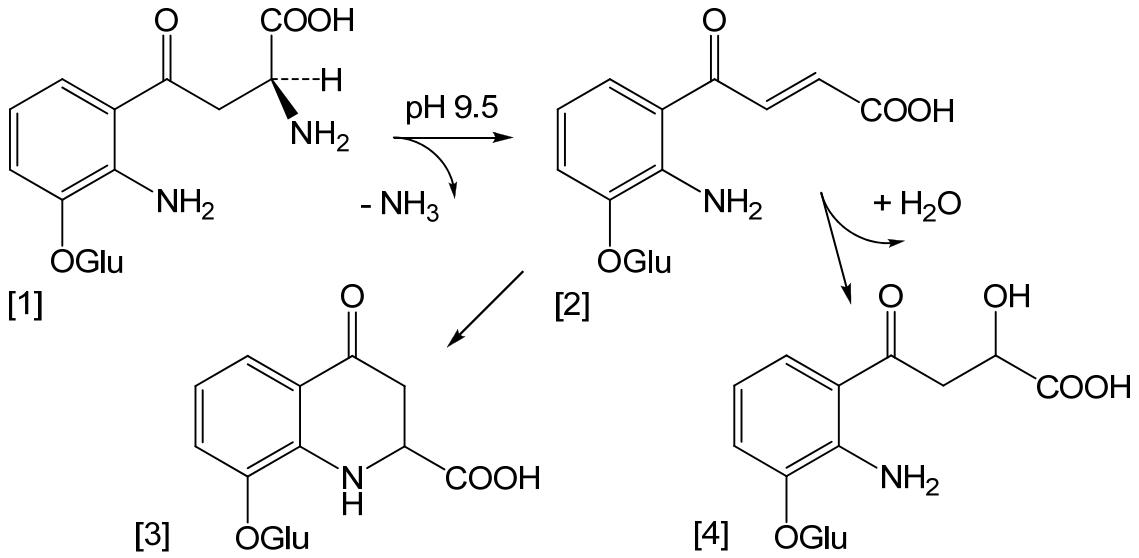


Scheme 2.7: Synthesis of 3OHKG from 3-hydroxyacetophenone.

2.5.6 Synthesis and characterisation of 3OHKG-Y, 3OHKG-W and 3OHKG-D

3OHKG-Y, 3OHKG-W and 3OHKG-D were synthesised from 3OHKG using a modified method of Tokuyama *et al.* [217]. 3OHKG (100 mg, 0.26 mmol) was dissolved in $NaHCO_3$ (15 mL, 0.60 M, pH 8.25, sparged with argon) then heated to reflux for 4 h under argon, in the dark. Reaction progress was monitored by TLC (R_f 0.89, RP, 20% CH_3CN). The solution was acidified to pH ~6 with 1 M HCl then lyophilised. Salts were removed using a Waters C18 RP Sep-Pak column using water/0.05% TFA, followed by 5% CH_3CN/0.05% TFA. Samples were purified by reversed phase HPLC (250 mm x 21.2 mm x 80 Å Phenomenex reversed phase preparative scale column using gradient elution (flow rate 8.0 mL/min) starting at 0% B (10 min), 0-40% B (80 min), 40-95% (4 min), 95% (2

min), 95-0% (4 min), 0% (30 min), with buffers A and B as stated above. Each compound, with the exception of AHAG, eluted as diastereomers with a ratio of ~1:1. These diastereomers were visible for during HPLC purification, with all 3OHKG derived compounds eluting as double peaks. This was further supported by the NMR data with H-6’ (on the glucose) producing two peaks of similar magnitude in each compound.



Scheme 2.8: Synthesis of 3OHKG UV filter metabolites
[1] 3OHKG deaminates at high pH forming [2] 3OHKG-D, which can ring-close *via* Michael addition to form [3] 3OHKG-Y or hydrate to form [4] 3OHKG-W.

2.5.6.1. Analytical data of 3OHKG-Y

3-Hydroxykynurenine *O*-β-D-glucoside yellow (8-(β-D-Glucopyranosyloxy)-1,2,3,4-tetrahydro-4-oxo-2-quinolinecarboxylic acid) $C_{16}H_{19}NO_9$ – (25.6 mg, 27%) as a pale yellow solid (~1:1 diastereomeric mixture). Calculated *m/z* $M+H^+$ is 370.1133. Found *m/z* $M+H^+$ is 370.1132. ESI HRMS 370.1132 ($M+H^+$, 11%), 392.0951 ($M+Na^+$, 18%), 324.1076 ($M+H^+$-HCOOH, 100%). ESI HRMS/MS of $M+H^+$, 370.1134 (100%), 208.0605 ($M+H^+$-Glu, 93%); ^{1}H NMR δ (400 MHz, CD_3OD) 7.43 (1H, d, *J* 8.1, H-5), 7.28 (1H, d, *J* 8.1, H-7), 6.72 (1H, dd, *J* 8.1, 8.1, H-6), 5.06 (0.5H, d, *J* 7.3, H-1’), 5.01 (0.5H, d, *J* 7.3, H-1’), 4.31 (1H, bd, *J* 6.9, H-2), 3.90 (1H, d, *J* 12.5, H-6’), 3.73 (1H, d, *J* 12.5, H-6’), 3.63 (1H, m, H-2’), 3.61 (1H, m, H-3’), 3.58 (1H, m, H-5’), 3.49 (1H, m, H-4’), 3.03 (1H, m, H-3), 2.91 (1H, m, H-3); ^{13}C NMR δ (CD_3OD), 198.0 (CO-4), 172.0 (CO_2H), 146.0 (C-8), 144.9 (C-8a), 144.7 (C-8a), 123.3 (C-7), 122.7 (C-7), 122.5 (C-5),

122.3 (C-5), 120.2 (C-4a), 118.8 (C-6), 102.9 (C-1'), 102.6 (C-1'), 77.6 (C-5'), 76.8 (C-3'), 74.1 (C-2'), 70.7 (C-4'), 61.8 (C-6'), 56.9 (C-2), 41.1 (C-3); λ_{max} 228, 268 and 376, λ_{min} 257 and 300 nm (**Figure 2.3**).

2.5.6.2 Analytical data of 3OHKG-W

3-Hydroxykynurenine *O*-β-D-glucoside water adduct

4-(2-Amino-3-hydroxyphenyl)-2-hydroxy-4-oxobutanoic acid *O*-β-D-glucoside $C_{16}H_{21}NO_{10}$ – (10.1 mg, 10%) as a pale yellow solid (~1:1 diastereomeric mixture). Calculated *m/z* $M+H^+$ is 388.1238. Found *m/z* $M+H^+$ is 388.1234. ESI HRMS/MS of $M+H^+$, 226.0710 ($M+H^+$-Glu, 100%), 208.0605 ($M+H^+$-OGlu, 22%); ^{1}H NMR δ (400 MHz, D_2O), 7.62 (1H, d, *J* 8.1, ArH-6), 7.31 (1H, d, *J* 8.1, ArH-4), 6.77 (1H, dd, *J* 8.1, 8.1, ArH-5), 5.03 (0.5H, d, *J* 7.3, H-1'), 4.68 (1H, bd, *J* 6.9, H-2), 3.92 (1H, d, *J* 12.5, H-6'), 3.76 (1H, d, *J* 12.5, H-6'), 3.63 (1H, m, H-2'), 3.62 (1H, m, H-3'), 3.59 (1H, m, H-5'), 3.54 (1H, m, H-4'), 3.50 (2x1H, m, H-3); ^{13}C NMR δ (CD_3OD), 201.3 (CO-4), 178.4 (C-1), 145.3 (ArC-3), 141.5 (ArC-2), 126.5 (ArC-6), 121.1 (ArC-4), 119.4 (ArC-1), 116.5 (ArC-5), 101.9 (C-1'), 76.6 (C-5'), 75.9 (C-3'), 73.3 (C-2'), 69.8 (C-4'), 67.6 (C-2), 60.9 (C-6'), 43.8 (C-3); λ_{max} 227, 260 and 363, λ_{min} 253 and 287 nm (**Figure 2.3**).

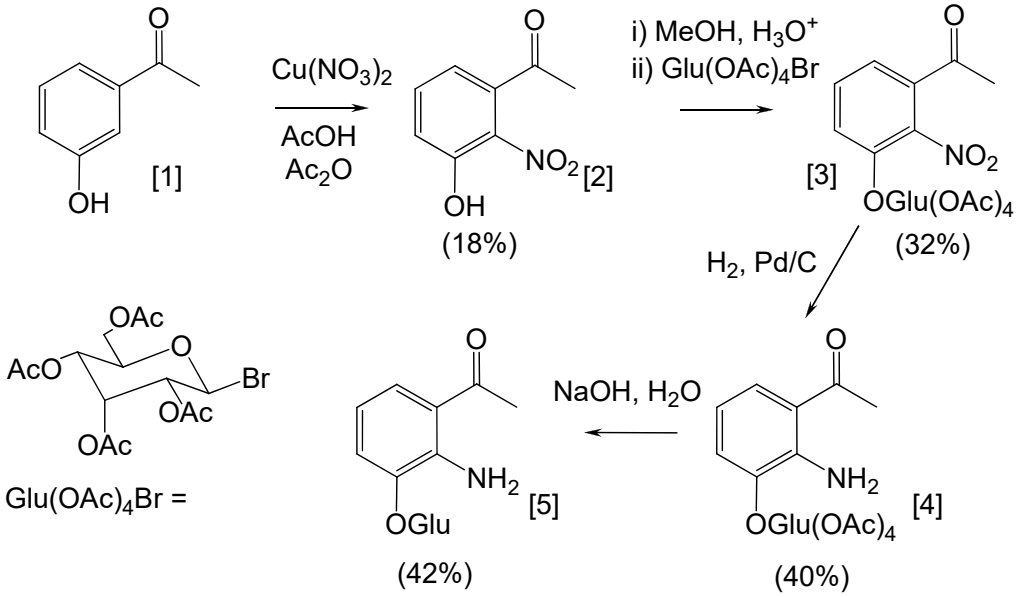
2.5.6.3 Analytical data of 3OHKG-D

4-(2-Amino-3-hydroxyphenyl)-4-oxobut-2-enoic acid *O*-β-D-glucoside $C_{16}H_{19}NO_9$ – (3.2 mg, 3.4%) as a medium orange solid. Calculated *m/z* $M+H^+$ is 370.1133. Found *m/z* $M+H^+$ is 370.1133. ESI HRMS/MS of $M+H^+$, 370.1133 ($M+H^+$, 100%), 208.0605 ($M+H^+$-Glu, 63%); ^{1}H NMR δ (D_2O) 7.73 (1H, d, *J* 15.6, H-3) 7.55 (1H, d, *J* 7.9, ArH-6), 7.25 (1H, d, *J* 7.9, ArH-4), 6.68 (1H, dd, *J* 8.0, 8.0, ArH-5), 6.64 (1H, d, *J* 15.8, H-2), 4.96 (1H, d, *J* 7.1, H-1'), 3.82 (1H, dd, *J* 11.8, H-6'), 3.68 (1H, dd, *J* 5.8, 6.0, H-6'), 3.56 (1H, m, H-2'), 3.54 (1H, m, H-3'), 3.50 (0.5H, m, H-4'), 3.47 (0.5H, m, H-5'), 3.45 (0.5H, m, H-5'), 3.41 (0.5H, m, H-4'); λ_{max} 234, 282 and 409, λ_{min} 257 and 340 nm (**Figure 2.3**).

2.5.7 Synthesis of AHAG

AHAG was synthesised using the method described by Kumar and Saha [218]. Catalytic reduction of the nitrated intermediate compound [3] was performed with a ThalesNano H-Cube Continuous Flow Hydrogenation Reactor using a 10%

ethanol/ethyl acetate mixture as the solvent and a palladium on carbon catalytic cartridge.



Scheme 2.9: Synthesis of AHAG from 3-hydroxyacetophenone.

2.5.7.1 Analytical data for AHAG

2-Amino-3-hydroxyacetophenone *O*-β-D-glucoside

$C_{16}H_{19}NO_9$ – as a white solid. Calculated *m/z* $M+H^+$ is 314.1234. Found *m/z* $M+H^+$ is 314.1233. ESI HRMS/MS of $M+H^+$, 152.0706 ($M+H^+$-Glu, 100%), 278.1024 (12%); ^{1}H NMR δ (400 MHz, D_2O), 7.65 (1H, d, *J* 8.1, ArH-6), 7.30 (1H, d, *J* 8.1, ArH-4), 6.74 (1H, dd, *J* 8.1, 8.1, ArH-5), 5.01 (1H, d, *J* 7.3, H-1'), 3.91 (1H, d, *J* 12.5, H-6'), 3.76 (1H, d, *J* 12.5, H-6'), 3.63 (1H, m, H-2'), 3.62 (1H, m, H-3'), 3.58 (1H, m, H-5'), 3.40 (1H, m, H-4'), 2.60 (1H, m, H-1); ^{13}C NMR δ (CD_3OD), 204.5 (CO), 145.5 (ArC-3), 139.5 (ArC-2), 127.3 (ArC-6), 121.0 (ArC-4), 120.3 (ArC-1), 117.3 (ArC-5), 101.8 (C-1'), 76.3 (C-5'), 75.6 (C-3'), 73.0 (C-2'), 69.6 (C-4'), 60.6 (C-6'), 28.1 (CH_3-1); λ_{max} 226, 260 and 363, λ_{min} 247 and 287 nm (**Figure 2.3**).

2.5.8 Extraction efficiency

The extraction efficiencies of two different methods for extracting UV filter where tested.

2.5.8.1 Method 1

For the UV filters 3OHKG, 3OHKG-W, 3OHKG-Y and AHAG, 100-200 mg of bovine lens tissue was mixed with an equivalent mass of 500 µg/mL aqueous UV filter solutions. The protein/UV filter mixture was homogenised and extracted (2 x 150 µL absolute ethanol) then centrifuged, filtered, lyophilised, resuspended into 150 µL of 1% (v/v) CH_3CN/water and analysed by RP LC-MS as described above, with quantification based on UV/Vis absorption of the eluted compounds. All tests were performed in triplicate [38].

2.5.8.2 Method 2

100-200 mg of bovine lens tissue was mixed with an equivalent mass of 500 µg/mL aqueous 3OHKG standard. The samples were then extracted with 5% KOH in 80% ethanol/water solution, with aliquots removed at 0, 8, 24, 32 and 48 h. The aliquots were then centrifuged to remove proteins, neutralised with 1 M HCl then analysed by RP LC-MS as described above [220].

2.5.9 Preparation of human lens proteins for LC-MS/MS analysis

Two normal human lenses (ages 60 and 61) and two cataractous human lenses (ages 50 and 70) were separated into the lens nucleus and cortex with a 5 mm cork-borer. The end sections of each nuclear core were shaved off and added to the cortex lens batch. The nuclei were first extracted in 100% ethanol followed by 80% ethanol (method used has been described previously) [68, 221]. The lens proteins were dissolved in guanidine hydrochloride (6 M, pH 5.5, 2 mg protein/mL), centrifuged (13,000 g, 20 min, 4 °C) and analysed by UV/Vis and fluorescence.

Two different normal human lenses (ages 67 and 69) were separated into cortex and nucleus as above. In addition, two different cataractous lenses (ages 60 and 85) were used as an unseparated whole lens. The normal lens nuclei and the whole cataractous lenses were separately homogenised in phosphate buffer (0.2 M, 0.5 ml, pH 7.4) and centrifuged (10,000 g, 30 min, 15 °C). The supernatant was removed and stored at 0 °C, while the pellets were again extracted. The pellets were subsequently homogenised in guanidine hydrochloride solution (6 M, pH 5.5, 0.5 mL) containing DTT (10 mM) and incubated at room temperature for approximately 10 min and then centrifuged (10,000 g, 30 min, 15 °C). The pellets were extracted one additional time. Both,

phosphate and guanidine hydrochloride supernatants were dialysed in phosphate buffer (0.1 mM, pH 6, 4 °C) for 24 h with two buffer changes and then lyophilised. The lyophilised proteins were re-dissolved into guanidine hydrochloride (6 M, pH 5.5, 2 mg protein/mL), centrifuged (13,000 g, 20 min, 4 °C) and analysed by UV/Vis and fluorescence. The normal lenses were obtained from the Sydney Eye Hospital Lions Eye Bank (Sydney, Australia) and cataractous lenses (designated as Type III–IV cataracts according to the Pirie / LOCSIII classification system [59, 60]) were obtained from K. T. Sheth Eye Hospital (Rajkot, India).

CHAPTER 3

UV IRRADIATION OF MODIFIED BOVINE LENS PROTEINS

Purpose

To investigate the reactivity of UV filter modified bovine lens proteins to UV light, and to determine if novel UV filter metabolites could be photosensitisers of oxidative damage.

Methods

A natural composition of bovine lens proteins (BLP) modified with UV filter compounds (Kyn, 3OHK, 3OHKG, 3OHKG-D, 3OHKG-Y, 3OHKG-W, AHAG and AHB) under physiological (pH 7.4) and basic (pH 9.5) conditions were exposed to UV light under varying conditions (aerobic and anaerobic, presence free UV filter) for varying periods of time. The UV light treated BLP were then analysed by SDS-PAGE electrophoresis to observe possible binding and protein aggregation.

Results

Different protein changes were observed between bound UV filters with an *o*-aminophenol and α,β-unsaturated carbonyl moieties (3OHK) and those with only an α,β-unsaturated carbonyl moiety (3OHKG, Kyn, 3OHKG-D) upon UV irradiation. No changes were observed with metabolites having only an *o*-aminophenol (AHB) upon irradiation. Evidence of cross-linking and non-disulphide binding was observed in the BLP modified with Kyn, 3OHK, 3OHKG and 3OHKG-D, though gel band formation and separation patterns of 3OHK were different to those of Kyn and 3OHKG. Irradiation (of at least 120 min) resulted in a significant loss of soluble protein, suggesting aggregation of modified BLP.

Conclusion

Covalent binding between BLP and a UV filter occurs mainly *via* the α,β-unsaturated carbonyl moieties, with binding being a prerequisite to photo-oxidative damage occurring upon irradiation with UV light. Photo-damage was only observed in samples modified with 3OHKG, 3OHK, Kyn and 3OHKG-D upon UV irradiation, with disulphide and di-Tyr bonds being observed. Binding that could not be accounted for by either bond type was also observed. No evidence of binding or protein damage was observed in samples treated with AHB, 3OHKG-Y, 3OHKG-W or AHAG upon irradiation, compared to irradiated untreated BLP.

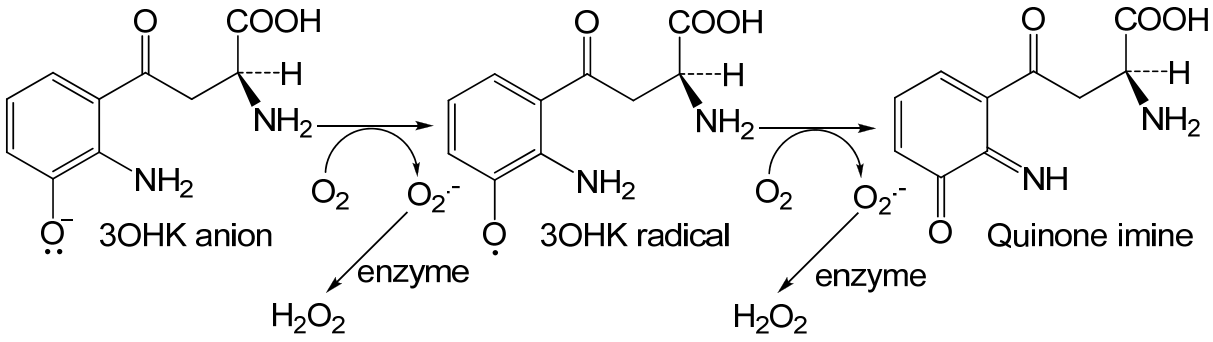
3.1 Introduction

The majority of all 300–400 nm UV radiation entering the eye, approximately 90-95%, is absorbed by UV filters [45, 181], which are synthesised in the lens in the anterior cortical epithelial cells [46]. The absorption maxima of these UV filters (and most of their metabolites) is in the 360-370 nm ultraviolet region [48, 68]. The human lens (like the lenses of most daylight active animals) is exposed to UV light throughout an individual's lifetime. UVA and long wavelength UVB light is high energy radiation that transmits through the human lens. It has previously been shown that this UV radiation damages human lens proteins in a process that is mediated through both singlet oxygen and superoxide intermediates [183, 185, 222]. Human lenses contain kynurenine (Kyn) based UV filters (*e.g.* 3OHKG, 3OHK, Kyn) to protect the lens against UV light damage, and the majority of all the harmful UV light above 300 nm is absorbed by them [45, 181]. Most UV light below 300 nm (approximately half of the UVB and all of the UVC spectra) is absorbed by the atmosphere with UVC reacting with oxygen (forming the ozone layer) and UVB being partly absorbed by ozone [223]. In the lens, the free UV filters have the correct properties for protecting the lens from UV light, namely absorbing the energy of UV light in the right wavelength range and subsequently being poor sensitisers of oxidant formation (*e.g.* singlet oxygen, hydroxyl and superoxide radicals) [203]. It is believed that these compounds protect the lens and eye from UV light damage [212]. As mentioned previously, ARN cataract does not normally start causing vision problems until a person is in their 50s or 60s. Over a person's lifetime, the levels of the beneficial free UV filters decrease, dropping most significantly after middle age whilst the levels of UV filters covalently bound to nucleophilic amino acids on the lens proteins rapidly increases [26, 224, 225]. The properties of the free UV filters has been thoroughly examined, and found to be protective to lens proteins, however it is still not understood clearly whether their reactivity changes once they bind to the lens proteins (crystallins). It is believed that the occurrences of decreasing free UV filter concentrations, increasing UV filter binding to lens proteins and the oxidation of lens proteins are inextricably linked to the development of ARN cataract [17, 224]. It is theorised that each occurrence has a cascading effect, which in essence causes a slow but deleterious chain reaction towards the formation of ARN cataract in the lens. In this study, as performed in previous lens protein modification studies, bovine lens proteins were

used as an analogue for human lens proteins as they have similar proportions of each main crystallin to humans and no native UV filter [50, 71].

3.1.2 Peroxide and oxidant formation

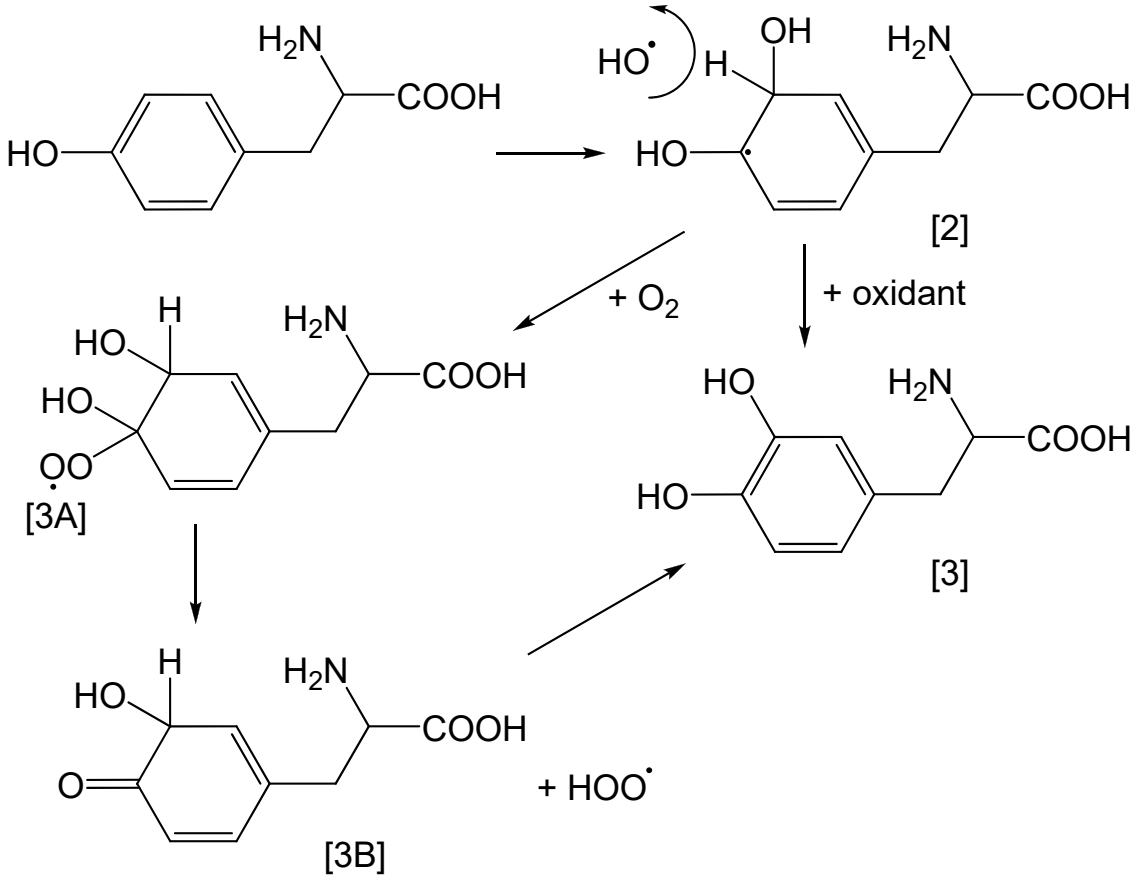
Some UV filters do not contribute to the oxidation of human lens protein because of their photo-physical properties. 3OHK absorbs UV radiation and has a fast decay which dissipates the UV energy, hence protecting the human lens against UV damage [181]. When bound to lens proteins, UV filters (3OHKG and 3OHK) and UV filter metabolites (like Cys-adducts) do not appear to have any significant role in the formation of peroxides when irradiated in their free state (unbound to protein) at concentrations up to 1 mM [50]. However, bovine lens proteins (BLP) modified with 3OHKG or 3OHK have been shown to generate a significant amount of peroxides when irradiated with UV light of 365 nm [50]. Testing with 3OHK is problematic due to its reactivity, particularly at concentrations higher than >1mM, as it can lead to oxidative stress and the formation of hydrogen peroxide [226, 227]. The latter may arise *via* the reduction of O_2 to $O_2^{\cdot-}$ by an excited state UV filter, after which superoxide dismutase converts the superoxide to H_2O_2 and O_2 [160, 228]. 3OHKG does not have the same reactivity as 3OHK, and much lower levels of peroxides are detected with free 3OHKG UV filter present with these being no different from those detected with the non-irradiated proteins. This suggests that significant photosensitisation only occurs with 3OHKG and 3OHK once the UV filters are covalently bound to lens proteins.



Scheme 3.1: Mechanism of H_2O_2 formation *via* 3OHK auto-oxidation [160].

In previous work, it has been shown that once protein-bound Kyn is exposed to 300-400 nm wavelengths of UV light, photo-oxidation occurs through the creation of singlet oxygen, hence resulting in oxidative damage to lens

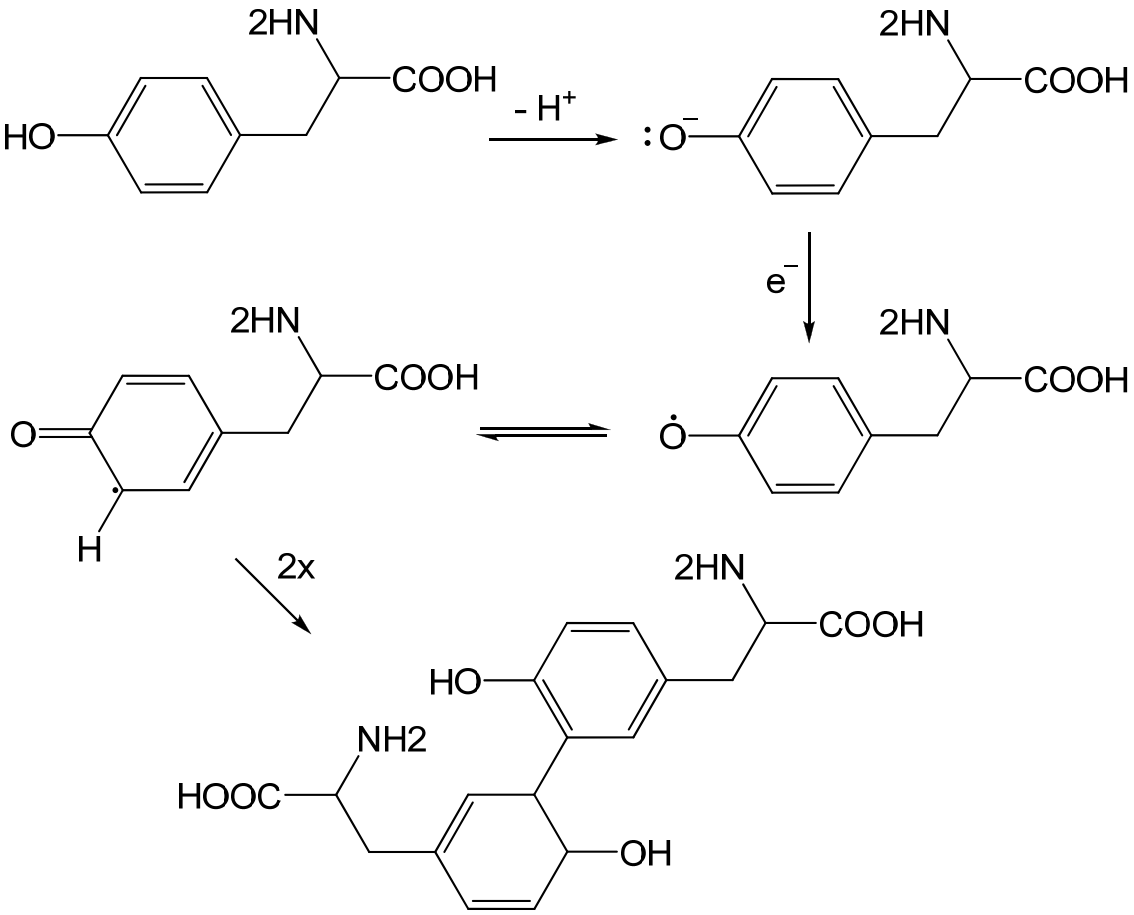
proteins [18]. Whilst in its free state, Kyn is protective to the lens proteins. Once it covalently binds to lens proteins, this results in the transformation of the otherwise protective compound into a species that upon UV illumination can result in the generation of H_2O_2, protein peroxides and the oxidation of protein tyrosine (Tyr) residues to di-tyrosine (di-Tyr) and 3,4-dihydroxyphenylalanine (DOPA) [18].



Scheme 3.2: Formation of L-DOPA from Tyr, with or without O_2 [229].

Photo-oxidation can occur either directly (Type I photo-oxidation) or indirectly (Type II photo-oxidation) [195, 230, 231]. In direct photo-oxidation, the energy from absorbed UV light remains in the absorbing species (whether a bound UV filter, or a reactive protein side-chain) causing it or a neighbouring residue to form a radical or other excited state species. When photo-oxidation occurs indirectly, the absorbed energy is first transferred to a ground state compound (*e.g.* oxygen), resulting in a secondary radical or excited state species (*e.g.* singlet oxygen, superoxide and hydroxyl radicals). These reactive oxygen species usually react quickly with the adjacent proteins or free amino acids, however they can also undergo alternative reactions to produce hydrogen peroxide or other free organic peroxides [16, 222, 232]. The human lens is predominantly made of protein, hence the

main sites of damage arising from the formation of reactive oxygen species will be the lens proteins and constituent amino acids [16, 233]. As there is little or no protein turnover in the lens, it is believed that the build-up of protein damage leads to the general malformation of the proteins (including denaturation, protein aggregation and conformational changes) and the loss of enzymatic activity (through reaction with enzyme binding sites and through the oxidation of amino acid side-chains) [137, 230].



Scheme 3.3: Formation of di-Tyr dimer from Tyr, *via* tyrosyl [229].

3.1.2.1 Peroxide formation upon UV irradiation

It is known that the concentration of peroxides formed by lens proteins bound to 3OHKG and Kyn is dependent on the concentration of the bound UV filter [50], with the greatest yields of peroxide obtained from illumination with shorter wavelengths of UVA light (305 nm cut-off filter) [50]. 3OHK modified BLP also forms peroxides, however it is less dependent on the 3OHK concentration, as 3OHK itself is prone to oxidation and the formation of other possible photosensitisers. Hence in this study, comparing the level of damage that

modified BLP generates upon irradiation in the presence/absence of oxygen was of key interest. Beyond peroxide formation from photosensitisers, some peroxide can be formed either purely from the ionisation energy of the UV light, or from other photosensitising compounds/amino acids that are already present in the lens proteins [18, 50]. When catalase is added to the sample undergoing irradiation, the levels of peroxide formation drop between 2.3-3.6 fold in 3OHK and 3OHKG modified BLP samples. The remaining peroxides are believed to be organic peroxides as catalase only enzymatically removes H_2O_2 ($H_2O_2 \rightarrow H_2O + ½O_2$) [234, 235]. Nevertheless, it is clear that the majority of the formed peroxides are from H_2O_2 in BLP bound to 3OHK and 3OHKG, hence free oxygen should be a factor in the level of damage observed [236, 237].

3.1.2.2 Singlet oxygen formation upon UV irradiation

In addition to peroxide formation, singlet oxygen is known to be formed upon UV irradiation [185]. However, due to its instability in water, experiments are typically performed in D_2O which extends the lifetime of singlet oxygen by approximately 10 fold [238-240]. D_2O is also known to affect the aggregation of hydrophilic proteins, particularly larger protein aggregates, so such experiments may have results that are unrepresentative of reactions in water [241].

With 3OHK modified BLP, when the samples are irradiated with sodium azide (10 mM), a singlet oxygen scavenger [242, 243], the levels of peroxide formation drop by approximately 2.4 fold. Without irradiation, sodium azide has no effect on peroxide levels, hence suggesting their formation is *via* the Type II route with singlet oxygen acting as the intermediate reactive oxygen species. Therefore, as the main means of radical formation is *via* the indirect route, the presence of oxygen gas with the modified proteins (and in the eye) is likely key to the level of potential damage experienced by the lens [120, 244], as it has been reported that the formation of singlet oxygen has a significant effect on the production of both free and protein-bound peroxides in the lens [245, 246]. In 3OHKG modified proteins, upon irradiation, peroxide formation is not significantly affected by the addition of sodium azide, showing that singlet oxygen formation may be restricted to 3OHK [39].

3.1.3 Aim of UV irradiation study

It is not understood which processes and which exact compounds cause the oxidation of the lens proteins, however it is believed that understanding what role the unstable UV filters have in the formation of ARN cataract is a key element of this research. The aim of this research was therefore to determine what effects recently described UV filter metabolites like 3OHKG-D, 3OHKG-Y, 3OHKG-W and AHAG may have on BLP, and which of them are responsible for the production of singlet oxygen, superoxide and hydrogen peroxide. Previously investigated UV filters (Kyn, 3OHK and 3OHKG) were also investigated under similar (or slightly modified) conditions for comparative purposes. Though most of the novel metabolites are unlikely to bind covalently to BLP, experiments were conducted to observe whether they had any protective capabilities (considering that they have similar UV absorbance patterns to the main UV filters). The main purpose of this investigation was to observe which UV filters and/or conditions led to protein-protein binding and aggregation whilst in the presence of UV light, and to what extent bound UV filters affected the level of observed damage.

3.2 Results

3.2.1 Modification of bovine lens proteins

A natural composition of bovine lens proteins (BLP), extracted and purified from calf lenses, were treated with 3OHKG, 3OHK, Kyn, 3OHKG-Y and AHB at both pH 7.4 and at pH 9.5 (to enhance deamination and encourage binding). At pH 7.4, UV filters/metabolites were incubated with BLP (1:5 mass ratio) for 7 days, whilst samples at pH 9.5 were incubated for 48 h. Both experiments were conducted in an anaerobic chamber. BLP were also treated with AHAG and 3OHKG-W at the higher pH 9.5 (only), whilst treatment with 3OHKG-D was at pH 7.4 (only) as it is already deaminated and highly reactive. The 3OHKG-D reaction was only done at 48 h due to its instability (it was expected to have either reacted or decomposed within 48 h). Kyn data was collected for comparison with the data of Parker *et al*. [18], whilst the 3OHK and 3OHKG experiments allowed comparison with the data reported by Mizdrak *et al*. [50]. Bovine lens proteins were used in this study as they lack any native UV filter-like compounds and provide a good model for human lens proteins as there is on average a 95% sequence homology between

the various individual bovine and human lens crystallins [149]. A more detailed analysis and characterisation of the various modified BLP is in Chapter 4.

3.2.2 Irradiation of BLP with UVA light

Proteins treated with the major human lens UV filters 3OHKG, 3OHK and Kyn, UV filter metabolites 3OHKG-D, AHB, 3OHKG-Y, 3OHKG-W and AHAG, and untreated controls were dissolved in un-buffered Milli-Q water (pH 7) then illuminated with a mercury arc UV filter lamp with a 305 nm filter. A 305 nm filter was used as it approximates the radiation range received from the sun on the Earth's surface. The wider UV range also maximised the effect of the irradiation in the limited timeframe of the experiment. Shorter wavelengths of light are more energetic and may potentially cause more damage when they hit proteins or cells, nevertheless they do not penetrate far and if not absorbed by the atmosphere are usually absorbed by the cornea [180]. Unfiltered irradiation was not used as the lamp produced UV wavelengths that are not naturally encountered on Earth (<250 nm) that may increase the risk of unintended damage occurring from direct ionisation of the proteins and oxygen present in the samples [18, 50]. Longer wavelengths penetrate further into the eye, and though they have less energy, they are of concern because they can reach the lens and cornea and hence cause damage in the more delicate parts of the eye [180]. In previous experiments, irradiation with a cut-off at 345 nm produced less peroxide, whilst irradiation with a 385 nm filter produced little more than a baseline quantity of peroxide [39] (**see Figure 3.17**).

The light source used for these experiments was an Osram (uncoated) 125 W broad spectrum mercury arc UV lamp. The light was filtered through a 305 nm cut-off filter, giving approximately 140 $\mu W/cm^2$ of UVB (280-315 nm) and 3299 $\mu W/cm^2$ of UVA (315 – 400 nm) measured using a Stellarnet Comet-SL-3C-50 Spectrometer, which is almost all UVA light (or above). The 305 nm filter had minimal effect on the light spectrum profile, as oxygen in the atmosphere absorbs most UV light of shorter wavelengths (than 305 nm) regardless. The filter was used to maintain consistency with previous experiments. In the 48 contiguous states of USA, spectrophotometric readings taken in a one year period indicate that an average of approximately 78 $\mu W/cm^2$ UVB and 6068 $\mu W/cm^2$ UVA light hits the earth's surface from the sun [247]. Being a similar distance from the equator as the middle point of the USA, it is expected that Sydney would

experience similar levels of solar irradiation, however these intensities do vary throughout the year [182, 202].

The UV lamp does produce more UVB light than the sun (approximately 79% more) at the irradiation distance of 15 cm. This is in part due to the close proximity of the lamp. However, from both sources, most of the intensity is from UVA light, which are the wavelengths of most concern in this research. The sun produces approximately 84% more UVA compared to this experimental setup.

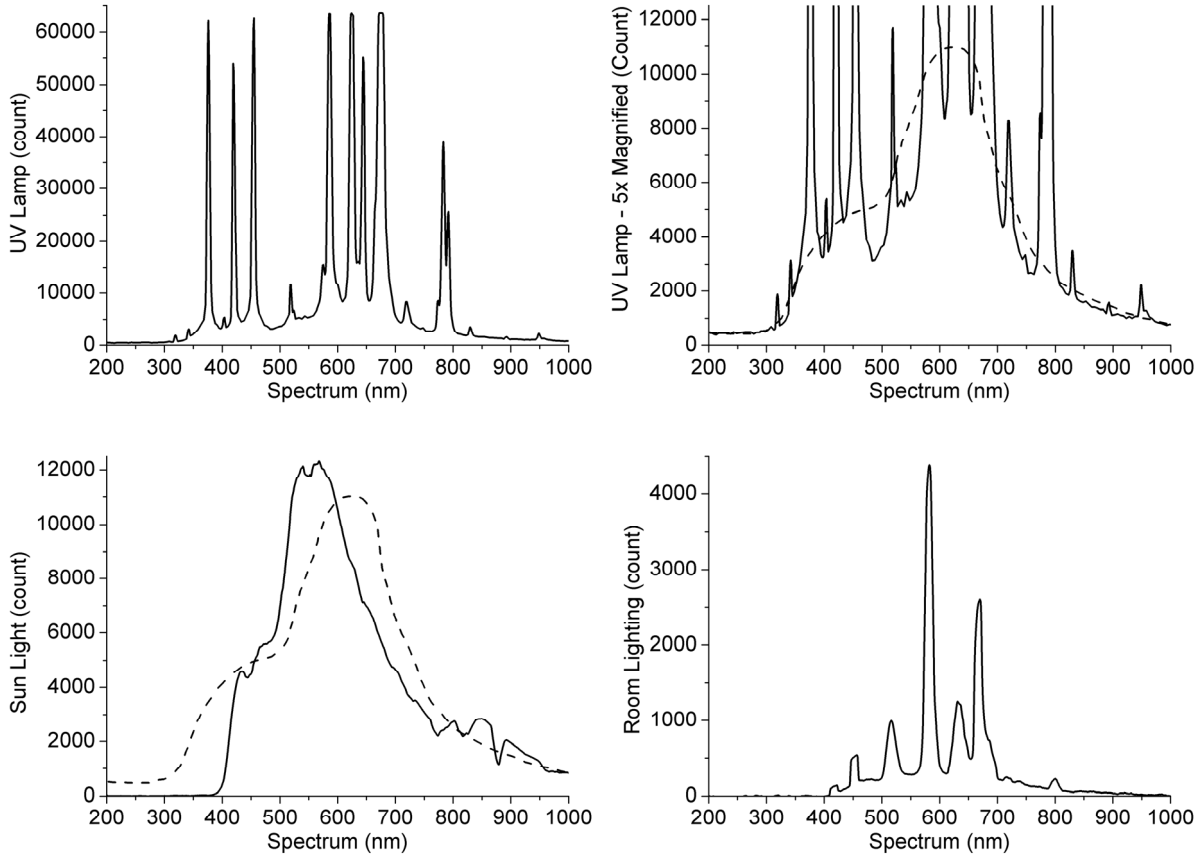


Figure 3.1: Electromagnetic light spectra.
125 W UV lamp (top left), 5x magnified UV lamp (top right) with approximate baseline average (dashed line), natural sunlight at 1pm on 20 November 2013 (bottom left) with superimposed UV lamp baseline (dashed line) and normal room fluorescent lighting (bottom right).

Though there are differences in the relative intensities of UVA and UVB between the UV lamp experimental setup and the sun, the lamp produces UV light of a similar magnitude to the sun in both the UVA and UVB ranges. In a person's normal environment, exposure to the sun's harmful UV rays is minimal and usually indirect (*i.e.* from UV reflective surfaces). These UV filter-BLP irradiation

experiments were designed to simulate many years of this indirect sun exposure to the human lens.

3.2.2.1 Modified lens proteins

Despite oxygen diffusing constantly into the eye, the human lens (and mammalian lens in general) is a very low oxygen environment [248, 249]. Hence experiments were performed under different oxygen levels to observe any differences in protein binding and damage due to interaction with oxygen. Samples were irradiated under four conditions: bubbled with nitrogen (simulated anaerobic), bubbled with air (aerobic), sealed and de-oxygenated with nitrogen (anaerobic) and sealed with ambient oxygen levels (partial aerobic). Unlike in the rest of the body, the lens does not have any blood vessels, hence in the human lens oxygen constantly diffuses into it to provide the oxygen it needs. This also creates a unique environment where oxygen, UV light and potential photosensitisers (in the form of protein bound UV filters) are in close proximity. The variation between younger and older/cataractous lenses is the presence of these protein-bound UV filters is mimicked through this experiment. It is known that peroxides and other reactive oxygen species are produced under these conditions, and that those reactive oxygen species levels increase over time as long as the irradiation continues [50]. It is also known that protein-bound 3OHK produces the highest levels of peroxide, with Kyn and 3OHKG each producing less than half the amount of peroxide when compared to 3OHK [50]. It is also known that these quantities are affected by the wavelength of the UV light (with the most produced when irradiated without a filter), hence a 305 nm filter was used to maintain a consistent light spectrum with natural sunlight.

3.2.2.2 Gel composition and irradiation of untreated BLP and control samples

Previously, the best gel band separation of modified lens proteins was generally obtained by separation on 12% Tris-Glycine gels with Coomassie Brilliant Blue staining used for band detection [18, 124]. Improved results were obtained by Mizdrak *et al.* through the use of silver staining [50], which is approximately 10x more sensitive at detecting proteins than Coomassie Brilliant Blue. Nevertheless, the gels still had a significant amount of baseline streaking for control proteins (untreated BLP), even in the samples reduced with the antioxidant β-mercaptoethanol.

In this study, initial experiments were thus run to test the suitability and quality of results obtained using a number of different available gels, beginning with Tris-glycine. Unless otherwise stated, all protein concentrations in gel experiments were performed at 1 mg/ml of BLP.

3.2.2.2.1 Tris-glycine gels

Untreated BLP, pH 7.4 and pH 9.5 control BLP and 3OHKG-D modified BLP were run on Bio-Rad premade 4-20% Tris-glycine gels with the recommended Tris-glycine SDS running buffer. Apart from the uneven electrophoresis experienced in the Bio-Rad (curved solvent front, due to greater flow through the gel middle), streaking was present in the controls when none was expected; lanes [5-7] and [9-12]. Likewise, the modified 3OHKG-D BLP in lanes [4], [8] and [12] did not resolve and were faint. Overall the gels displayed good protein banding, but further work was not performed on Tris-glycine gels due to these problems.

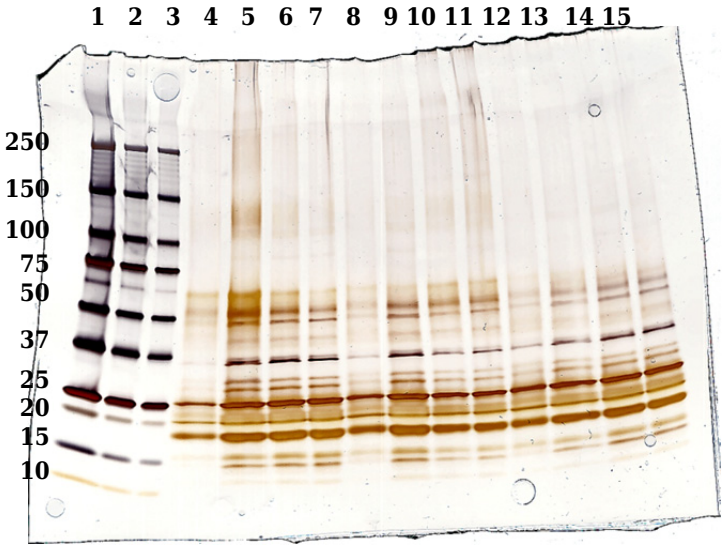


Figure 3.2: Bio-Rad 4-20% Tris-glycine gel run with Tris-glycine SDS buffer.
Lanes: Molecular weight marker [1] 8 µl; [2] 4 µl; [3] 2 µl
3OHKG-D pH 7.4 BLP [4] 12 µl; [8] 12 µl; [12] 12 µl
Untreated BLP [5] 12 µl; [6] 8 µl; [7] 6 µl
Control pH 7.4 BLP [9] 12 µl; [10] 8 µl; [11] 6 µl
Control pH 9.5 BLP [13] 12 µl; [14] 8 µl; [15] 6 µl

3.2.2.2.2 Tris-acetate gels

An alternate gel system was available from NuPAGE, with Novex premade 3-8% Tris-acetate being one of the two gel compositions tested. Gels were run with the

recommended Tris-acetate buffer. The design of the gel housing (L shaped gel), gives a straight solvent front, hence neat banding towards the bottom of the gel. Untreated BLP were run in the gels under both normal/non-reduced (left) and reduced (right) conditions. Similar to the Tris-glycine gels there was a significant amount of streaking present, particularly in the pH 9.5 BLP samples. This may arise, at least in part, from the lower polyacrylamide percentages used, which reduced the level of banding at higher molecular masses. Interestingly, running the control BLP under reduced conditions not only removed most of the apparent cross-linking in the higher molecular mass region, it also regenerated some of the individual bands visible in the untreated BLP, but which became cross-linked in the control BLP. Though overall the lower mass proteins produced neat bands, and produced very consistent gels, further work was not performed on Tris-acetate gels as higher gels than 7% were not available from NuPAGE and significantly greater separation was not expected.

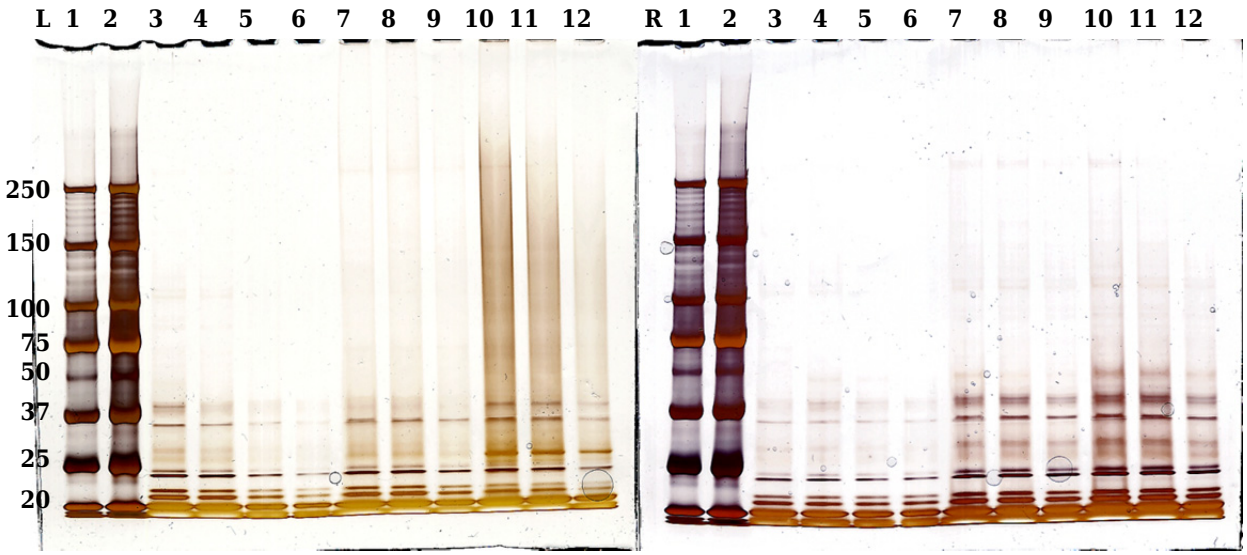


Figure 3.3: NuPAGE 3-8% Tris-acetate gel run with Tris-acetate buffer. Same lane order was run on both the left (normal) and right (reduced) gels. Lanes: Molecular weight marker [1] 4 µl; [2] 2 µl Untreated BLP [3] 16 µl; [4] 16 µl; [5] 12 µl; [6] 8 µl Control pH 7.4 BLP [7] 16 µl; [8] 12 µl; [9] 8 µl Control pH 9.5 BLP [10] 16 µl; [11] 12 µl; [12] 8 µl

3.2.2.2.3 Bis-Tris gels

The NuPAGE gel system is based on a Bis-Tris buffer system with 4-12% polyacrylamide gels run with either MES or MOPS buffer. These gels also have an

L-shaped design, hence they run with a straight solvent front. MES buffer is advertised as producing better separation of lower mass proteins, and it did, though the bands were less well resolved from each other. MOPS buffer resulted in less separation of the lower mass bands, however it generally produced neater bands and resulted in less cross-linking being apparent in control samples where it was not expected. The best resolution and separation was produced with Bis-Tris gels using MOPS buffer. Though some cross-linking was still apparent in the higher molecular mass (when not expected), using MOPS buffer produced sharper bands than MES and also gave good separation from 250 down to 10 kDa. All the irradiated proteins (modified, controls and untreated) were therefore run on MOPS buffered Bis-Tris gels for consistency.

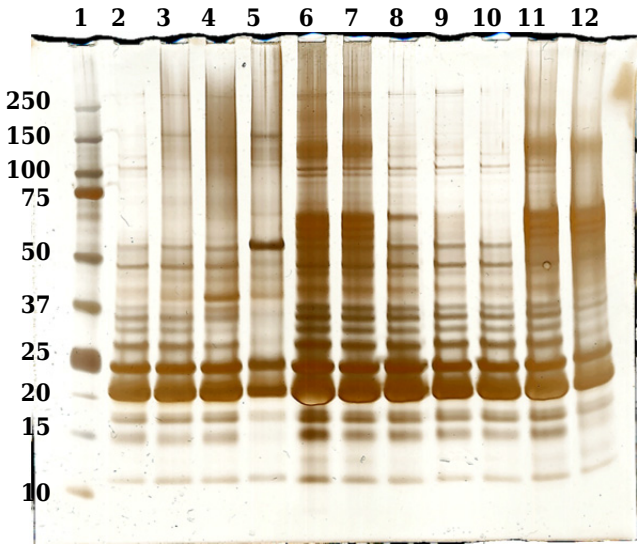


Figure 3.4: NuPAGE 4-12% Bis-Tris gel in MES buffer.
Lanes: Molecular weight marker [1] 4 µl
Control pH 9.5 BLP [2] 6 µl; [3] 8 µl; [4] 12 µl
3OHKG-D pH 7.4 BLP [5] 4 µl
Untreated BLP (2 mg/mL) [6] 16 µl; [7] 12 µl; [11] 8 µl; [12] 6 µl
Control pH 7.4 BLP [9] 12 µl; [10] 8 µl; [11] 6 µl

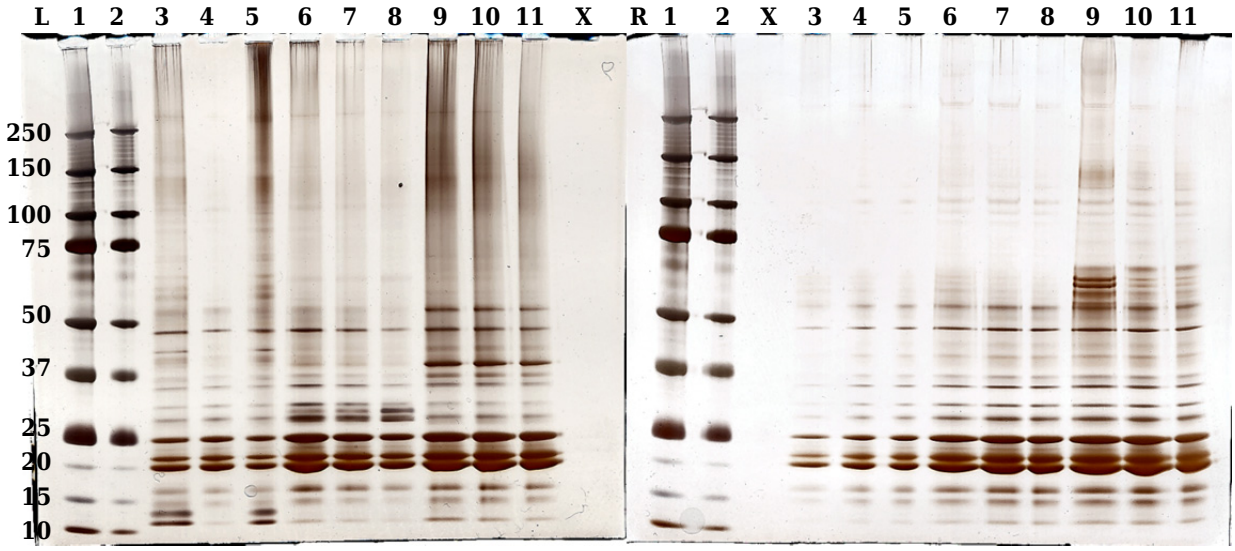


Figure 3.5: NuPAGE 4-12% Bis-Tris gel in MOPS buffer.
Same lane order was run on both left (normal) and right (reduced) gels. Lane X was empty.
Lanes: Molecular weight marker [1] 4 µl; [2] 2 µl
Untreated BLP [3] 16 µl; [4] 8 µl; [5] 12 µl
Control pH 7.4 BLP [6] 16 µl; [7] 12 µl; [8] 8 µl
Control pH 9.5 BLP [9] 16 µl; [10] 12 µl; [11] 8 µl

3.2.2.2.4 Irradiation of untreated BLP samples

Untreated BLP were irradiated from periods of 0 through to 120 min under multiple conditions: bubbled with N_2 gas (to remove oxygen); left in the open air; bubbled with air; and/or sealed in a bottle cuvette (after de-oxygenation with N_2 gas). Despite the varying conditions, and the different lengths of time irradiated, there were no consistent differences between untreated irradiated BLP and negative controls. There were likewise few differences between the samples run under non-reducing and reducing conditions.

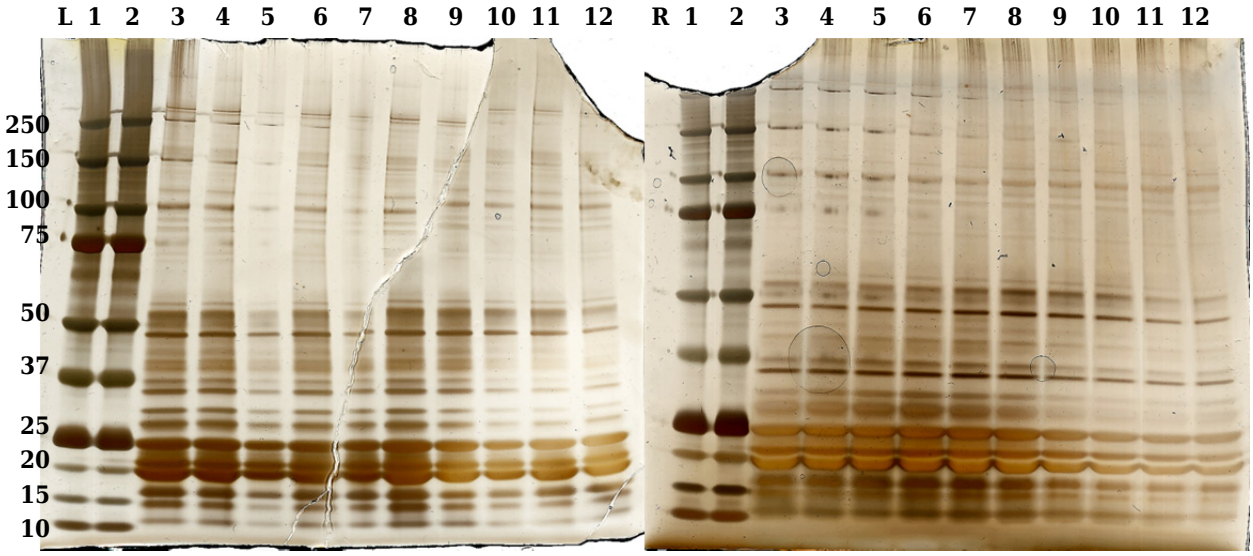


Figure 3.6: Untreated BLP run on a Bis-Tris gel with MOPS buffer. Samples were irradiated for up to 120 min and separated under (left) normal (right) reducing conditions, using the same lane order. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).
Lanes: Molecular weight marker [1] 2 µl; [2] 2 µl
N_2 Bubbled BLP [3] 0 min; [4] 15 min; [5] 30 min; [6] 60 min; [7] 120 min
Air Open BLP [8] 0 min; [9] 15 min; [10] 30 min; [11] 60 min; [12] 120 min

As four different samples of BLP were irradiated under differing conditions and no evidence was found of any binding, cross-linking or damage in the cells, the strong suggestion is that UV irradiation alone does not bring about any visible changes in lens proteins, at least not ones observable upon separation with electrophoresis. No difference was observed between samples that were bubbled continuously with nitrogen gas and those left open to the air or even those bubbled with air. An almost identical result was observed for samples that were bubbled with air and those degassed with nitrogen and irradiated in a sealed container. The only conclusion being that either 120 min is insufficient to cause any observable damage in bovine lenses, or that due to the lack of a photosensitiser, UV light does not directly cause observable damage to lens proteins. The latter possibility is most likely, in that lens proteins are transparent to visible light so they should also be (at the very least) partially if not completely transparent to higher frequency UV light (*e.g.* UVA light). The presence of UV

filter compounds in human (and other species) lenses adds weight to this possibility.

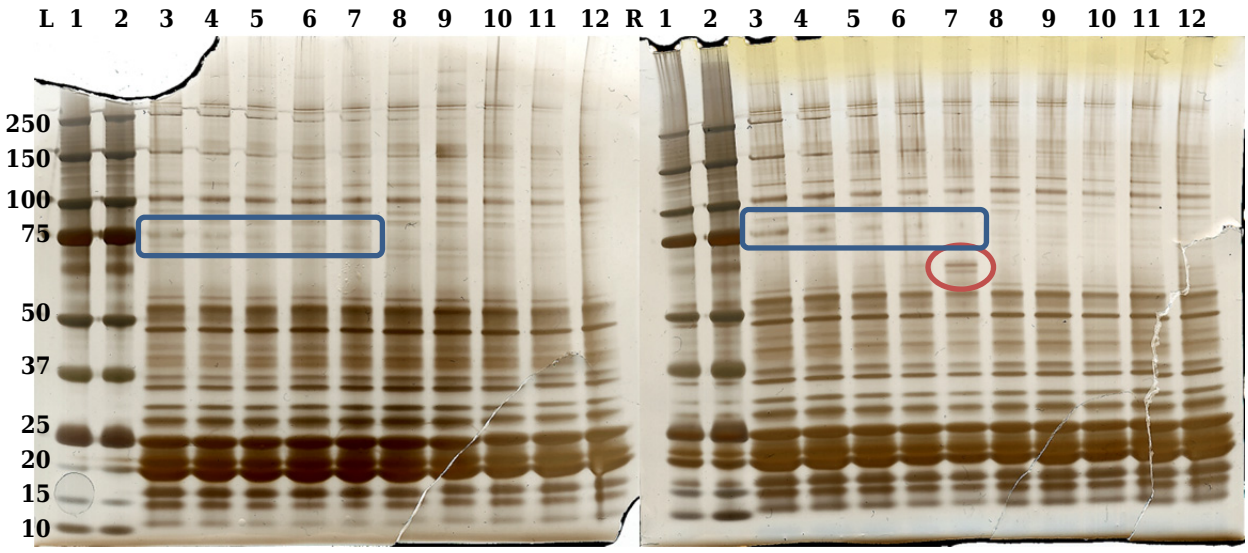


Figure 3.7: Untreated BLP run on a Bis-Tris gel with MOPS buffer. Samples were irradiated for up to 120 min and separated under (left) normal (right) reducing conditions, using the same lane order. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).
Lanes: Molecular weight marker [1] 2 µl; [2] 2 µl
Air Bubbled BLP [3] 0 min; [4] 15 min; [5] 30 min; [6] 60 min; [7] 120 min
N_2 Sealed BLP [8] 0 min; [9] 15 min; [10] 30 min; [11] 60 min; [12] 120 min

Likewise the gels of the negative controls (**see Appendix A**) produced almost identical banding and had an almost identical appearance to those run under both normal and reducing conditions after irradiation. The only observed difference was in the air bubbled reduced gel, where two new bands were visible after 120 min of UV irradiation (**Figure 3.7, marked with red oval**). There was also a weak band at 75 kDa that was present at 0 min, but gradually faded after 120 min irradiation (**Figure 3.7, blue rectangle**) however the band was not very clear. Regardless, it appears from these results that, on its own, irradiation with UV light does not cause any observable differences in bovine lens protein. Hence, it is believed that a photosensitiser (in the form of a bound UV filter) is needed for any observable changes to occur upon irradiation with UV light.

3.2.2.3 Irradiation of BLP modified with Kyn

In addition to being a major UV filter in the human lens, Kyn also reacts readily with BLP (at pH 9.5). Irradiation was performed on both samples modified at pH 7.4 (**see Appendix B**) and at pH 9.5 (**Figure 3.8**). BLP modified with Kyn were irradiated for 120 min (with samples taken at 0, 15, 30, 60 and 120 min) under two conditions: bubbled with air (to simulate the availability of oxygen to the lens in the body) and bubbled with N_2 gas (after being deoxygenated for ~10 min) to see if oxygen had any affect.

In the oxygenated samples, the greatest differences were observed at pH 9.5, as was expected. There is a steady increase in the level of non-reversible binding (lanes 2 through to 6) in the 37 kDa and above region (**Figure 3.8, blue rectangle**), which is most visible in the 60 and 120 min lanes (lanes 5 and 6). This cross-linking is retained under reducing conditions, strongly suggesting that covalent bonds other than disulphides are being formed. Likewise there are only a few faint narrow bands that are comprised of multiple protein fragments bound together with disulphide bonds in both the air and nitrogen irradiated samples under normal running conditions. In the nitrogen bubbled samples, more of these bands remained as the irradiation continued (at just above the 50 kDa mark, Figure 3.9, red oval), which in the air bubbled samples drop to only one band at ~50 kDa (marked with an arrow). Under reducing conditions, all of these bands were recovered (from the break-up of disulphide bonds) thus forming 4 bands above the 50 kDa marker (**Figure 3.8, red oval**). The other strong indicator of non-disulphide binding, particularly under oxygen and upon extended irradiation, was the almost complete loss of bands in the 100-250 kDa regions (**Figure 3.8, green trapezium**). On the gels run under normal (non-reduced) conditions, these bands were almost invisible. However the evidence of non-disulphide binding that is dependent on the presence of oxygen is most evident in the reduced gels. In the air bubbled reduced sample, only the time 0 (non-irradiated sample, Figure 3.9 marked with an A) retained the bands of the larger proteins. 15 min of irradiation under oxygen caused an observable decrease in these bands, whilst upon irradiation in the absence of oxygen (N_2 bubbled), those bands were still present at 60 and 120 min of irradiation (**Figure 3.8, marked with a B**).

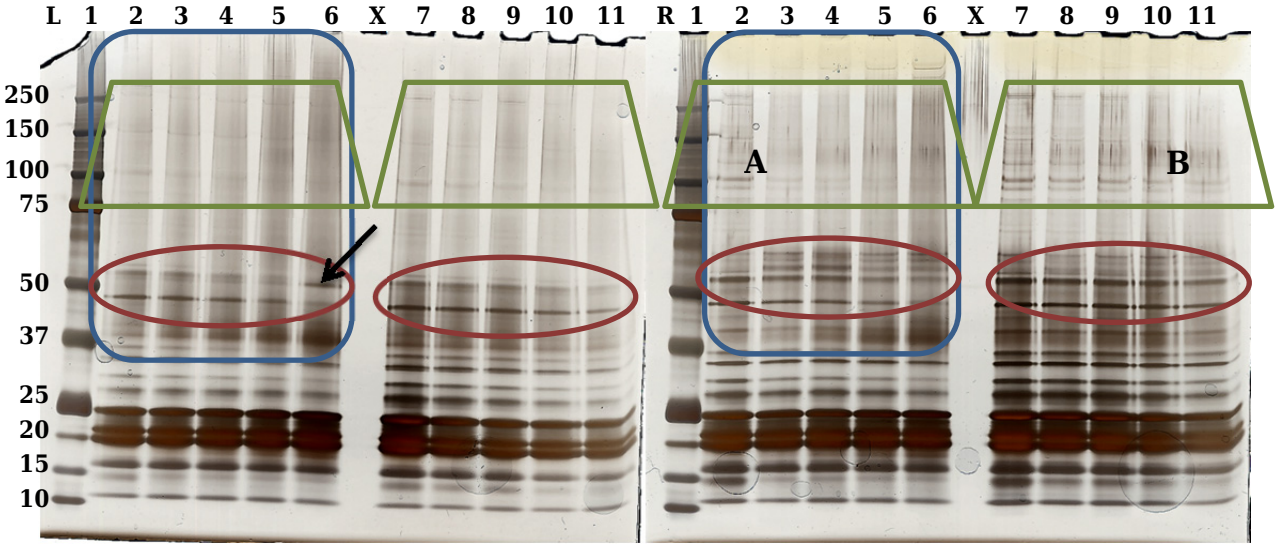


Figure 3.8: Kyn pH 9.5 BLP run on a Bis-Tris gel with MOPS buffer. Samples were irradiated for up to 120 min and separated under (left) normal and (right) reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).
Lanes: Molecular weight marker [1] 2 µl
Air Bubbled BLP [2] 0 min; [3] 15 min; [4] 30 min; [5] 60 min; [6] 120 min
N_2 Bubbled BLP [7] 0 min; [8] 15 min; [9] 30 min; [10] 60 min; [11] 120 min

Upon irradiation, the Kyn modified BLP experienced changes over time that comprised of both reversible disulphide bonds (reversible under reduced conditions, hence not present in the right gel) and irreversible ones which are visible as denser cross-link streaks in lanes 5 and 6 in both gels (clustered partly at 37 kDa, and generally in the 50 kDa and above region). It should be noted that the Kyn non-irradiated controls and Kyn pH 7.4 irradiated samples showed little to no change over the 120 min period (*i.e.* stayed the same as time 0). They are shown in the appendix (**see Appendix C**).

3.2.2.4 Irradiation of BLP modified with 3OHK

The BLP modified with 3OHK produced the most surprising results, in that upon irradiation the air bubbled samples experienced a reduction in the amount of detectable protein. This loss of protein (visualised as ‘fading’ bands) was

consistent between both the normal and reduced air bubbled gels (**Figure 3.9**). Seemingly, the only factor in this loss of detectable protein was the presence of oxygen, which is indicative of how unstable 3OHK is in oxygen (even when bound to proteins). The loss of these proteins is believed to be due to the formation of very large insoluble protein aggregates, which adhered strongly to the cuvettes upon irradiation whilst being bubbled with oxygen. The difference between 3OHK and Kyn is that the changes over time (from 0-120 min) affect all of the bands (from 10-250 kDa) proportionally, suggesting that the damage/binding is non-specific and thus involves the formation of non-disulphide bonds. This loss of observable protein was not present in the control samples.

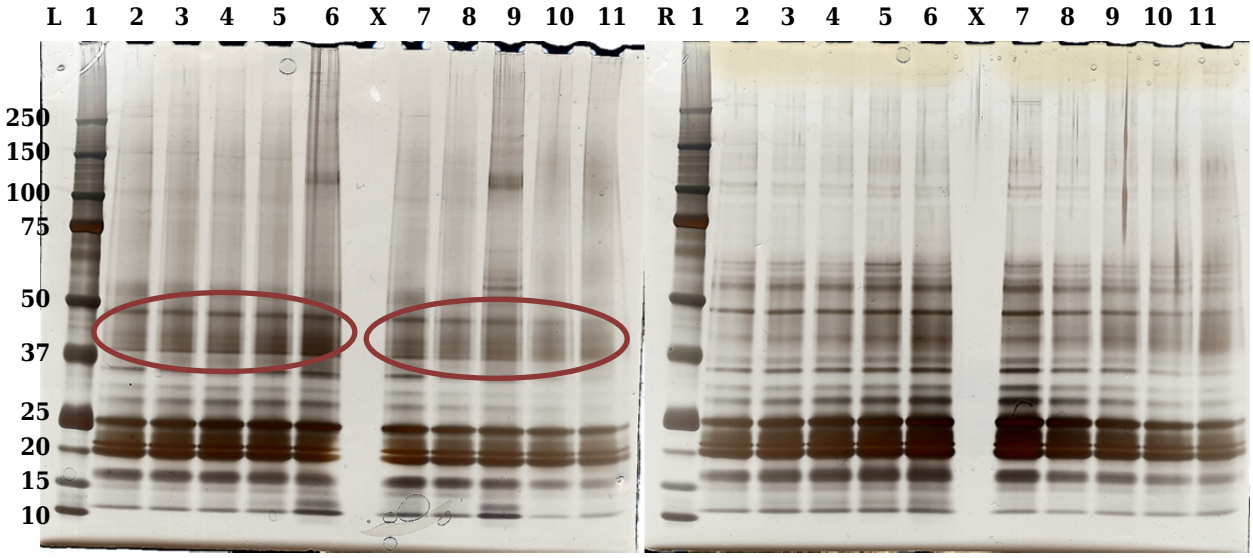


Figure 3.9: 3OHK pH 9.5 BLP run on a Bis-Tris gel with MOPS buffer. Samples were irradiated for up to 120 min and separated under (left) normal and (right) reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).
Lanes: Molecular weight marker [1] 2 µl
N_2 Bubbled BLP [2] 0 min; [3] 15 min; [4] 30 min; [5] 60 min; [6] 120 min
Air Bubbled BLP [7] 0 min; [8] 15 min; [9] 30 min; [10] 60 min; [11] 120 min

The formation of disulphide bonds and the general loss of band definition in the 30-60 kDa region (**Figure 3.9, red oval**) occurred only in the irradiated samples, with the bands being retained in all the controls (both run under normal and under reducing conditions). It appears that regardless of whether oxygen is present or not, irradiation induces some level of (mainly) reversible binding to occur in 3OHK modified BLP.

3.2.2.5 Irradiation of BLP modified with 3OHKG

As the most abundant UV filter present in the human lens, the activity of 3OHKG modified BLP was of considerable interest. 3OHKG pH 9.5 modified BLP were irradiated under 4 different conditions: bubbled with air, bubbled with N_2 gas, open to the air or de-oxygenated and sealed in a bottle cuvette. The same varying conditions were used for 3OHKG pH 7.4 modified BLP. As the changes over time in those gels were minimal, they along with the control gels are only shown in the appendix (**see Appendix E**).

The changes detected with 3OHKG modified BLP were mostly comparable to those of Kyn, with all samples irradiated displaying some level of change over 120 min. As previously, the control samples had the least observable changes over time. In samples exposed to oxygen (**Figure 3.10, lanes 7-11 and Figure 3.11, lanes 2-6**), the level of cross-linking increased with time, with the greatest amount of cross-linking present in the samples irradiated for 120 min. This cross-linking consisted of both reversible and non-reversible bonds, with the cross-linking at 120 min not changing when run under reducing conditions. When run under normal conditions, the pH 9.5 modified 3OHKG BLP displayed a significant amount of smearing (most likely from the formation of disulphide bonds). Almost all of that smearing was removed through reduction in the 0-60 min samples, however running under reducing conditions had almost no effect on the 120 min samples suggesting that some different or strong bond formation is occurring after that period of irradiation. As the differences were most apparent in the reduced samples, the remaining comparisons focus on the reduced gels (**Figures 3.10 and 3.11, right gel**).

In an almost identical manner to Kyn, air bubbling resulted in the detection of bands in the higher mass regions of the gel (50-250 kDa) in the time 0 min sample (not irradiated) (**Figure 3.10, lane 7**). Nitrogen bubbled irradiated samples also retained their higher mass bands for longer, with some visible at all different time samples (**Figure 3.10, blue rectangle**). The air bubbled samples lost almost all their higher mass bands (**Figure 3.10, red oval**), whilst those in the 120 min samples were not visible due to excessive cross-linking. There was still some darkening and smearing of the nitrogen bubbled, nitrogen sealed and open air samples, however most of it was removed by running the gels under reducing conditions. Open air irradiated samples retained some cross-linking

even upon being reduced, with the 120 min sample again showing the most protein damage (**Figure 3.11, lane 6 under normal, left, and reduced, right, conditions**). Under these lower oxygen conditions, there was still loss of the higher mass bands (**Figure 3.11, 50-250 kDa**), however to a much lower degree than the actively air bubbled samples. Again, there was a much greater retention of the higher mass bands, the amount of fading in the higher mass bands being proportional to how much oxygen was available to the irradiated sample.

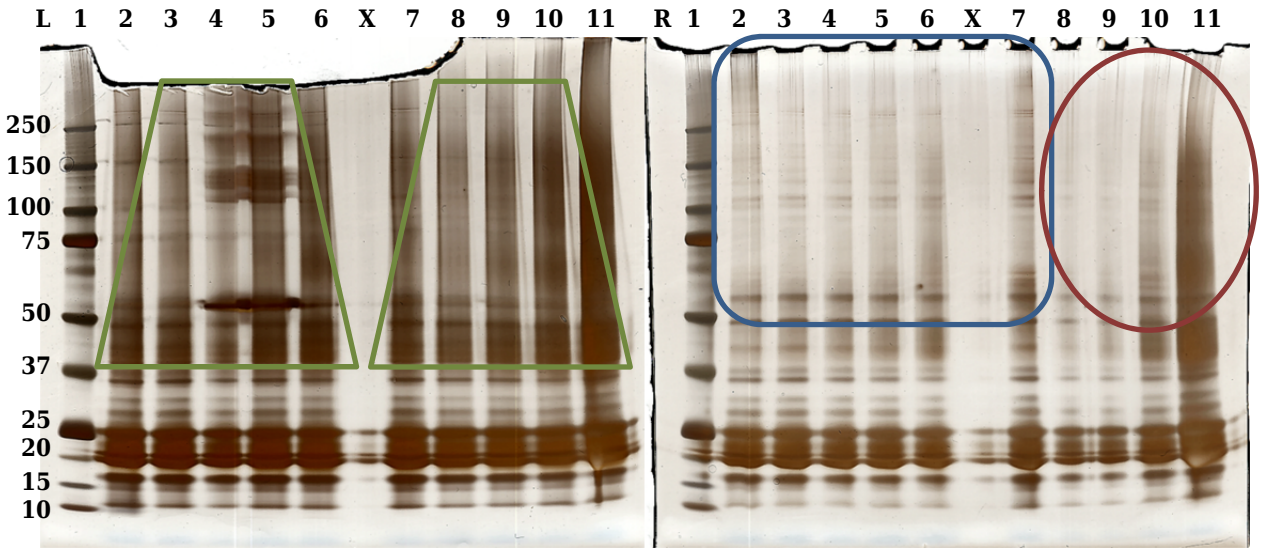


Figure 3.10: 3OHKG pH 9.5 BLP run on a Bis-Tris gel with MOPS buffer. Samples were irradiated for up to 120 min and separated under (left) normal and (right) reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).
Lanes: Molecular weight marker [1] 2 µl
N_2 Bubbled BLP [2] 0 min; [3] 15 min; [4] 30 min; [5] 60 min; [6] 120 min
Air Bubbled BLP [7] 0 min; [8] 15 min; [9] 30 min; [10] 60 min; [11] 120 min

Damage to lens proteins appears to be dependent on at least three factors. There appears to be a requirement for a photosensitiser of UV light, they require UV light (usually over an extended period of time) and they require the presence of oxygen, which it is believed leads to the formation of reactive oxygen species that can cause permanent, irreversible damage to the proteins. In a similar sequence of experiments to those run at pH 9.5, the 3OHKG BLP modified at pH 7.4 produced very similar cross-linking and proportions of protein damage (with the most at 120 min), however the extent of damage was less than the pH 9.5

samples. This is consistent with the lower amount of UV filter binding at physiological pH values (**see Appendix E, control and pH 7.4 gels**).

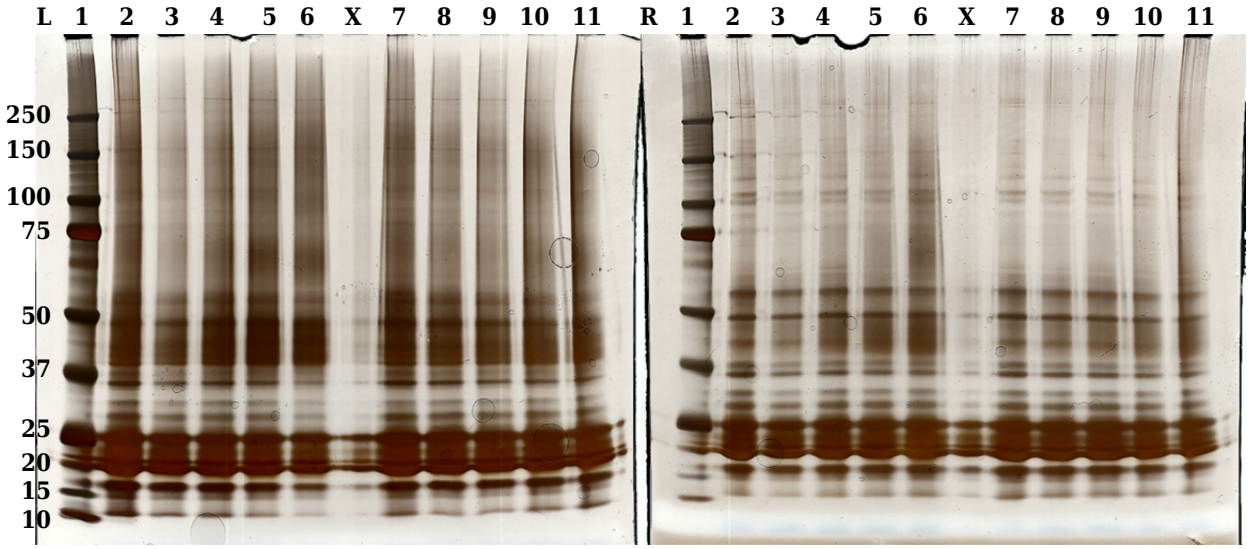


Figure 3.11: 3OHKG pH 9.5 BLP run on a Bis-Tris gel with MOPS buffer. Samples were irradiated for up to 120 min and separated under (left) normal and (right) reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).
Lanes: Molecular weight marker [1] 2 µl
Air Open BLP [2] 0 min; [3] 15 min; [4] 30 min; [5] 60 min; [6] 120 min
N_2 Sealed BLP [7] 0 min; [8] 15 min; [9] 30 min; [10] 60 min; [11] 120 min

3.2.2.6 Irradiation of BLP modified with 3OHKG-D, 3OHKG-W, AHB, AHAG and 3OHKG-Y

3.2.2.6.1 Irradiation of BLP modified with 3OHKG-D

Due to its inherent reactive properties from being an α,β-unsaturated carbonyl system, 3OHKG-D was bound to BLP at a pH of 7.4 rather than pH 9.5. It has already been shown that 3OHKG-D can form in the lens from 3OHKG, and it is believed to be typically present in minute quantities in the lens [219]. Despite the neutral pH, BLP modified with 3OHKG-D were expected to produce protein damage similar to that observed in BLP modified by 3OHKG at pH 9.5.

BLP modified with 3OHKG-D were irradiated for 120 min whilst either air or nitrogen bubbled. Both the air and nitrogen bubbled samples produced heavy

streaking in the 37-250 kDa regions of the normal gels (**Figure 3.12**). The controls and reduced air bubbled gels had little or no cross-linking, with moderately high non-disulphide binding apparently being present in the reduced nitrogen bubbled gels. This is in contrast to the Kyn and 3OHKG BLP modified samples, where the reduced gels produced relatively clear bands. The main change observed was the loss of higher mass bands in the air bubbled gels, with three clear bands present in lane 7 (**Figure 3.12 right, 30, 45 and 50 kDa**) at time 0, which changed to one weak band at 30 kDa after 120 min irradiation in air (lane 11). These results are similar to those observed in the 3OHK modified BLP, where the amount of detectable protein diminished proportionally to the time of UV exposure in the presence of oxygen. Again, the loss of protein suggests the formation and precipitation of large protein aggregates, which were not observed (or present) in the SDS-PAGE gels, however they were not as a result of any of the other processes performed on the modified BLP.

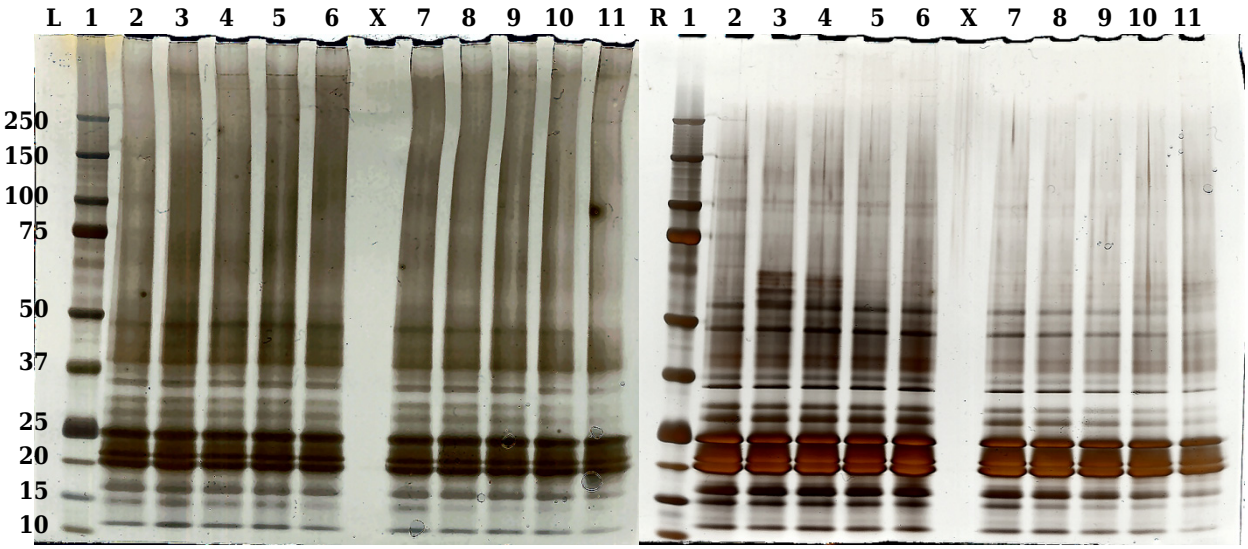


Figure 3.12: 3OHKG-D pH 7.4 BLP run on a Bis-Tris gel with MOPS buffer. Samples were irradiated for up to 120 min and separated under (left) normal and (right) reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).
Lanes: Molecular weight marker [1] 2 µl
N_2 Bubbled BLP [2] 0 min; [3] 15 min; [4] 30 min; [5] 60 min; [6] 120 min
Air Bubbled BLP [7] 0 min; [8] 15 min; [9] 30 min; [10] 60 min; [11] 120 min

Non-irradiated controls run for the 3OHKG-D BLP produced gels were very similar to those of the nitrogen bubbled irradiated gels. It is clear that for the

3OHKG-D modified, as well as the 3OHK modified BLP, extended UV irradiation in the presence of oxygen, leads to observable protein levels diminishing proportionally to the length of exposure. This loss of protein is believed to be as a result of large protein aggregates forming, and either being retained in the cuvette upon sampling or by simply not being detectable *via* SDS-PAGE and silver staining. Controls for 3OHKG-D modified BLP have been included in the appendix (**see Appendix F**).

3.2.2.6.2 Irradiation of BLP modified with 3OHKG-W

3OHKG-W samples were treated at pH 9.5, and irradiated whilst air bubbled or nitrogen bubbled. As one of the more stable 3OHKG derivative metabolites, no changes were hypothesised to occur upon upon irradiation.

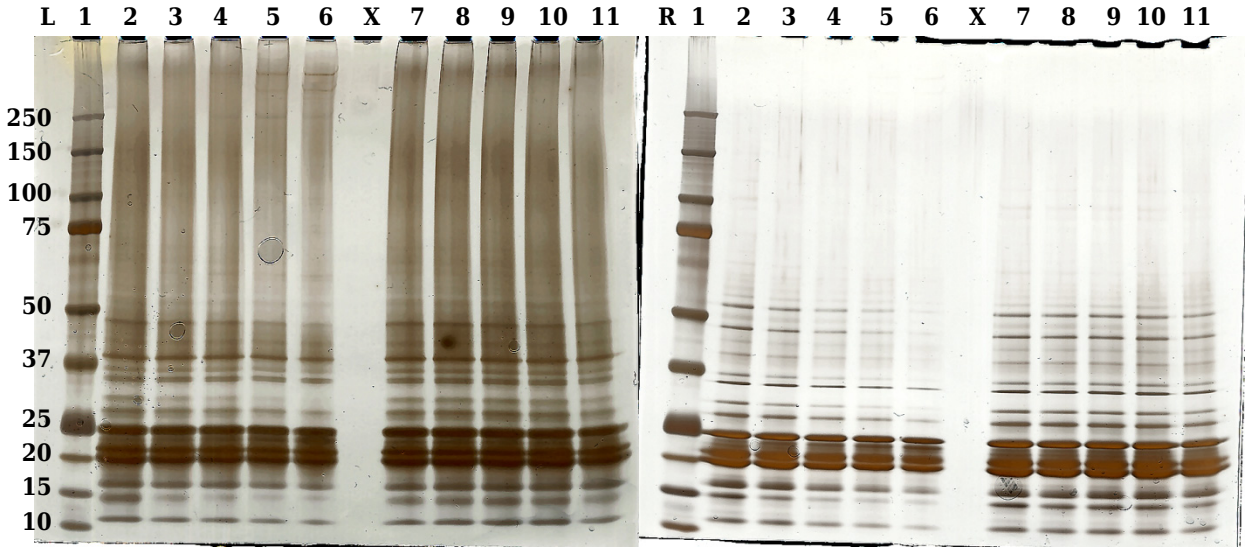


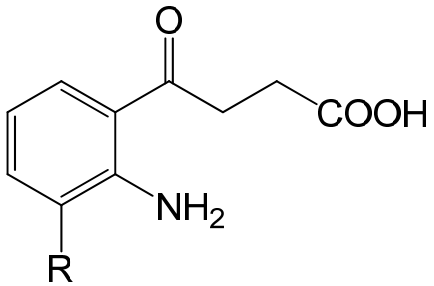
Figure 3.13: 3OHKG-W pH 9.5 BLP run on a Bis-Tris gel with MOPS buffer. Samples were irradiated for up to 120 min and separated under (left) normal and (right) reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).
Lanes: Molecular weight marker [1] 2 µl
Air Bubbled BLP [2] 0 min; [3] 15 min; [4] 30 min; [5] 60 min; [6] 120 min
N_2 Bubbled BLP [7] 0 min; [8] 15 min; [9] 30 min; [10] 60 min; [11] 120 min

Irradiation of BLP modified with 3OHKG-W produced gels that were very similar to the untreated control BLP, which was expected as 3OHKG-W was not expected to bind to BLP due to the reactive amino group (on its parent 3OHKG) being substituted by a less reactive hydroxyl group. There was, however, some fading in

the air bubbled reduced samples (**Figure 3.13, lanes 2-6 right gel**). The same amount of fading was not present in the gels run under normal conditions, so this fading is most likely a minor variation in the luminescence of the gels upon scanning. Control gels are in the appendix (**see Appendix F**).

3.2.2.6.3 Irradiation of BLP modified with AHB

AHB, and the related compound AHA, do not bind covalently to proteins due to the lack of the amino group, although they do appear to associate non-covalently with lens crystallins [39]. Despite this, AHB (but not AHA) can lead to the formation of hydrogen peroxide. This is only observed when the metabolite is at higher concentrations (>1 mg/mL) and is observed in both irradiated and non-irradiated samples suggesting that like 3OHK (though not as strongly as 3OHK), AHB has a damaging effect when present in higher concentrations. This is believed to be due to the *o*-aminophenol moiety undergoing auto-oxidation, leading to $O_2^{\cdot-}$ formation and hence H_2O_2 [160, 228]. Analogous experiments with AHA instead of AHB do not give rise to a significant increase in peroxide levels over control values. AHA lacks the *o*-aminophenol moiety which is of major importance for peroxide formation. This data also sheds light on the fact that the deamination of Kyn (and subsequent reduction to AHA) is most likely a protective mechanism in human lenses as significantly higher levels of peroxides are produced from Kyn-modified proteins when compared to lens proteins non-covalently modified with AHA.



Scheme 3.4: AHA and AHB (R=H AHA and R=OH AHB)

AHB pH 9.5 treated BLP samples were irradiated in the presence of either bubbled air or nitrogen. When run under normal conditions, there was a significant amount of streaking/smearing in the upper portions of the gels (**Figure 3.14, 75-250 kDa**), however none of the smearing was conserved on the

reduced gels and it is believed to be an artefact of modification in the pH 9.5 buffer. A similar level and type of smearing was present in the pH 9.5 unmodified control BLP (**Figure 3.6, lanes 9-11**) and this was confirmed by the AHB control BLP gels (**see Appendix G**) which also displayed streaking/smearing in the normal gels but no such smearing in the reduced gels. Previous studies have shown that AHB displays some level of non-covalent binding to BLP [39], in addition to causing a change in the treated proteins absorbance profile, however these are insufficient indicators for susceptibility to photo-oxidation upon irradiation. BLP modified with AHB at pH 7 were not separated *via* SDS-PAGE.

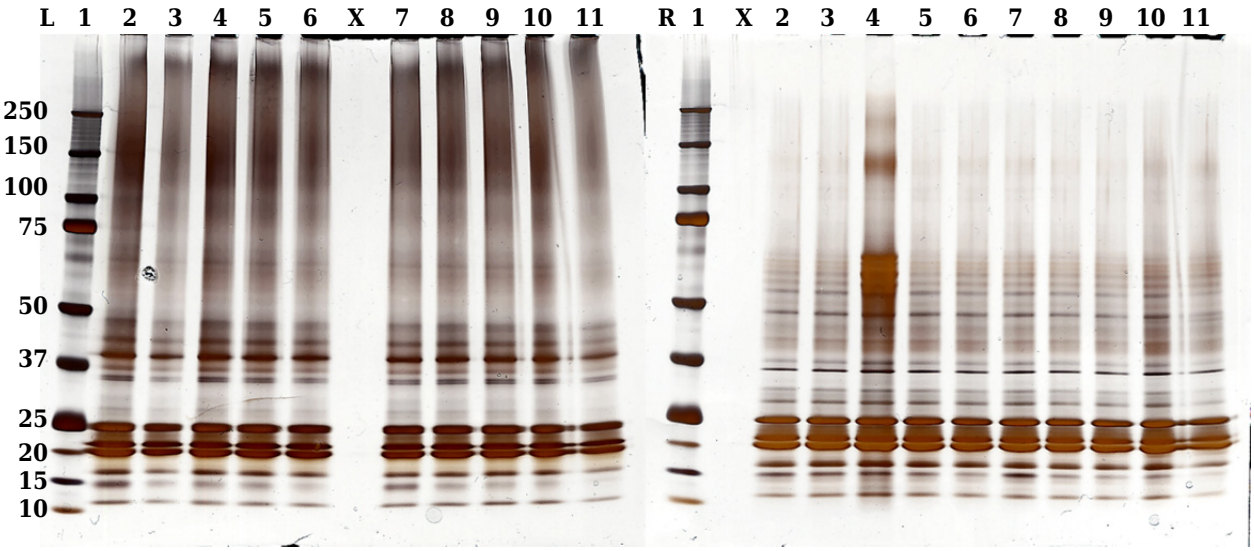
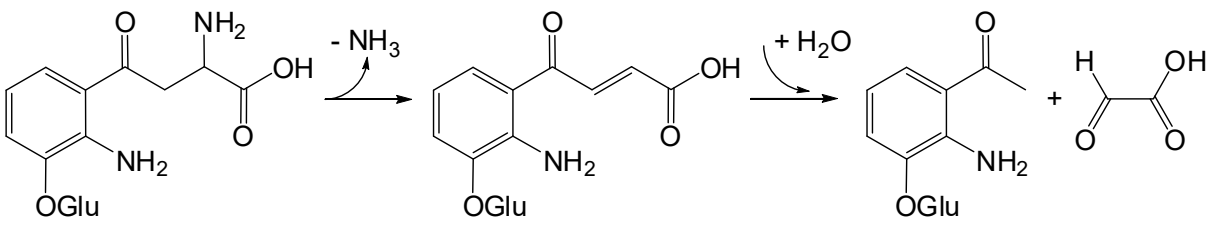


Figure 3.14: AHB pH 9.5 BLP run on a Bis-Tris gel with MOPS buffer. Samples were irradiated for up to 120 min and separated under (left) normal and (right) reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).
Lanes: Molecular weight marker [1] 2 µl
N_2 Bubbled BLP [2] 0 min [3] 15 min [4] 30 min [5] 60 min [6] 120 min
Air Bubbled BLP [7] 0 min [8] 15 min [9] 30 min [10] 60 min [11] 120 min

3.2.2.6.4 Irradiation of BLP modified with AHAG



Scheme 3.5: Possible AHAG formation via 3OHKG reverse Aldol reaction [68]

AHAG, as it does not have the functional side-chain of 3OHKG, cannot form the reactive α,β-unsaturated carbonyl system, and hence is a relatively stable UV filter-like compound. Unlike 3OHKG-D, AHAG has an absorbance profile that is very similar to 3OHKG, that is, one that is characteristic of a UV filter. No changes were expected to the BLP modified with AHAG upon irradiation, however the samples were run for completeness of results.

AHAG treated BLP were irradiated for 120 min whilst bubbled with air or with nitrogen. There was some gel malformation evident in the normal condition gels (**Figure 3.15**), however there were no substantial difference between any of the lanes. On the gels run under reducing conditions, all the lanes appear identical to each other regardless of the irradiation exposure time or whether under aerobic, anaerobic or control conditions (**see Appendix G, control gels**). Essentially, AHAG modified BLP gave the same SDS-PAGE gels as untreated BLP.

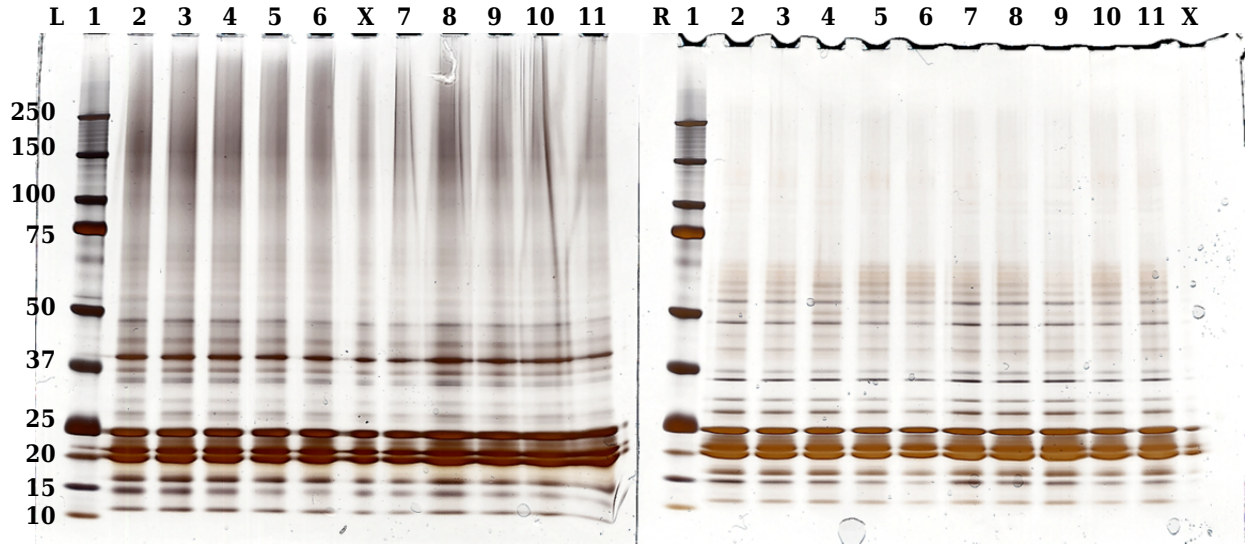


Figure 3.15: AHAG pH 9.5 BLP run on a Bis-Tris gel with MOPS buffer. Samples were irradiated for up to 120 min and separated under (left) normal and (right) reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).
Lanes: Molecular weight marker [1] 2 µl
Air Bubbled BLP [2] 0 min [3] 15 min [4] 30 min [5] 60 min [6] 120 min
N_2 Bubbled BLP [7] 0 min [8] 15 min [9] 30 min [10] 60 min [11] 120 min

3.2.2.6.5 Irradiation of BLP modified with 3OHKG-Y

3OHKG-Y pH 9.5 treated BLP were irradiated for 120 min whilst open to the air or bubbled with nitrogen. Like the untreated pH 9.5 control of BLP, there was a moderate amount of streaking in the higher mass regions of the normal gels (**Figure 3.16, 75-250 kDa**), however all of this was lost under reducing conditions and again it is believed to be an artefact of treatment at pH 9.5. In the reduced gels, all the samples produced identical bands, which is indicative of 3OHKG-Y having little or no effect on the structure of the lens proteins, whether in the presence of oxygen or whether irradiated with UV light.

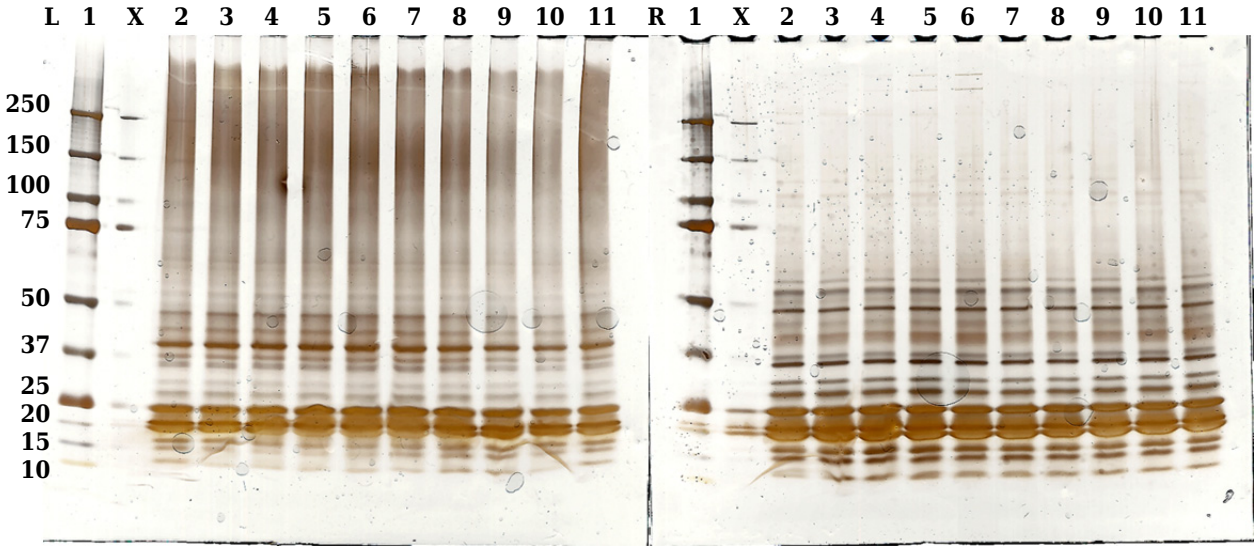


Figure 3.16: 3OHKG-Y pH 9.5 BLP run on a Bis-Tris gel with MOPS buffer. Samples were irradiated for up to 120 min and separated under (left) normal and (right) reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).
Lanes: Molecular weight marker [1] 2 µl
Air Open [2] 0 min [3] 15 min [4] 30 min [5] 60 min [6] 120 min
N_2 Bubbled [7] 0 min [8] 15 min [9] 30 min [10] 60 min [11] 120 min

The BLP modified at pH 7.4 were not run as different results to pH 9.5 were unlikely. Control and 3OHKG-Y pH 9.5 treated BLP (irradiated whilst air bubbled) were also run under normal and reducing conditions. No changes were observed. These gels are presented in the appendix (**see Appendix H**).

3.3 Discussion

A major indicator of advanced age related nuclear cataract is the aggregation of water insoluble protein clusters in the lens as a result of protein-protein cross-linking [134, 250, 251]. These aggregates can reach a diameter of some 350 nm and are up to 10^6 kDa [252]. This in contrast to unmodified α-crystallins from healthy lenses, which have a diameter of 15 nm and average 800 kDa [252, 253]. It is believed that cross-linking between lens proteins may be initiated by subjecting the UV filters 3OHKG, 3OHK and Kyn, along with the UV filter metabolites 3OHKG-D and AHB, to short wavelength UVA light. As healthy lenses age the concentration of both αA, αB and other larger mass crystallins diminish due to progressive truncation, with high a corresponding increase of small peptides [254]. The increase in crystallin fragments results in increased exposure of reactive Cys, His and Lys residues, hence providing a greater opportunity for unstable UV filters to bind to lens proteins. Unfortunately this due to relative youth of bovine lenses (<2 years old), this could not be performed in bovine model. Though ARN cataract may (literally) take a lifetime to form, in the absence of the usual protective antioxidants/enzymes (*e.g*. glutathione), and with a strong broad spectrum UV lamp, it may be possible to replicate cross-linking to a small extent *in vitro* over a short period of time with subsequent observation of the large aggregates through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

A comparison with natural sunlight is also important. Upon measuring the emission spectrum of the lamp with a 305 nm cut-off filter, a similar spectrum to natural sunlight could be reproduced, the main variations being a major emission peak at 376 nm (in addition to the multiple emission peaks that a mercury arc lamp produces in the visible light wavelengths). The profile is largely similar in magnitude, with both having a gradual increase of intensities from 400 to 600 nm, and a gradual drop through to 800 nm. The maxima of sunlight is at approximately 550 nm compared to the 650 nm of the lamp, and both have similar levels of infrared emission above 800 nm.

An interesting observation was made with the ambient room lighting in that approximately 0.25 μW/cm^2 of UVB and 0.65 μW/cm^2 of UVA light was measured. It was initially believed that these small readings were merely instrument noise, however the emission spectrum of the room lighting had peaks at 320, 342 and 376 nm, the same as the mercury-arc lamp used. The UV lamp used in this experiment operates the same as regular fluorescent and compact fluorescent

globes, however at a much higher wattage and without the UV absorbing phosphorous coating. Though not a focus of this study, cheap, poorly coated or damaged fluorescent light globes may be a potential source of UV light damage to the human lens [255-258].

SDS-PAGE was performed on modified and untreated bovine lens proteins samples under varying conditions. Proteins were irradiated over a time course of 120 min, with samples taken periodically. Samples were irradiated in the presence and absence of oxygen (by displacing it with nitrogen), as well as under deoxygenated/sealed conditions. Comparisons were made between untreated and unexposed controls, as well as negative controls over the same time course. Samples were separated on both reducing and non-reducing gels (*i.e.* with or without the reductant β-mercaptoethanol, respectively), to identify cross-linking by protein disulphide bonds, which can be disrupted upon being subjected to electrophoresis. Modern standardised preformed gels were used, though the principles of separation are the same as those described by Laemmli [259]. As the quantities of the target proteins were expected to be small, silver staining was used to observe the extent of proteins separation/binding as it is generally accepted to be one of the more sensitive staining techniques [50].

The main form of bonding in cross-linked proteins is the disulphide bond between cysteine residues, however other non-disulphide cross-links were also evident (*e.g.* di-Tyr) [50]. In general, the level of cross-linking in the experiments conducted was found to be greatest in samples that had been illuminated with UV light, with the largest level of cross-linking being observed in the proteins modified with 3OHK and 3OHKG-D and 3OHK. Of these, Kyn modified BLP displayed less cross-linking under all conditions. The other UV filter metabolites (AHB, 3OHKG-Y, 3OHKG-W and AHAG) showed little difference to the control samples upon illumination. Though previous data have suggested that the actual level of cross-linking is minimal in these relatively short periods of illumination (up to 120 min) [200, 260], in this study cross-linking was detected with the highly sensitive silver staining technique rather than the less sensitive Coomassie blue staining techniques used by Parker *et al*. and Korlimbinis *et al.* [200, 260]. The order of detectable aggregates was also the same between the non-reduced and the reduced SDS-PAGE gels.

When run under reducing conditions, disulphide bonds are cleaved and formation of further bonds between protein cysteine residues is inhibited by the

reducing agent (in this case β-mercaptoethanol). In the lens, this function is believed to be performed by glutathione. Despite the reduction of the expected cysteine cross-links, there is evidence of protein aggregation in these experiments, which suggests the formation of di-Tyr bonds (which are not reduced by β-mercaptoethanol) [261]. Though the formation of di-Tyr bonds is possible, di-Tyr cannot account for all the observed cross-linking particularly when 3OHK and 3OHKG/3OHKG-D modified BLP are considered. Previous studies have shown marked differences in the levels of di-Tyr detected *via* reversed phased HPLC between these two UV filters, yet both display similar levels of binding *via* SDS-PAGE [39, 261]. Other cross-linking reactions may occur between the proteins, of which intramolecular binding between 5-cysteinyl-DOPA residues (generated from the oxidation product of DOPA, DOPA quinone and cysteine) upon UV irradiation is one possibility [262, 263]. It is also possible that UV generated products, particularly those formed from $^1O_2$ interaction, may also be involved in the cross-linking of the modified proteins with evidence having been reported for cross-links involving other Tyr-derived products, His, Trp and Lys residues [232, 245, 264-270]. Hence, it is important to investigate whether UV filters can bind to His and Lys protein residues alongside with Cys residues and whether those bound products also have potential photosensitising capabilities.

3.3.1 Effect of oxygen on UV irradiation

It is known that the presence of oxygen, particularly the replenishment of oxygen, leads to greater production of peroxides most likely through the indirect (Type II) route of peroxide formation. A nitrogen atmosphere, or a sealed oxygen depleted environment, also results in the formation of peroxides but to a lesser extent. The effect of oxygen being greater with 3OHKG and 3OHK modified BLP than with Kyn was observed in this study and is consistent with previous literature [50].

3.4 Conclusion

In this study, it was proposed that exposure to UV light of UV filter modified lenses is a major contributor to the accelerated development of cataract in human lens. This phenomenon was readily modelled by incubating BLP in the presence of UV filters and their metabolites, initially with the aim of studying the reactivity of 3OHKG, 3OHKG-D, 3OHKG-Y, 3OHKG-W, AHAG, AHB and AHA to BLP. To compare with previously reported results, experiments with Kyn and 3OHK as

positive controls, and unmodified BLP as a negative control, were simultaneously conducted. The second aim was to determine what effect UV light (and under what condition) had on these modified lens proteins.

The covalent binding of a UV filter to BLP is believed to occur mainly *via* the α,β-unsaturated carbonyl moieties, with the binding being a prerequisite to additional photo-oxidative damage occurring upon irradiation with UV light in the 300-400 nm range. Upon analysis by SDS-PAGE electrophoresis, binding, cross-linking and photo-oxidative damage was observed for kynurenine based UV filters (Kyn, 3OHK, 3OHKG, 3OHKG-D). No evidence of binding or photo-oxidative damage was observed for samples treated with 3OHKG-Y, 3OHKG-W, AHAG and AHB upon irradiation.

From these results, further evidence of the four main categories of protein-protein cross-linkage in cataractous human lens was observed; disulphide bonds between cysteine residues (which were directly observed in this study), di-Tyr bonds between Tyr residues (suggested by the literature) [50] formation of dehydro-2-imidazolone and 2-imidazolone from histidine (His) [271] and a less understood category of bonds involve Lysine (photo-damage where the other three categories are unlikely or not possible). As has also been demonstrated between partially modified BLP (under pH 7.4) and highly modified BLP (pH 9.5), the binding of unstable UV filters to lens proteins changes the photochemistry of those compounds from largely inert compounds (at least for 3OHKG and Kyn) to efficient photosensitisers of damaging reactive species. This nexus between the level of binding and the level of damage in the lens is also played out in cases of ARN cataract, where the gradual decrease in the levels of the protective free UV filters corresponds to a gradual increase of UV filter binding and hence increased damaged from photo induced reactive oxygen species [26, 224]. Likewise, the decrease in antioxidants in older lens (analogous to the SDS-PAGE gels of non-reduced UV irradiated modified BLP) leads to a cycle of greater and greater lens damage from initially the binding of the UV filters and then from the oxidant that they create.

There are potentially three major main sources of peroxide formation in the lens, with the two key processes being the formation of singlet oxygen and superoxide (both of which are converted to hydrogen peroxide) and the second being the auto-oxidation of the *o*-aminophenol moiety present on 3OHK and its derivative metabolites like AHB. 3OHK appears to be the most efficient sensitiser

of singlet oxygen formation and appears to be as effective as 3OHKG despite its much lower concentration in the lens. The third and least understood process involves peroxide formation mediated by other species either already present in or bound to the protein (possibly involving Lys and His interaction with the UV filters). This third means is indirectly evident, as the level of peroxide formation (mainly hydrogen peroxide) do not correlate with the formation of other protein oxidation products like DOPA and di-Tyr bonds [50, 172]. It appears that there must be some form of alternate means of energy transfer from the excited state UV filter chromophores possibly to free oxygen in the lens, however also possibly through the direct route straight to neighbouring protein residues, for example as mediated by 3OHKG-adducts in the formation of phenoxyl radicals, resulting in di-Tyr [245, 246, 272].

Regardless of what contribution all these processes have on protein aggregation and cross-linking, these are the consequences of UV filter instability in ARN cataract. Binding to and malformation of protein greatly increases and protein fragmentation decreases if not ceasing all together in highly developed forms of cataract. There are multiple pathways in the formation of protein cross-links, of which the involvement of Lys and His must also be considered. The common elements are that they involve photosensitisation of protein bound UV filters in the presence of UV light and that all of them contribute to the modification of the lens proteins. Hence understanding all the possible means by which UV filters (and other compounds present in the lens) can induce cross-linking and protein aggregation is necessary to be able to determine the exact pathways of cross-linking. This may provide some insight into how protein damage can be controlled, prior to the spiralling feedback loop of ARN cataract development begins in older age.

3.5 Experimental

3.5.1 Materials and apparatus

Organic solvents and acids were all of HPLC grade and purchased from Sigma-Aldrich (NSW, Australia). Milli-Q® water, purified to 18.2 MΩ cm^{-2}, was used for the preparation of all solutions. DL-Kynurenine sulphate salt (≥ 95%), 3-hydroxy-DL-kynurenine, formic acid (> 99%), MES and MOPS stock solutions, 4-12% SDS-Page gels and deuterium oxide (D_2O, 99.9%) were purchased from Sigma-Aldrich.

Bovine lens proteins (BLP) were purified from calf lenses, and contained their natural composition of crystallins (**see 4.5.7 Preparation of proteins from bovine lenses**).

3.5.2 Modification of BLP with UV filters

BLP (10 mg/mL) were treated with UV filters (2 mg/mL) in oxygen depleted Na_2CO_3/$NaHCO_3$ buffer (50 mM, pH 9.5) or in NaH_2PO_4/Na_2HPO_4 buffer (100 mM, pH 7.4) as described previously [18, 124, 225]. The reaction mixtures were incubated at 37 °C for 48 h (pH 9.5; Kyn, 3OHK, 3OHKG, AHB, 3OHKG-Y, 3OHKG-W, AHAG; and pH 7.4; 3OHKG-D) or 7 days (pH 7.4; Kyn, 3OHK, 3OHKG, AHB, 3OHKG-Y) in an anaerobic chamber. The mixtures were dialysed at 2 °C for 48 h in oxygen depleted NaH_2PO_4/Na_2HPO_4 buffer (10 mM, pH 7.4) in a 1 litre Schott bottle (with 3 buffer changes in that period). The coloured proteins (pale yellow, brown or brown/red) mixtures were then lyophilised and stored under nitrogen at -20 °C until used.

3.5.3 UV light irradiation procedure

Modified, control and untreated BLP were dissolved at two concentrations (0.5 mg/mL and 1 mg/mL) in water and then irradiated with a 125 W broad-spectrum mercury arc lamp, filtered with a 305 nm filter. Irradiation was performed from a fixed point 15 cm from the samples. Both the light intensity (W/cm^2) and emission spectra were recorded on a Stellarnet Somet-SL-3C-50 Spetrophotometer from Warsash Scientific (Sydney, Australia). Emission spectral data are shown below (filter data provided by Jasminka Mizdrak).

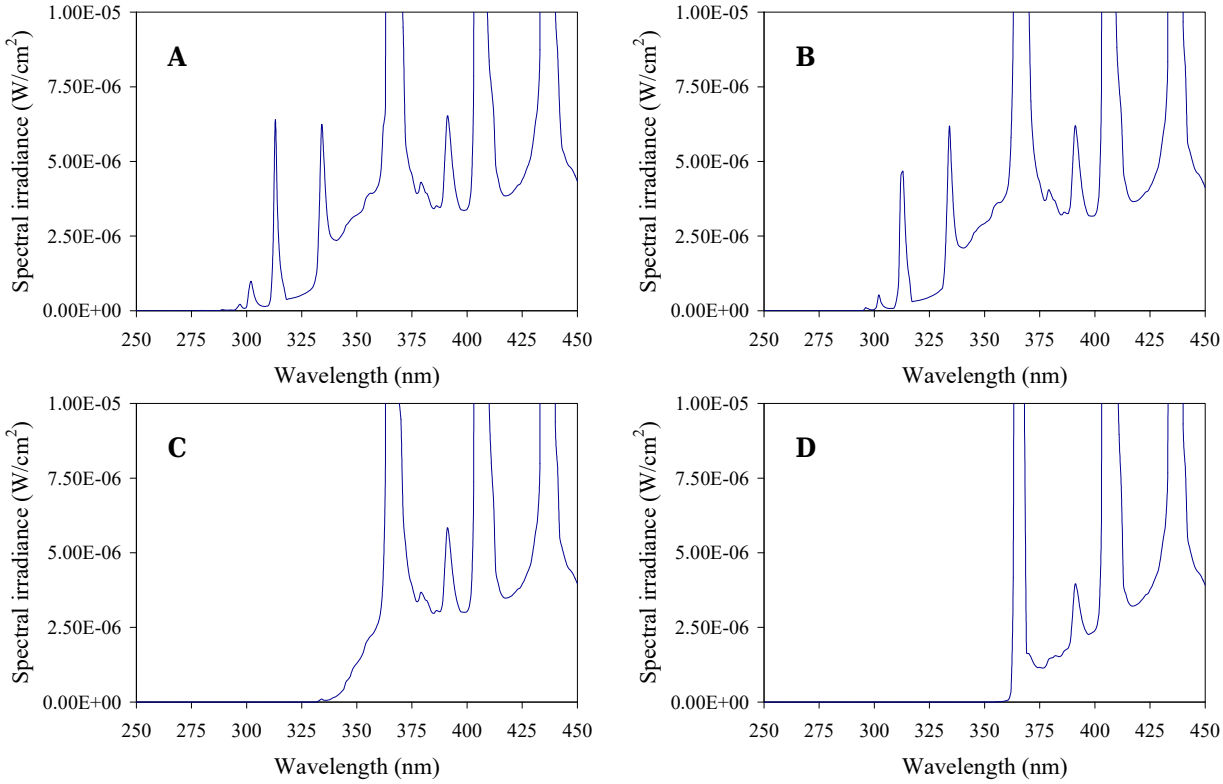


Figure 3.17: 125 W mercury arc lamp radiation spectra of various filters. A) No filter; B) 305 nm; C) 345 nm; D) 385 nm. Data and diagram courtesy of Jasminka Mizdrak.

Samples were irradiated in 1 x 1 x 4 cm (4 mL) quartz cuvette bottles sealed with a septum. Cuvettes were supported by an open sided stand to ensure reproducible experimentation (thank you to Christopher Gad for designing and 3D printing the stand). The septum was pierced with needles for the controlled delivery of gases (air or N_2). Samples prepared for illumination were irradiated under varying conditions including (1) continuous bubbling with N_2 (2) continuous bubbling with air (21% oxygen/nitrogen mixture) (3) degassed with N_2 (for 10 min) and sealed (4) under ambient oxygen conditions and sealed. Irradiation was performed in a fume cupboard, and samples did not require cooling due to the constant forced airflow of the running fume cupboard, which remained at ~20 °C. Samples were irradiated for 15, 30, 60 and 120 min with aliquots of the samples taken at each point. Each aliquot was wrapped in aluminium to prevent any further light irradiation and then placed in a -20 °C freezer. The following picture shows the irradiation setup.

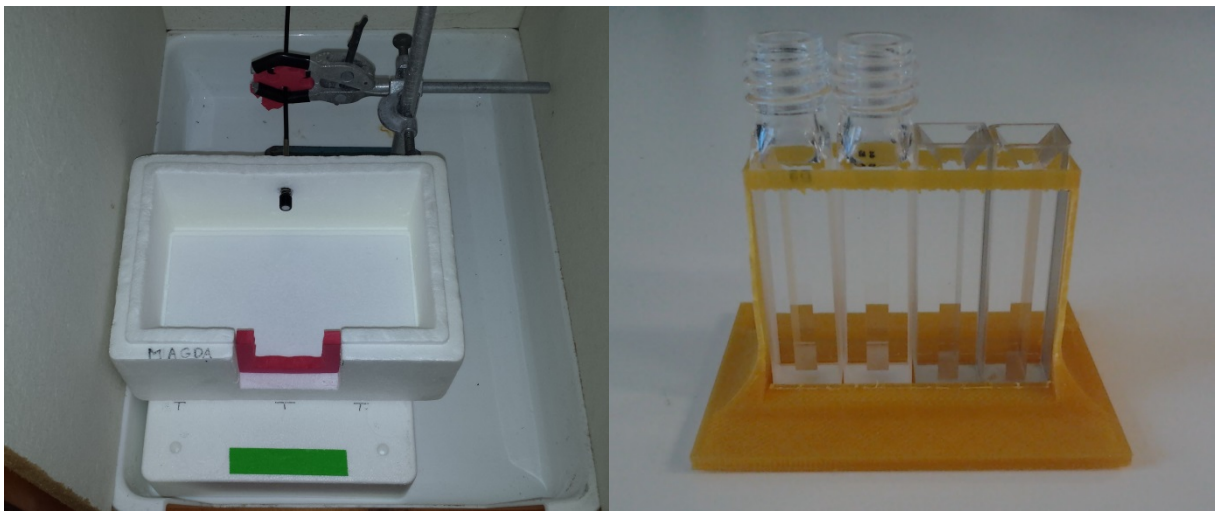


Figure 3.18: Irradiation apparatus set up, and 3D printed cuvette stand. Filter was fitted into the red foam and the cuvette stand was placed directly behind the filter inside the Styrofoam box.

3.5.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
Proteins (modified, untreated and controls) were separated through electrophoresis using a standardised modern method based on the methods described originally by Laemmli [259]. Samples were separated using the Novex NuPAGE SDS-PAGE Gel system with NuPAGE 4-12% Bis-Tris precast running gels using MOPS (3-(*N*-morpholino)propansulfonic acid) as a running buffer using the standardised protocol of the system/gel manufacturer [273]. Both illuminated samples (15, 30, 60 and 120 min), control samples (allowed to stand at room temperature with identical periods of time) were diluted with 4 x concentrated loading buffer (5:15 µL), heated (95°C, 5 min), cooled, centrifuged down for 3 s and loaded (16 µL) onto the 4-12% Bis-Tris gels. Samples were run under mild conditions (150 V for 45 min). Both, illuminated and non-illuminated untreated BLP were used for controls. Samples were run solely with loading buffer (normal conditions) or with β-mercaptoethanol (5%) added to the gels for running under reducing conditions. Coloured molecular weight markers (Bio-Rad broad range Kaleidoscope SDS-PAGE standards, 7,100 - 209,000 Da, Sigma-Aldrich) were loaded in lane 1 or 2 (and an additional lane when space was available). Bands were then detected by silver staining, and the resulting gels were digitised on a Bio-Rad Gel Doc 1000 system.

CHAPTER 4

ANALYSIS OF LENS PROTEINS MODIFIED WITH KYNURENINE BASED HUMAN LENS UV FILTERS AND METABOLITES

Purpose

This chapter describes the characterisation by UV/Vis, fluorescence and LC-MS/MS analysis of bovine lens proteins modified/treated with the major human lens UV filters 3OHKG, 3OHK and Kyn and the metabolites 3OHKG-D, 3OHKG-Y, 3OHKG-W, AHAG and AHB. Amino acid residues involved in covalent binding of UV filters/metabolites were also detected and identified.

Methods

Treatment of the bovine lens proteins was performed through incubation separately with 3OHKG, 3OHK, Kyn, 3OHKG-D, 3OHKG-Y, 3OHKG-W, AHAG and AHB at pH 7.4 and 9.5 (**Chapter 3**). Treated BLP that showed modification were characterised *via* UV/Vis analysis and 3D fluorescence through comparison with mixtures of BLP and free UV filter/metabolites for evidence of binding. The amino acid adducts were identified *via* total synthesis and quantified following enzymatic hydrolysis of the modified lens proteins with Pronase through LC-MS/MS spectroscopy.

Results

3OHKG was found to react with lens proteins at Cys and His residues at both pH 7.4 and 9.5, with the greatest level of binding on Cys at pH 9.5. Low levels of binding were detected at the Lys residues at pH 9.5, but near detection limit levels at pH 7.4. Comparative studies with Kyn, 3OHK and 3OHKG-D similarly resulted in modification at Cys residues, some binding at His and low binding at Lys. 3OHKG-Y unexpectedly produced very low binding to Cys at both pH 7.4 and pH 9.5. The extent of modification was found to be significantly higher at pH 9.5 in almost all cases. 3OHKG, 3OHKG-D, 3OHK and Kyn modified lens proteins were found to be coloured and fluorescent, resembling those from aged and age related nuclear cataractous lenses. AHB treated at pH 9.5 showed some UV/Vis and fluorescence, however no covalent binding was detected. The metabolites

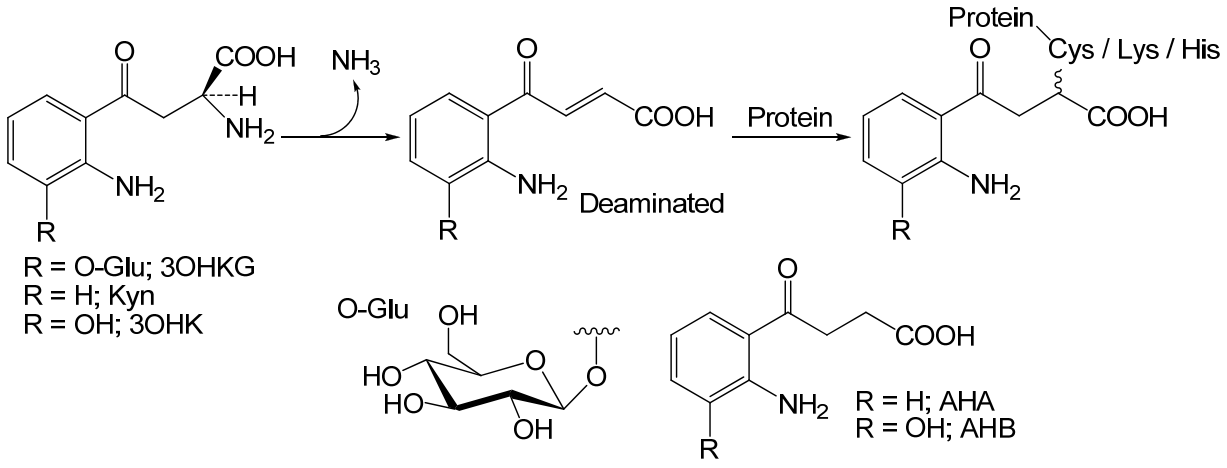
AHB, 3OHKG-W and AHAG did not produce any detectable amino acid adducts.

Conclusion

UV filters which did not form α,β-unsaturated carbonyl compounds of lens proteins, did not produce significant covalent modification of lens proteins. UV/Vis and fluorescence of treated BLP generally gave a good indication of whether covalent binding of a UV filter had occurred, with Cys, His and Lys binding to 3OHKG, 3OHK, Kyn and 3OHKG-D being detected and quantified.

4.1 Introduction

The major UV filters 3-hydroxykynurenine-*O*-β-D-glucoside (3OHKG), and 3-hydroxykynurenine (3OHK) and kynurenine (Kyn) can all deaminate to reactive α,β-unsaturated carbonyl compounds, and hence are intrinsically unstable under physiological conditions [46, 212, 274]. These α,β-unsaturated carbonyl compounds can readily react with nucleophiles in the human lens, including the functional groups of free amino acids and proteins [50]. The most likely amino acid targets of these reactions are the thiol and amine nucleophiles such as cysteine, His and Lys residues on the human lens proteins, specifically lens crystallins [225, 260].



Scheme 4.1: Binding of UV filters Kyn, 3OHK, 3OHKG and structure of AHB.

Previous studies have observed that the deaminated metabolite derived from Kyn, 3OHK and 3OHKG reacts more readily *in vitro* and *in vivo* with Cys residues than any other amino acid in human lens crystallins [124]. His and Lys binding has been speculated but not detected directly in previous studies [124, 225]. The extent of protein modification that takes place in the lens is dependent on the

age of the individual, with the level of damage caused by the unstable UV filters increasing significantly after middle age in people with genetically normal and healthy lenses [160, 224]. Along with the increase of UV filter related damage, three other important changes occur concurrently in the lens from middle age, all exacerbating the amount of damage and binding. First the levels of free UV filters decrease [26, 224], with the main UV filter 3OHKG levels declining at a rate of approximately 12% per decade. Second, the levels of the major antioxidant glutathione also begin to decrease [26, 139], and related to the decrease in glutathione, thirdly the levels of the glutathione adduct of 3OHKG (GSH-3OHKG) and other UV filter metabolites begin to increase in concentration [51, 68, 219]. In other *in vivo* studies it has been found that in older lenses, 3OHKG binds predominantly to the Cys residues of γS-crystallins, with a small amount of binding to the βB1-crystallins [72]. In the human (and generally the mammalian) lens, the α-crystallins are the largest and most abundant group of crystallins, with the two component polypeptides αA- having two Cys residues and αB-crystallins having no Cys residues in their respective 173 and 175 amino acid residue structures [275, 276]. The lower abundance γS- and βB1-crystallins have 7 and 3 Cys residues, respectively, hence providing more potential binding sites for the unstable UV filter molecules [72, 149].

Previously, Kyn and 3OHK were both shown to preferentially bind with Cys residues and that binding can occur at physiological pH, though a higher pH (9.5) encourages deamination and accelerates the rate of binding to Cys [124, 277]. Hence, it is hypothesised that at higher pH values, binding to other reactive amino acids, namely His and Lys (as well as Cys) may increase to a detectable level. Kyn and 3OHK have a similar structure to 3OHKG, however the amino acid sites involved in 3OHKG binding to bovine lens proteins (BLP) *in vitro* has not been previously investigated.

The main aim of this chapter was to investigate the structural and spectral (UV/Vis, fluorescence, LC-MS/MS) properties of BLP modified with 3OHKG, 3OHK, Kyn or the recently identified novel UV filter metabolites 3OHKG-D, 3OHKG-Y, 3OHKG-W, AHAG and AHB (**Chapter 2**). The amino acids involved in their covalent binding to lens crystallins were also identified and quantified [68, 81, 278].

4.2 Results

4.2.1 Treatment of bovine lens proteins

In this study, BLP were incubated with 3OHKG, 3OHK, Kyn, AHB, 3OHKG-Y, 3OHKG-W, AHAG and the reactive metabolite 3OHKG-D. Though no reaction was expected with some of the metabolites (*i.e.* 3OHKG-Y, 3OHKG-W and AHAG, as they lack the side-chain amino group and thus the capability of forming α,β-unsaturated carbonyl compounds) it was of interest to determine if they could still bind to the proteins or elicit a physical change in the lens proteins upon incubation. This study would therefore ascertain the relative importance of the aromatic group and the α,β-unsaturated carbonyl moiety in protein binding.

The modification of BLP with Kyn and 3OHK at pH 7.4 and 9.5 have been investigated previously by Garner [38], Aquilina [72], Parker [18], Korlimbinis [224] and Mizdrak [50]. pH 7.4 is important as it is physiologically relevant, whilst pH 9.5 was employed as the higher alkalinity results in significantly greater deamination of the UV filters and facilitates nucleophilic attack by the amino or thiol amino acid side-chains on lens proteins [51]. BLP were used in this study as a model for human lens proteins as there is a large degree of sequence homology between bovine and human crystallins (94% for αA and 97% for αB) [149]. In addition, bovine lenses lack any UV filter compounds (or UV filter-like compounds) and are therefore an excellent model for examining the effects of exogenous UV filters. This lack of UV filters could be attributed to the domestication and selective breeding of cattle by humans as long lived vision would not produce any survival benefit to their relatively short and protected lives, however the lack of UV filters is common to most mammals except for primates and grey squirrels [64, 279]. Modification of the BLP was under similar conditions to previous studies [18, 50, 225]. BLP and each UV filter/metabolite individually were dissolved in deoxygenated phosphate buffer at pH 7.4 or bicarbonate buffer at pH 9.5 and incubated in the dark at 37 °C in an anaerobic chamber. As stated in chapter 3, 3OHKG-D did not require incubation at pH 9.5 for protein modification as it is already deaminated and highly reactive.

4.2.1.1 Colour of modified BLP

Upon incubation, the 3OHK and AHB incubations developed a red-brown colouration. Most of the colour diffused into the buffer during subsequent

dialysis. The resultant 3OHKG, 3OHKG-Y and 3OHKG-W modified proteins changed in colour from colourless or pale yellow to a moderate yellow colour at both pH 7.4 and pH 9.5. Treatments with Kyn, 3OHKG-W and AHAG produced very little change in the treated protein's colour. The 3OHK, 3OHKG-D and AHB reactions produced the greatest colour changes, suggesting (though not confirming) that physical modification may have occurred. At pH 7.4, 3OHK coloured the crystallins red-brown, whilst at pH 9.5 it produced a brown (no red tinge) product. 3OHKG-D produced a bright orange/yellow product, whilst AHB gave the proteins a slight reddish tinge at both pHs, though stronger at a pH of 9.5.

Figure 4.1: UV filter modified BLP at pH 7.4 and pH 9.5
(Left to right) Control pH 7.4, Control pH 9.5, Kyn pH 7.4, Kyn pH 9.5, 3OHK pH 7.4, 3OHK pH 9.5, 3OHKG pH 7.4, 3OHKG pH 9.5, 3OHKG-Y pH 7.4, 3OHKG-Y pH 9.5, 3OHKG-W pH 9.5, AHAG pH 9.5 and 3OHKG-D pH 7.4

4.2.1.2 UV/Vis and fluorescence of modified BLP

The modified BLP samples were characterised by UV/Vis and 3-D fluorescence spectroscopy (**Table 4.1**). The untreated, control and modified proteins were dissolved in 6 M guanidine hydrochloride for spectroscopic analysis, with all readings being performed in quartz cuvettes. Guanidine hydrochloride has an absorption maximum at ~250 nm, with relatively high absorbance at 280 nm (protein absorbance peak) but low absorbance at greater than 300 nm. Its fluorescence maxima is at λ_{ex} 315 / λ_{em} 366 nm, with a minor excitation peaks at λ_{ex} 370 / λ_{em} 440 nm, though it did not interfere with the absorbance and fluorescence measurements of the protein samples. At 366 nm, the absorbance maxima of BLP modified with 3OHKG were very similar to those of free 3OHKG (λ_{max} 365 nm) and Cys-3OHKG (λ_{max} 370 nm) [50], however the fluorescence was different to Cys-3OHKG, with no fluorescence at λ_{ex} 337/λ_{em} 438 nm at either pH

examined. It is believed that the absence of UV filter-like fluorescence is possibly due to the absence of Cys-3OHKG dimers or aggregates which normally form under aqueous conditions, hence its fluorescence is quenched [200]. The results were similar for BLP modified with Kyn. BLP treated with 3OHK had both absorbance maxima at λ_{max} 367 nm, and at 440 nm suggesting that these particular chromophores were likely to be arising from oxidative products of 3OHK (some of which also have an absorption maxima at approximately 440 nm [158, 168, 247].

Table 4.1: Absorbance and fluorescence data for UV filter and metabolite treated BLP in 6 M guanidine hydrochloride.

Bound UV filter	Absorbance (λ_{max}, nm)		Fluorescence ($\lambda_{ex}/\lambda_{em}$, nm)	
	pH 7.4	pH 9.5	pH 7.4	pH 9.5
Controls	278	278	280/555	280/555
Kyn	368	368	380/485	370/455
3OHK	367, 440	367, 440	345/490	350/510
3OHKG	365	370	370/450	370/480
3OHKG-Y	370 (low)	370 (low)	370/420	370/420
AHB	360, 400 (low)	328, 400, 440	330/365 380/475	330/365 390/490
3OHKG-W	N/A	370 (low)	N/A	370/425
AHAG	N/A	370 (low)	N/A	370/420 400/460
3OHKG-D	370	N/A	370/505	N/A

It is known that *o*-aminophenols are highly reactive and readily oxidised to quinone imines, even in the presence of trace levels of oxygen. Alkaline conditions can accelerate the rate of such oxidation compared to physiological pH [160]. The fluorescence profile of 3OHK treated BLP also varied with the incubation pH. This is probably due to the formation of pH dependent fluorophores, for example Cys-3OHK has excitation maxima at λ_{ex} 340 and 405, and emissions at λ_{em} 520 and 475 nm. This is in contrast to oxidised 3OHK protein-bound adducts, for example

benzimidazole and benzoxazolone adducts, which have an excitation peak at λ_{ex} 390 and emission at λ_{em} 490 nm [158, 167]. The protein extracts of aged human lenses have similar fluorescence profiles (λ_{ex} 340-360 and λ_{em} 420-440 nm) [57], whilst cataractous human lenses have excitation/emission peaks in a higher range (λ_{ex} 420-435 and λ_{em} 500-520) which are more consistent with the metabolite 3OHKG-D [280]. More recently, Ranjan *et al*. confirmed that there are age related increases in non-Trp fluorescence with excitation peaks λ_{ex} 312/365 nm, and emission peaks in the λ_{em} 380-470 nm range of intact lenses, with a gradual shift in absorbance between 210-470nm [281].

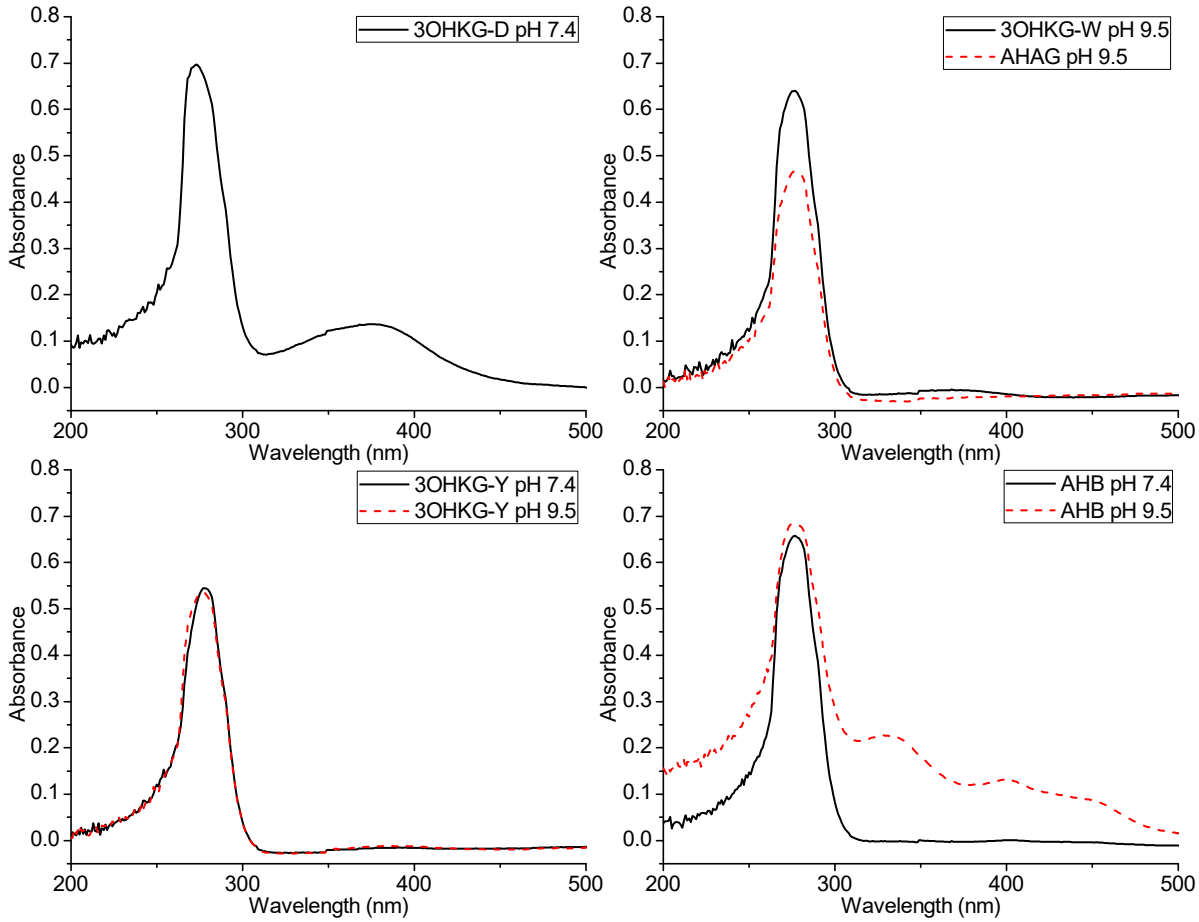


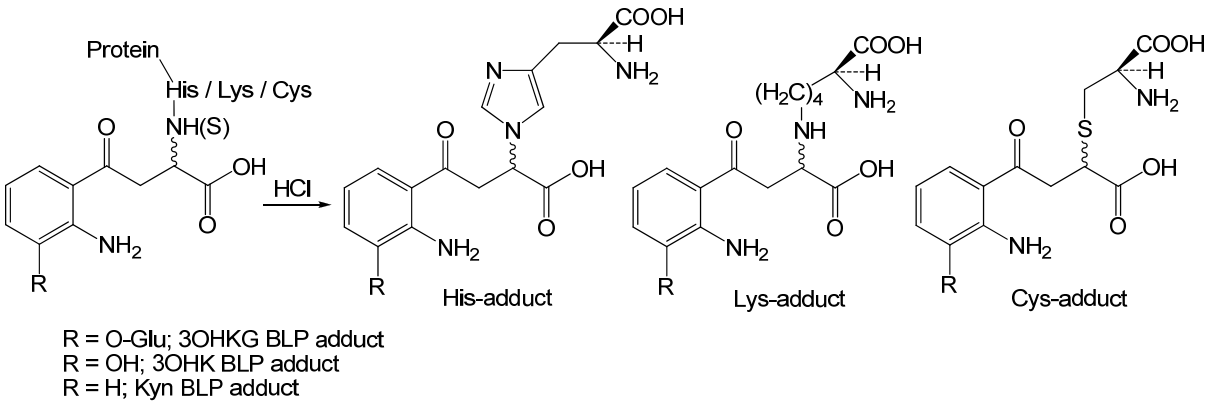
Figure 4.2: UV/Vis spectroscopy profiles of 3OHKG and 3OHK metabolites BLP modified at pH 7.4 and 9.5.

BLP treated with AHB at pH 9.5 showed free AHB-like absorbance and fluorescence. This was unexpected as AHB cannot form an α,β-unsaturated carbonyl or reactive quinone imine due to the lack of an amine group on the 2nd

carbon. UV/Vis spectra of treated AHB lens proteins did not show any distinct absorbance or fluorescence patterns for the free AHB molecule (*i.e.* λ_{max} 369, λ_{ex} 346 and λ_{em} 435 nm). In the absence of free AHB (and with the characteristic higher absorbance range of oxidised *o*-aminophenol products), this may be due to the interaction or formation of oxidised xanthommatin-related products by AHB [159, 161, 165, 282]. Similar to 3OHK, the fluorescence profiles of AHB treated BLP appear to vary slightly with pH. Mizdrak *et al.* had previously reported similar non-covalent binding evidence for the related compound 4-(2-aminophenyl)-4-oxobutanoic acid (AHA), however these were not investigated in this study [50].

4.2.1.3 Analysis of modified BLP

In previous studies, the major sites of protein modification by Kyn and 3OHK were determined by acid hydrolysis of the modified proteins and comparison of the amino acid adducts formed with authentic standards of Kyn/3OHK/3OHKG amino acid adducts [38, 124, 225, 277]. Due to the acid lability of the glucoside moiety, acid hydrolysis would lead to 3OHK amino acid adducts from 3OHKG modified BLP. Though it would be possible to indirectly quantify the amount of protein-bound 3OHKG, and to determine the amino acid residues it was bound to (by testing for and using 3OHK amino acid standards), a gentler means of hydrolysis would be preferred so that the intact 3OHKG amino acid adduct could be recovered. Likewise, the harsh destructive nature of HCl hydrolysis would likely have an adverse effect on any weak covalently or non-covalently bound UV filters. Gentler hydrolysis was proposed through the use of an enzyme preparation called Pronase.



Scheme 4.2: Structures of Pronase hydrolysis products of BLP modified with 3OHKG, 3OHK and Kyn UV filter.

Pronase consists of a mixture of proteases isolated from the extracellular fluid of the bacterium *Streptomyces griseus*. Through the manufacturers own testing, Pronase hydrolyses proteins (on average) at 80% of the efficiency of standard acid hydrolysis with 6 M HCl [283]. This loss in efficiency is more than made up for by the ease of the subsequent processing and clean-up of the sample, particularly for LC-MS/MS analysis. To provide a comparison to previous studies, Kyn and 3OHK treated BLP were hydrolysed using Pronase to their constituent free amino acids. In order to quantify the extent of protein modification, synthetic standards of Kyn, 3OHK and 3OHKG adducts were made with each the three amino acids Cys, His and Lys. Free cysteine base was reacted directly with Kyn, 3OHK or 3OHKG by refluxing under argon in pH 9.5 sodium bicarbonate buffer (to accelerate formation of the deaminated compounds) and then purified through reversed phase HPLC.

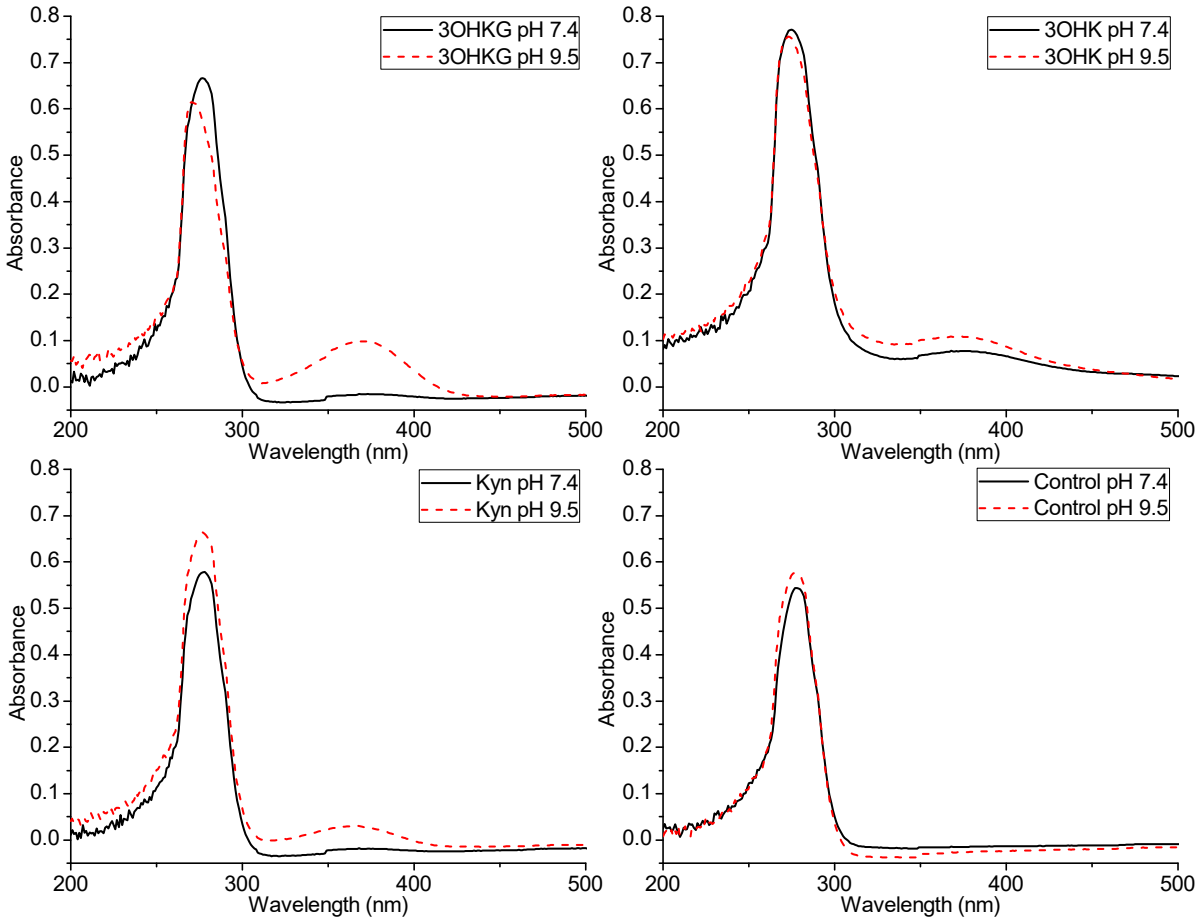


Figure 4.3: UV/Vis profiles of BLP modified with the major UV filters, 3OHKG, 3OHK and Kyn, and untreated control BLP at pH 7.4 and pH 9.5.

His and Lys standards were made in the same manner except that the UV filters (Kyn, 3OHK and 3OHKG) were bound to BOC protected His or Lys [225]. BOC protected amino acids were used to increase the synthesis efficiency by ensuring binding to the functional group of His and Lys. After reversed phased HPLC purification, the BOC protected amino acid adducts were deprotected by refluxing them under nitrogen in 1% formic acid in a modified green chemistry method of Zinelaabidine *et al.* [284]. Pronase hydrolysis was performed on untreated BLP, control BLP and the UV filter modified BLP. To minimise oxidation, samples were degassed and de-oxygenated by bubbling with nitrogen prior to digestion. Samples were first analysed through direct injection in the MS/MS for potential masses of interest, with subsequent analyses performed by reversed phase LC-MS/MS with the eluent monitored at 360 nm [124, 285]. The

identity of the amino acid adducts was confirmed by spiking with the synthetic standards and by ESI LC-MS/MS.

4.2.1.3.1 Untreated BLP

Untreated BLP were hydrolysed with Pronase under varying conditions to investigate the performance of Pronase on lens crystallins. Quantification was through derivatisation using *o*-phthaldialdehyde (OPA), and then measuring fluorescence after separation on a UPLC [235, 286]. Pronase is stable at up to 60 °C and provides significantly better performance at higher temperatures [283], nevertheless temperatures above 40 °C were not used for the modified BLP due to concerns about the stability of the modified amino acid residues, particularly the Cys adducts. Higher temperatures also increased the likelihood of concentration calculation errors due to greater evaporation, and this was partly evident by the larger standard deviations where (except for Arg, Tyr and Lys) every amino acid had a greater standard deviation at 50 °C than at 40 °C.

Untreated BLP were tested under the recommended protocol (40 °C, 72 hours) as well as for different lengths of time (24, 48 and 72 hours), temperatures (40 and 50 °C) and concentrations (0.5, 1 and 2 mg/mL). Another factor that was investigated was the age/length of time that the BLP were in solution (*i.e.* frozen storage). At the higher temperature, approximately 81% of the protein digestion took place in 24 hours, rising to approximately 95% at 48 hours. Higher temperatures also increased the efficiency of the digestion, from an average of 65% at 40 °C to 72% at 50 °C. A higher starting concentration of BLP also resulted in better digestion, particularly at 50 °C (69% for 1 mg/mL, compared to 78% at 2 mg/mL), however samples digested at 50 °C generally had a higher standard deviation compared to those performed at 40 °C. Another significant, though difficult to measure, factor that affected the efficiency of the digestion was the age/quality of the in-solution proteins. Both modification and storage time length in solution decreased the efficiency of protein digestion.

Table 4.2: Amino acid residue (molar) percentages of untreated BLP.

	40 °C			**50** °C			**Combined Data**		
Amino Acid	**Avg**	**Std Dev**	**Std Dev % of Avg**	**Avg**	**Std Dev**	**Std Dev % of Avg**	**Avg**	**Std Dev**	**Std Dev % of Avg**
Asp	3.5%	0.2%	4.5%	3.4%	0.1%	2.2%	3.4%	0.1%	3.5%
Glu	5.1%	2.4%	45.9%	6.0%	3.0%	49.6%	5.5%	2.6%	46.6%
His	2.7%	0.1%	3.4%	2.9%	0.1%	5.1%	2.8%	0.1%	5.2%
Ser	23.0%	0.5%	2.2%	22.0%	0.6%	2.7%	22.5%	0.7%	3.2%
Arg	5.5%	0.3%	5.4%	5.2%	0.2%	4.6%	5.3%	0.3%	5.6%
Gly	1.5%	0.4%	24.4%	1.1%	0.8%	70.9%	1.3%	0.6%	47.5%
Thr	2.9%	0.4%	12.7%	2.4%	0.6%	26.7%	2.7%	0.6%	21.6%
Ala	2.4%	0.5%	21.7%	1.9%	0.8%	43.1%	2.2%	0.7%	32.6%
Tyr	5.4%	0.3%	6.3%	6.0%	0.3%	5.7%	5.7%	0.5%	8.0%
Trp	3.2%	0.4%	11.1%	3.5%	0.8%	23.9%	3.4%	0.6%	19.3%
Met	1.9%	0.1%	3.1%	1.9%	0.1%	4.1%	1.9%	0.1%	3.5%
Val	4.9%	0.5%	9.4%	5.1%	0.6%	12.5%	5.0%	0.5%	10.7%
Phe	14.2%	0.9%	6.1%	13.7%	1.3%	9.8%	13.9%	1.1%	8.0%
Ile	4.2%	0.4%	9.7%	4.4%	0.5%	11.2%	4.3%	0.4%	10.3%
Leu	15.1%	0.4%	2.9%	15.7%	1.2%	7.8%	15.4%	0.9%	5.9%
Lys	4.5%	0.2%	4.1%	4.9%	0.2%	3.8%	4.7%	0.3%	6.0%

Freshly dissolved BLP had an average digestion efficiency of 70%, whilst storage/freezing in-solution for 2 months reduced that efficiency to approximately 64%. The combination of modification/treatment and in-solution storage reduced that efficiency down to an average of 54% for the modified BLP. Hence, digestion of the modified BLP was performed at 40 °C for 72 hours, to reduce any potential breakdown of the target amino acid adducts and to maintain the highest level of consistency in the amino acid residue proportions. Storage and freeze/thawing of the reaction mixtures were also kept to a minimum to maintain efficiency where possible.

4.2.1.3.2 3OHKG

Of the three major UV filters tested (3OHKG, 3OHK and Kyn), BLP modified with 3OHKG at pH 9.5 had the most modified amino acid residues detected upon digestion and separation through LC-MS/MS. The most abundant residue was 3OHKG-Cys, with approximately 63700 pmol detected per mg of modified protein, which had a retention time of 9.7 min and was identified by its detecting the

adduct's MS/MS fragments at the expected elution time of the compound (*m/z* 491.1, fragments to 473.1 (100%) and 311.1 (25%)). 3OHKG-Cys residues were also detected in proteins modified with 3OHKG at pH 7.4, however at a 21-fold lower abundance (approximately 2920 pmol/mg modified protein). The level of modification at pH 7.4 was similar to the reported average concentration found in a normal human lens nucleus (1307 pmol/mg, Korlimbinis *et al*.) [224]. 3OHKG-His (*m/z* 525.2, fragments to 363.1 (100%) and 208.0 (20%)) and 3OHKG-Lys (*m/z* 516.2, fragments to 370.1 (100%) and 314.1 (35%)) were also detected at much lower levels than (3840 and 2550 pmol/mg protein at pH9.5). At a modification pH of 7.4, only 3OHKG-His was detected at 1070 pmol/mg.

LC-MS/MS quantification of modified amino acid residues was performed on three separate occasions (with a correction factor applied based on a caffeine standard). The best results were obtained upon quantification within 24 hours of Pronase digestion. Interestingly, 3OHKG-Cys was the most stable of the detected adducts, and even at pH 9.5 it was still detectable in quantities of greater than 53% after more than 72 hours of processing (by the third quantification). 3OHKG-His detection levels diminished rapidly, with approximately 16% detectable after 72 hours of processing on the same sample, whilst 3OHKG-Lys was not detected in the second and subsequent quantification experiments. The rapid instability of the individual modified amino acid residues in the digestion mixtures was in contrast to the stability of the pure synthetic amino acid-adducts. The instability is likely due to reactive components in the complex digestion mixture

Table 4.3: Binding of 3OHKG (pmol/mg protein) to amino acid residues.

BLP Modification	Adduct	pH	*m/z*	Retention time	Retention Time (Std)	pmol/mg Protein
3OHKG	3OHKG-Cys	7.4	491.1	9.75	9.73	**2920**
3OHKG	3OHKG-Cys	9.5	491.1	9.75	9.76	**63700**
3OHKG	3OHKG-His	7.4	525.2	3.93	4.07	**1070**
3OHKG	3OHKG-His	9.5	525.2	4.00	4.07	**3840**
3OHKG	3OHKG-Lys	7.4	516.2	0	3.49	**N/D**
3OHKG	3OHKG-Lys	9.5	516.2	3.79	3.51	**2550**

4.2.1.3.3 3OHKG-D

As it is already deaminated, hence a reactive compound, modification of BLP with 3OHKG-D was only performed at pH 7.4. Reactions with 3OHKG-D are inherently problematic due to its instability, however analysis of modified BLP gave results consistent with its role as the intermediate compound when binding occurs with 3OHKG. All three amino acid adducts (Cys, His and Lys) were present in greater quantities than the pH 7.4 3OHKG samples, with 3OHKG-His being present at a greater quantity than for pH 9.5 3OHKG BLP. These results also highlight that deamination of 3OHKG (and by analogy the other UV filters, Kyn and 3OHK) is the only limiting factor of protein binding/modification and that binding can readily occur *in vivo* under normal physiological conditions.

Table 4.4: Binding of 3OHKG-D (pmol/mg protein) to amino acid residues.

BLP Modification	Adduct	pH	*m/z*	Retention time	Retention Time (Std)	pmol/mg Protein
3OHKG-D	3OHKG-Cys	7.4	491.1	9.76	9.77	**4720**
3OHKG-D	3OHKG-His	7.4	525.2	3.94	4.09	**4620**
3OHKG-D	3OHKG-Lys	7.4	516.2	3.65	3.50	**675**

Otherwise, the 3OHKG-D modified adducts behaved in a similar manner to those made from BLP modified with 3OHKG at pH 9.5, with the adduct quantities quickly diminishing over time (*e.g*. 3OHKG-Lys was not detected on the second and subsequent quantifications of the same sample). The ratio of 3OHKG-Cys to 3OHKG-His was very different at approximately 1:1, compared to 17:1 ratio for pH 9.5 3OHKG. Though all the UV filters are substantially more reactive with Cys residues, than with His or Lys, this lower level of binding is probably due to the near neutral pH at which they were modified, which would have reduced the level of denaturing in the BLP hence exposing fewer cysteine residues to potential modification.

4.2.1.3.4 3OHK

Reversed phase LC-MS/MS analysis of 3OHK modified BLP revealed that greater modification occurred at pH 9.5 compared to pH 7.4. This was consistent with the initial deamination (just like for 3OHKG) being the critical step in the reaction process [51]. With 3OHK, most binding occurred at Cys residues at both pHs.

Binding at pH 9.5 was two orders of magnitude greater than at pH 7.4. The Cys adducts showed absorption maxima at 268 and 368 nm, which is consistent with the authentic synthetic standard of Cys-3OHK [124]. ESI LC-MS/MS analysis of the sample produced a prominent ion at *m/z* 329, with fragmentation consistent with the Cys-3OHK standard, as reported with previous analysis of the adduct [124].

LC-MS/MS analysis showed a molecular ion at *m/z* 354.2 that further fragmented to ions similar to those detected for an authentic standard of Lys-3OHK (203.1 (100%) and 147.0 (35%)). The concentration of this adduct was very low at pH 7.4 (246 pmol/mg protein), and was close to the detection limit of the instrument. These low quantities of Lys-adduct were similar to those found for 3OHKG and 3OHK modified BLP. Ions corresponding with the His adduct were also found at the known retention time of the authentic standard. Modified His adducts were present, though at very low concentrations (491 pmol/mg protein at pH 7.4 and 1860 pmol/mg protein at pH 9.5) in the modified protein samples. In previous attempts to detect His or Lys adducts, their quantities were thought to be present at levels below the limits of instrument detection [72, 287]. At pH 7.4, Cys binding by 3OHK was similar to 3OHKG and 3OHKG-D (with approximately similar concentrations detected). At pH 9.5, there was a 10-100x increase in adduct concentration for 3OHKG (from physiological pH), but only a 2-4x fold increase for 3OHK, suggesting that pH has a stability impact on 3OHK. In contrast to 3OHKG, the 3OHK-Lys residues were marginally more stable, with them being still quantifiable on the third quantification run. 3OHK-Cys and 3OHK-His were not detectable after 72 hours of processing (3rd quantification), and were only detected at miniscule quantities after 24 hours.

Table 4.5: Binding of 3OHK (pmol/mg protein) to amino acid residues.

BLP Modification	Adduct	pH	*m/z*	Retention time	Retention Time (Std)	pmol/mg Protein
3OHK	3OHK-Cys	7.4	329.1	8.03	7.97	**2350**
3OHK	3OHK-Cys	9.5	329.1	7.82	8.03	**5120**
3OHK	3OHK-His	7.4	363.1	3.99	4.16	**491**
3OHK	3OHK-His	9.5	363.1	4.15	4.11	**1860**
3OHK	3OHK-Lys	7.4	354.2	3.58	3.52	**245**
3OHK	3OHK-Lys	9.5	354.2	3.63	3.53	**5900**

Mizdrak *et al.* observed other distinct peaks found in RP-HPLC traces of 3OHKG and 3OHK modified BLP (**see Appendix J)** [39]. However, due to the low LC-MS/MS yields obtained for these compounds, they could not be identified further. Their presence in both 3OHKG and 3OHK modified lens proteins combined with their LC-MS/MS profiles and the UV/Vis spectra, strongly suggests that they are derived from 3OHK. Equivalent peaks were not observed upon Pronase digestion.

4.2.1.3.5 Kyn

Similarly to 3OHKG, Kyn had a strong preference for binding to Cys, however Kyn-Cys (*m/z* 313.1, fragments 294.1 (100%) and 202.0 (40%)) adducts were only detected in the pH 9.5 Kyn modified BLP (25100 pmol/mg protein)) with negligible amounts detected at pH 7.4. Higher levels of Kyn-Cys residues have been reported in previous studies [18, 200], however these variations in the degree of modification (at both pH 7.4 and pH 9.5) were possibly due to variations in the amount of available oxygen in the treatment reactions. Hence, with the anaerobic conditions used for modification in this study, without the increased deamination caused by a higher pH, there was close to no binding of Kyn to the BLP at pH 7.4. At both pHs, the level of Kyn-His (*m/z* 347.1, fragments 301.1 (100%) and 156.0 (35%)) detected was low at 634 pmol/mg protein (pH 7.4) and 914 pmol/mg (pH 9.5). The detected levels of Kyn-Lys (*m/z* 338.2, fragments 203.0 (100%) and 147.0 (20%)) were close to the detection limit at both pH values. The level of His and Lys binding at pH 7.4 is consistent with reported binding in human lenses (71 year old lens, 700 pmol/mg Kyn-His and 120 pmol/mg Kyn-Lys, Vazques *et al*.) [225]. Though Kyn binds preferentially to Cys residues, the low level of observed Kyn-Cys at pH 7.4 suggests that the reaction is rate-limited by the level of deamination. Deamination, however, does not appear to be as significant for binding to His residues. This strongly suggests that other factors affect the level of binding to Cys, primarily the position of the reactive amino acid residue in the protein conformation. The Kyn-Cys adduct is also known to be very unstable, so the low peaks may signify a high level of decomposition at pH 7.4 [225]. Higher pH values, in addition to encouraging deamination, also denature the BLP and allow reactions to occur with Cys that might otherwise be protected by the protein's structure.

Table 4.6: Binding of Kyn (pmol/mg protein) to amino acid residues.

BLP Modification	Adduct	pH	*m/z*	Retention time	Retention Time (Std)	pmol/mg Protein
Kyn	Kyn-Cys	7.4	313.1	0	11.25	**42**
Kyn	Kyn-Cys	9.5	313.1	11.44	11.39	**25100**
Kyn	Kyn-His	7.4	347.1	6.55	6.43	**634**
Kyn	Kyn-His	9.5	347.1	6.64	6.62	**914**
Kyn	Kyn-Lys	7.4	338.1	4.42	4.44	**163**
Kyn	Kyn-Lys	9.5	338.1	4.52	4.47	**293**

4.2.1.3.6 AHB

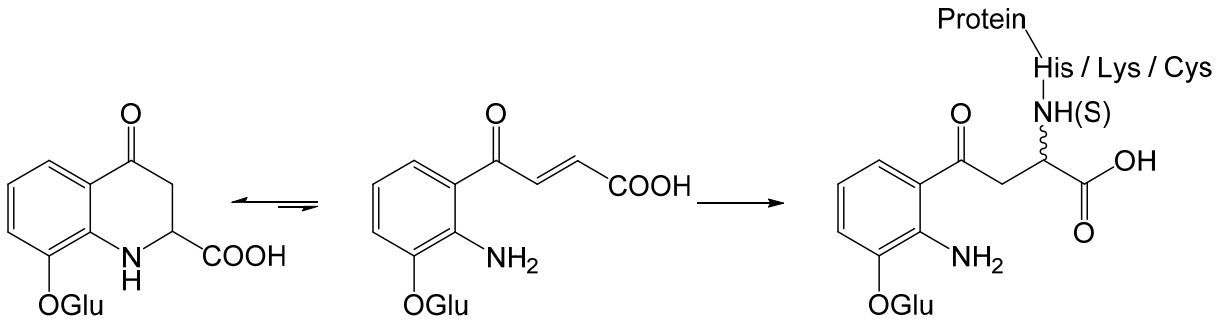
AHB mass spectral analysis was performed *via* direct injection and liquid chromatography. No definite AHB adducts were detected. In previous work by Mizdrak *et al.*, RP-HPLC and LC-MS/MS analysis of AHB-treated BLP revealed AHB (0.03 mol AHB/mol protein at pH 7.4 and 0.09 mol AHB/mol protein) and additionally 3 prominent single peaks at both pH 7.4 and 9.5 [39]. Mizdrak also observed unidentified peaks that exhibited absorbance at approximately 400 nm, of which one had a molecular ion of *m/z* 399. This molecular ion was previously observed when AHB was incubated at both physiological (pH 7.4) and under basic conditions (pH 9.5, not shown) [81]. As only minute amounts could be isolated, further analysis or identification of these peaks was not attempted.

As AHB contains a reactive *o*-aminophenol moiety similar to 3OHK (**Scheme 4.1**), it was investigated to determine if oxidative products of AHB might be involved in protein colouration. AHB was incubated on its own at pH 9.5 for 24 hours at 37 °C in nitrogen-bubbled buffer, with BLP added at that time point. The incubation was continued for a further 24 h. No obvious differences between this sample and the previously described AHB-treated BLP sample (pH 9.5 for 48 h) were observed spectrally and upon hydrolysis. This suggests that either the oxidised species are not involved in binding to BLP under the experimental conditions investigated, or due to their instability they do not surive the hydrolysis process. Likewise, the results obtained in the current study suggest some form of non-covalent binding between AHB and BLP when incubated at pH 9.5 (**Figure 4.2**), however a fragment ion at *m/z* 399 (consistent with AHB binding to amino acid binding) could not be found. Nevertheless, a fragment ion

at *m/z* 373.1 was found at pH 7.4 and in greater quantity at pH 9.5. This mass fragmented with MS-MS to ions of 354.1 (100%, mass of 3OHK-Lys), 254.2 (80%) and 147.2 (25%, further fragmentation ion of 3OHK-Lys). This compound has potential for further analysis, however as a logical reaction path for AHB binding with an amino acid was not obvious, no further investigation of this (potential) AHB adduct was carried out.

4.2.1.3.7 3OHKG-Y

3OHKG-Y BLP reaction digests gave masses consistent with those of 3OHKG-His (*m/z* 525.2) and 3OHKG-Cys (*m/z* 491.1). Due to its very low quantity and short retention time (which caused it to elute with most of the amino acids, hence obscuring its peak), the potential 3OHKG-His compound could not be analysed further by tandem mass spectrometry. The potential 3OHKG-Cys adduct was also difficult to investigate due to a similar mass compound co-eluting with it in the digested pH 9.5 proteins. Fortunately, this obstructing compound was not present in the pH 7.4 reaction mixtures. At this pH an *m/z* 491.1 molecular ion was detected having a retention time (9.89 min) and fragmentation pattern (473 (100%) and 311 (40%)) consistent with that of the 3OHKG-Cys synthetic standard. The concentration of the adduct was 436 pmol/mg protein (7-fold lower than pH 3OHKG BLP, and 11-fold lower than pH 7.4 3OHKG-D). A possible explanation for the (putative) presence of 3OHKG-Cys is that 3OHKG-Y must somehow undergo a reverse Michael addition, forming the reactive 3OHKG-D. Similar reverse reactions have been observed with Kyn [288]. This reaction does not appear strongly pH dependent, as a similar (if not lower) quantity of this *m/z* 491.1 was found in the pH 9.5 3OHKG-Y modified BLP.



Scheme 4.3: Proposed reverse Michael addition of 3OHKG-Y

4.2.1.3.8 3OHKG-W and AHAG

Despite repeated efforts, no adducts were detected for 3OHKG-W or AHAG BLP incubated samples. These two metabolites display the greatest level of stability amongst all the UV filters and their metabolites, hence modification to the lens proteins was not expected to occur.

4.2.1.3.9 Removal of unbound material from modified proteins

BLP treated with 3OHKG, AHB and AHA at pH 9.5 for 48 h were dissolved in guanidine hydrochloride, filtered and the filtrate extracted with acetonitrile as per the method of Mizdrak [39]. Guanidine hydrochloride has chaotropic properties, hence can disrupt non-covalent bonds (*e.g.* hydrogen bonds) and is used to denature proteins [289, 290]. The results confirmed that 3OHKG was covalently bound to lens proteins *via* Cys residues and that dialysis after the protein modification was sufficient to remove all unbound UV filters, as no free 3OHKG was detected. However, following acid hydrolysis of the 3OHKG modified BLP, Mizdrak found that the Cys adduct was collected at approximately 63% of the expected initial amount of bound UV filter (a slightly lower recovery of 43% was found for the 3OHK adduct) [39]. This was also supported by the remaining yellow-brown and pale yellow colour of the 3OHK and 3OHKG treated BLP, upon guanidine hydrochloride treatment. This loss of Cys-adducts can be explained by the instability of the Cys adducts during the harsh experimental conditions of hydrolysis with HCl. Hence Pronase digestion of the modified BLP was used as an alternative, and separation of the Pronase digested filtrate showed a complete absence of UV filters or their derivatives, confirming that there was no free UV or non-covalently bound UV filters in the treated proteins after dialysis.

To ensure free AHB and AHA from AHB- and AHA-treated BLP (respectively) did not remain due to insufficient dialysis, additional extractions were performed for AHA and AHB treated BLP as for 3OHKG. AHA and AHB modified BLP used in this study were dissolved in guanidine hydrochloride and then extracted with acetonitrile with recovery of ~72% and ~53%. Modified AHA and AHB BLP were prepared by Jasminka Mizdrak [39]. These guanidine hydrochloride washed AHA and AHB treated BLP were then acid hydrolysed and were found to contain no detectable amounts of AHA and AHB or any other compounds that have significant absorption at 360 nm. This data also suggests that oxidative products of AHB may not be involved in covalent protein

modification and strongly support the hypothesis that AHA and AHB are non-covalently bound to lens proteins and that the original dialysis method had not been sufficient to remove all of the AHA and AHB. The apparent binding of AHB to BLP was not expected as AHB lacks the two main binding requirements; the *o*-aminophenol moiety and an ability to form an α,β-unsaturated carbonyl compound. Though satisfactory extraction could be performed on AHA and AHB treated BLP, for consistency with the other metabolite treatments, in this study Pronase digestion was also performed for AHA and AHB treated BLP.

4.3 Discussion

There is now a substantial amount of evidence in support of the non-oxidative modifications that occur in proteins as a result of reaction with the Trp-derived UV filters, in both normal and cataractous human lenses [3, 50, 72, 124]. This process is largely responsible for the age-dependent lens colouration (and the associated decrease in the perception of colour) in persons who are developing age related nuclear cataract [3]. There are multiple possibilities as to why this arises, the main contributing factors being: the intrinsic instability of the UV filters [51], the development of a barrier to diffusion within the lens [67], and the decrease of antioxidants in the lens [27, 76].

The major human lens UV filters 3OHKG, Kyn and 3OHK were found to react with BLP, at both pH 7.4 and 9.5, and to give coloured (λ_{max} ~365 nm for Kyn and 3OHKG, and ~365 and 440 nm for 3OHK) and fluorescent (λ_{ex} 345-380 and λ_{em} 450-510 nm) modified proteins. These UV filter modified BLP bear many similarities to older and age related nuclear cataractous lenses, as has been reported in previous studies [39]. LC-MS/MS analysis of hydrolysed Kyn, 3OHK, 3OHKG and 3OHKG-D treated BLP showed that these UV filters are covalently bound to lens proteins predominantly *via* Cys amino acid residues under basic conditions (pH 9.5), with varied binding concentrations at pH 7.4. Consistently higher quantities of the Cys adducts were seen at pH 9.5, which is likely to be due to the greater rate of UV filter deamination under these conditions, but also possibly due to the denaturing caused by the higher pH exposing structurally protected amino acid residues. This was evidenced by the levels of binding observed in the 3OHKG-D pH 7.4 modified BLP, which although exposed to a highly reactive metabolite of 3OHKG, did not result in binding levels substantially different from 3OHKG at pH 7.4. Nevertheless, where a length of time given to

the experiment that was comparable to the time that it takes ARN cataract to develop (years/decades) it is expected that increased levels of binding would also be observed for the physiological pH experiments.

Previous studies have shown that the UV filters and related compounds (3OHKG, Cys-3OHKG adduct, AHA and AHB) have variable stability under acid hydrolysis of modified BLP, with the loss of glucoside on 3OHKG and other glucosylated metabolites being the most affected (<50% recovery, 3OHKG or Cys-3OHKG) [39]. It was decided to use methods that avoided the harsh conditions of refluxing in HCl at 110 °C. Instead, Pronase enzymatic digestion (in pure H_2O at 40 °C) achieved a similar level of hydrolysis and a higher recovery amount. Likewise, protocol for BOC deprotection included refluxing under highly acid conditions [81, 225]. In this study, the synthetic standards were refluxed in moderately acidic (pH 2.5) formic acid/water, which was modelled on a green method suggested by Zinelaabidine *et al.* [284]. In part due to the mild and anaerobic conditions use for BLP binding, it was possible to detect and analyse both Lys and His adducts in this study, even though they had not been directly detected in previous studies [38, 224, 225]. In comparison to levels of UV filter-modified proteins detected in older human lenses, BLP modified at pH 7.4 showed similar levels of binding to those found in the nucleus of healthy older lenses [224]. These results add further support to the use of BLP in experiments as a human analogue. At pH 9.5 however, BLP in this study were modified far more heavily than has been found *in vivo* [224].

Table 4.7: Comparison of protein bound UV filters to *in vivo* studies.

UV Filter	Amino Acid	Concentration (pmol/mg protein)			
		BLP pH 7.4	Light Cataract	Dark Cataract	Normal Lens
Kyn	Cys	42	12 [224]	6 [224]	37 [224]
	His	634	-	-	700 [225]
	Lys	163	-	-	120 [225]
3OHK	Cys	491	-	-	9 [224]
3OHKG	Cys	2920	277 [224]	153 [224]	1307 [224]

Other amino acids may also be modified, but in significantly lower levels compared to the Cys, His and Lys modifications found in this study. For example,

modification of Arg residues by 3OHK has been determined previously, however this was not observed in the current study [166]. As eluded to previously, binding is likely to be influenced by the tertiary structure of the protein, the accessibility of residues for the reactions, the nucleophilicity of the amino acid residues and the stability of the amino acid adduct. The nucleophilicity of amino acids is related to their pK_a values [291]. If pK_a values are considered, the order of reactivity of His, Lys and Cys towards deaminated UV filters at physiological pH would be expected to be His (pK_a 6.0), followed by Cys (pK_a 8.33) and then Lys (pK_a 10.53), although pKa values, in particular for Cys residues on proteins, are known to vary significantly and may depend on the local environment [225]. In totality, the data obtained in this study is consistent with the Cys residues present in BLP being better nucleophiles than both the His and Lys residues under the investigated experimental conditions, resulting in faster reactions with the side-chains of the deaminated UV filters and metabolites.

All analyses of both AHB and AHA modified BLP showed no evidence of covalent binding of these metabolites to proteins. It is believed that there may be some interactivity with the hydrophobic sites on BLP, though this would require additional investigation. As previously postulated by Mizdrak *et al.* these results support the theory that the reduction of the deaminated Kyn to AHA acts as a defence mechanism of the lens, as it prevents Kyn from binding to lens proteins and thus decreases the photosensitiser activity of oxidative damage on lens proteins [81]. Similar results were demonstrated in the experiments of Taylor *et al*. where upon the inclusion of NADH in the mixture containing Kyn and BLP, the level of binding decreased by approximately 50% [69]. Hence, it is clear that the deamination of the side-chain, with the formation of an α,β-unsaturated carbonyl compound is a prerequisite to lens protein damage and potentially ARN cataract. Nevertheless, though the binding was non-covalent, both AHA and AHB seem to bind very strongly to BLP and (particularly AHB at pH 9.5) could only be removed completely by denaturing the proteins in guanidine hydrochloride. The spectral results obtained with AHB were even more interesting, as even though no binding could occur through the side-chain of AHB, the treated proteins did undergo changes in absorbance (λ_{max} 328, 368 and 400 nm) and fluorescence (λ_{ex} 380-390 and λ_{em} 475-490 nm) properties, though only at pH 9.5. These changes were comparable to those seen for 3OHK modified BLP (λ_{max} 367 and 440 nm; λ_{ex} ~350 and λ_{em} 490-510 nm) suggesting that some part of the change in absorbance and

fluorescence properties of 3OHK modified lens proteins is due to some reaction other than covalent binding to the protein, and possibly related to oxidation of the *o*-aminophenol moiety in both (3OHKG and AHB). Previously, RP-HPLC analysis of acid-hydrolysed AHB-treated BLP showed some evidence of three additional highly coloured compounds that exhibited long wavelength absorbance, which appear to be dimerised decomposition products of AHB and exhibit a molecular ions of *m/z* ~399 [81], however these compounds where not detected in this study. Likewise, the hydrolysis experiments displayed no free or protein bound oxidative products of 3OHK in the 3OHK modified samples, implying that binding to the deaminated side-chain is a favoured result and that the *o*-aminophenol moiety has a much lower reactivity with BLP. Alternatively, the oxidation species may not be recoverable due to their instability or may form multiple different products (hence diffusing any potential peaks).

4.4 Conclusion

There are a number of key factors that appear to be involved in the covalent binding of human lens UV filters to the lens proteins in which they are present. A few are general in nature, such as the nucleophilic nature of the target amino acid, and the general stability of the UV filter and formed adduct. The most important factor appears to be the lability at which the UV filter deaminates. Likewise, it also appears that changes in pH affect the accessibility of the residues for the reactions. Experimental modification at pH 9.5 was primarily done to encourage deamination, however a clear side-effect of this was the denaturing of the BLP which allowed access to additional reactive residues, particularly the more reactive Cys residues. Combining the binding studies with the observed protein damage (**Chapter 3**) data, in all experiments covalent binding is a very strong prerequisite for photo-oxidative damage to occur through UV light irradiation. Covalently binding Kyn based UV filters to lens proteins yields efficient photosensitisers that may exacerbate UV-induced photo-damage to the lens and retina (through the formation of H_2O_2 and other peroxides) [50]. Unbound, and non-binding UV filters have no apparent effect on the level of cross-linking and aggregation in the protein (**Chapter 3**). The fluorescence and UV/Vis profiles of treated BLP are generally good indicators of binding, with AHB the only exception. AHB treated BLP produced UV/Vis and fluorescence changes at pH 9.5, though upon UV irradiation no cross-linking was observed *via* SDS-PAGE

(**Chapter 3**), nor was any potential amino acid adduct detected *via* LC-MS/MS. Hence, for a UV filter to act as a photosensitiser of oxidative damage, results in this study confirm that it must be bound to the target protein. Though it was not investigated in these experiments, it would be of interest to see what structural differences there are on binding to each of the different amino acid residues. This could be a project for potential future studies.

While the exact chemical reactions involved have not been determined yet, a working model for the process that leads to ARN cataract has been proposed in this chapter, with the importance of the α,β-unsaturated carbonyl firmly established as a prerequisite to a continuous build-up of UV induced lens protein damage. This study has also provided a basis for further investigations to define the role of Kyn, 3OHK and 3OHKG and particularly of its metabolites (3OHKG-D) in the changes that occur in the human lenses with age and in the onset of age related nuclear cataract. Further experiments are needed to investigate the mode of binding of 3OHK and AHB products to lens proteins, and to find out the importance of the *o*-aminophenol moiety in protein modification and possibly ARN cataract formation. Additionally, further experiments are needed to investigate if binding to lens proteins *via* the *o*-aminophenol moiety occurs with prolonged incubation time. This could be determined through comparative protein binding experiments between a compound having both the *o*-aminophenol and α,β-unsaturated carbonyl moieties and a compound lacking one of these moieties, such as in the case of 3OHK versus AHB and Kyn versus AHA (not experimented with in this study), nevertheless the complexity of the possible modifications precluded it from being carried out in this study. Hence, the importance of the *o*-aminophenol (as opposed to the α,β-unsaturated carbonyl moiety) in protein modifications needs to be investigated further.

4.5 Experimental

4.5.1 Materials and apparatus

All organic solvents and acids used were of HPLC grade (Sigma-Aldrich, NSW, Australia). Milli-Q® H_2O (purified to 18.2 MΩ cm^2) was used in preparation of all solutions. Dulbelcco's phosphate-buffered saline (PBS), without calcium and magnesium, consisted of KCl (2.7 mM), KH_2PO_4 (1.4 mM), NaCl (137 mM) and Na_2HPO_4 (7.68 mM) [292]. Pre-washed chelex resin was added to the PBS buffer (2

g/L) and left for 24 h prior to use. The pH of PBS was adjusted to 7.0 with 1 M NaOH. DL-Kynurenine sulphate salt (Kyn, ≥ 95%), 3-hydroxy-DL-kynurenine (3OHK), BOC protected amino acids (N-α-t-BOC-L-histidine (t-BOC-His), N-α-t-BOC-L-lysine (t-BOC-Lys)), L-cysteine (Cys), trifluoroacetic acid (TFA, > 99%), formic acid (98%), thioglycolic acid, phenol, sodium azide (NaN3), guanidine hydrochloride, β-mercaptoethanol, (> 98%), 500 µM Amino acid standard solution, *o*-phthaldialdehyde (OPA) solution, all buffer salts (*e.g.* 100 mM Na_2HPO_4/NaH_2PO_4 and 50 mM Na_2CO_3/$NaHCO_3$), dithiothreitol (DTT), sodium acetate salt (> 98%), tetrahydrofuran (THF), methanol (MeOH), acetic acid (AcOH) and dialysis tubing (molecular mass cut-off 20,000 Da) were purchased from Sigma-Aldrich. Cys-Kyn [225], 4-(2-aminophenyl)-4-oxobutanoic acid (AHA), 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid (AHB), 3-hydroxykynurenine-*O*-β-D-glucoside (3OHKG) and cysteinyl-3-hydroxykynurenine-*O*-β-D-glucoside (Cys-3OHKG) were synthesised as reported previously [81, 125, 216]. Thin-layer chromatography (TLC) plates were of normal phase 60 F_{254} (Merck, Germany) and developed using BAW (12:3:5 *n*-butanol/AcOH/H_2O, v/v) for the mobile phase. TLC plates were visualised under UV light (254 and 365 nm) and sprayed with ninhydrin (ninhydrin [0.2%, w/v] in *n*-butanol [94.8%, v/v] and AcOH [5%, v/v]) where indicated [293]. Vivaspin (6 mL) protein concentrators (molecular mass cut off 10,000 Da) were purchased from Crown Scientific. CD_3OD (99.8%) was purchased from Cambridge Isotope Laboratories. Pronase enzymes (preparation at 2 mg/mL) were purchased from Roche Australia. A Labconco FreeZone 12 plus freeze drier (0.04 mBa, -80 °C) from Crown Scientific was used for lyophilisation of aqueous solutions. The reactions under vacuum were conducted using a vacuum pump at ~80 mBar. Anaerobic experiments were performed in a Forma Scientific Anaerobic System (Anaerobic Chamber), model 1029. All protein samples and digest filtrations were performed with a 0.2 µm VWR Centrifugal Filter, spun for 4 min at 10,000 RPM (~9,500 g) in an Eppendorf benchtop Microcentrifuge at 4 °C.

4.5.2 UV/Vis and fluorescence measurements

UV/Vis absorbance spectra were obtained using a Varian DMS 90 UV/Vis spectrometer with quartz cuvettes (1 mL). Fluorescence spectra were recorded on a PerkinElmer LS55 Luminescence spectrometer using SUPRASIL® PerkinElmer fluorescence cells (0.3 or 3 mL). Slit widths were 10 nm for

excitation and emission. Guanidine hydrochloride (6 M, pH 5.5, 2 mg protein/mL) and PBS (pH 7.0, 0.5 mg amino acid adduct/mL) were used as solvents. Samples were analysed against a blank solution of guanidine hydrochloride or PBS.

4.5.3 Reversed phase HPLC (RP-HPLC)

RP-HPLC was performed on a Shimadzu HPLC equipped with LC-10ADvp pumps, a SIL-10Avp autoinjector and SPD-M10Avp diode array detector. The preparative purifications and basic analyses were performed on a Phenomenex (Luna, 100 Å, 10 μm, 15 x 250 mm, C18) column fitted with a Phenomenex (Synergy Fusion, 100 Å, 4 μm, 10 x 10 mm, C18) guard column. The following mobile phase system; buffer A (H_2O/0.05% TFA, v/v) and buffer B (100% CH_3CN). A flow rate of 8 mL/min was kept constant throughout all preparative purification. The eluents were monitored at 254 and 360 nm. Preparative purification of the 3OHKG (Cys-3OHKG, *t*-Boc-His-3OHKG and *t*-Boc-Lys-3OHKG), 3OHK (Cys-3OHK, *t*-Boc-His-3OHK and *t*-Boc-Lys-3OHK) and Kyn (Cys-Kyn, *t*-Boc-His-Kyn and *t*-Boc-Lys-Kyn) amino acid adducts were purified with a mobile phase gradient as follows: 0-10 min (0% buffer B), 10-40 min (50% buffer B), 40-45 min (50% buffer B), 45-50 min (90% buffer B) and 50-70 min (0% buffer B). The analytical separation of protein Pronase hydrolysates was conducted with a mobile phase gradient as the methods paper by Hawkins *et al.* [235].

4.5.4 Nuclear magnetic resonance (NMR) spectroscopy

^1H, ^{13}C, COSY (^1H-^1H correlation spectroscopy), HSQC (^1H-^{13}C heteronuclear single quantum correlation) and HMBC (^1H-^{13}C heteronuclear multiple bond correlation) NMR experiments were acquired on a Bruker Avance 400 spectrometer (^1H, 400 MHz; ^{13}C, 100 MHz) at 25 °C. NMR spectra were run in CD_3OD. The solvent signal was used as the internal reference (^1H - 4.79 ppm for D_2O and 3.31 ppm for CD_3OD, ^{13}C – 49.0 ppm for CD_3OD) [294]. Resonances are quoted in ppm and coupling constants (*J*) are given in Hz.

4.5.5 Mass spectrometry (LC-MS/MS)

Samples were separated using a Thermo Finnigan Surveyor HPLC system with a Phenomenex Synergi 4μ Fusion-RP 80 column (150 mm x 2.0 mm x 80 Å; 30 °C) coupled to a Thermo Finnigan LCQ Deca XP Max ion trap with electrospray

ionisation in positive ion mode. Samples were eluted at a flow rate of 0.2 mL/min using a gradient system with 100% buffer A (5 min), before a linear increase to 40% buffer B over 40 min, then increasing to 95% B over 2 min, washing with 95% B for 1 min, before returning to 100% A over 2 min and re-equilibration with 100% A (buffer A: 0.05% (v/v) formic acid in water; buffer B: 100% CH_3CN). Eluted compounds were detected by UV absorbance at 360 nm, and MS selected reaction monitoring (SRM) of expected ion fragments (**Table 4.3 - 4.6**). Mass spectrometer conditions were: electrospray needle voltage, 4.5 kV, sheath gas flow rate 42, sweep gas flow rate 24 and a capillary temperature of 275 °C. The sheath and sweep gases were N_2, while the collision gas was helium. Spectra were acquired in positive continuum mode and monitored at 360 nm.

Synthetic compounds were resuspended in aqueous 50% CH_3CN/0.05% formic acid (v/v) and analysed by electrospray ionisation tandem MS (MS/MS) in positive ion mode *via* direct injection. MS settings were the same as for LC, except sheath gas flow was set to 18 and sweep gas was set to 0. Ions were subjected to a range of collision energy settings (typically between 28-35 eV). All measurements were done in positive mode (unless otherwise stated). Samples were flow injected by a syringe pump into the Thermo Finnigan electrospray source at 3 µL/min.

4.5.6 Synthesis and characterisation of amino acid adducts

Synthesis of 3OHKG, 3OHK and Kyn amino acid adducts were conducted following a modified method of Vazquez *et al.* and Korlimbinis *et al.* [124, 225]. A similar method was used for all three UV filters whereby 3OHKG (~30 mg, 78 mmol), 3OHK (~19 mg, 183 mmol) or Kyn sulphate (~25 mg, 82 mmol) were mixed with L-cysteine (~100 mg, 826 mmol) in an approximate 10:1 ratio, or either *N*-α-*t*-Boc-L-histidine (~100 mg, 392 mmol) or *N*-α-*t*-Boc-L-lysine (~100 mg, 406 mmol) at a 5:1 ratio. Each of the mixtures were then each dissolved into argon-gassed (~20 min) Na_2CO_3-$NaHCO_3$ buffer (10 mL, 100 mM, pH 9.5). The pH was adjusted to pH 9.5 by dropwise addition of 1 M NaOH. The light yellow solutions were bubbled with argon for ~10 min, sealed in a reactor carousel vessel and incubated in the dark at 37 °C with stirring. The progress of the reactions was monitored by normal phase TLC using a mobile phase of BAW (12:3:5 *n*-butanol/AcOH/H_2O, v/v). After 24 h, another approximately 100 mg of either L-cysteine (~100 mg, 826 mmol), *N*-α-*t*-Boc-L-histidine (~100 mg, 392

mmol) or *N*-α-*t*-Boc-L-lysine (~100 mg, 406 mmol) were added to each of the respective reaction vessels, the pH was readjusted to 9.5 by dropwise addition of NaOH (1 M) for all reactions and upon argon bubbling were again incubated in the dark at 37 °C. After 72 h, the dark yellow (L-cysteine) and dark brown (*N*-α-*t*-Boc-His and *N*-α-*t*-Boc-Lys) reaction mixtures were acidified to pH 6 by dropwise addition of 1 M AcOH and lyophilised. RP-HPLC preparative purification afforded the various *N*-α-*t*-Boc-L-histidine, *N*-α-*t*-Boc-L-lysine- and Cys adducts as summarised in the table below. All compounds were separated out as light yellow solids, except for Cys-3OHK which was dark yellow/brown in colour. Apart from Kyn-Cys, all compounds separated out as ~1:1 diastereomeric ratios. Kyn-Cys separated out as three products (two approximately 1:1 diastereomers, plus a third compound with an earlier retention time). ^{1}H NMR, ^{13}C NMR, LC-MS/MS, UV/Vis and fluorescence measurements for the Cys-Kyn and Cys-3OHK were in agreement to those reported previously [124, 295]. *R_f data: N*-α-*t*-Boc protected 3OHK amino acid adducts (modified method of Vazquez 2002) (15.7 mg, 15.2%, R_f 0.33), *N*-α-*t*-Boc-L-lysine-3OHK (8 mg, 21%, R_f 0.37) and Cys-3OHK (13.7 mg, 49%, R_f 0.22). The *N*-α-*t*-BOC protection on the amino acid adducts was removed through acid hydrolysis under mild conditions, to prevent further hydrolysis of the glucoside on 3OHKG, using a method of Zinelaabidine *et al.* [284]. The protected amino acid adducts were each individually refluxed in degassed 1% (pH 2.3) formic acid at a concentration of ~1 mg/mL under an atmosphere of nitrogen for 2 h, in the dark.

After hydrolysis, the moderately yellow solutions were cooled to room temperature and the pH was checked to monitor whether deprotection was complete (pH ~2.6 all samples). The pH was not adjusted further, and the mixtures were then lyophilised and subsequently purified (above 95% purity) *via* reversed phase HPLC to give above 95% purity for the adducts Cys-3OHK (13.7 mg), Lys-Kyn (8.7 mg), His-Kyn (11 mg), Lys-3OHK (5.9 mg), His-3OHK (6.3 mg), Cys-3OHKG (16.3 mg), Lys-3OHKG (13.8 mg) and His-3OHKG (14.9 mg). Cys-Kyn (N/A) was very unstable and was decomposing whilst purified by HPLC.

Table 4.8: Yields of synthetic standards and intermediates

Compound	Protected Mass (mg)	Yield (%)	De-protected Mass (mg)
Cys-Kyn	-	-	-
Cys-3OHK	N/A	49	13.7
Cys-3OHKG	N/A	43	16.3
N-α-*t*-Boc-Lys-Kyn	11.6	32	8.7
N-α-*t*-Boc-Lys-3OHK	8.0	21	5.9
N-α-*t*-Boc-Lys-3OHKG	16.8	35	13.8
N-α-*t*-Boc-His-Kyn	14.7	40	11.0
N-α-*t*-Boc-His-3OHK	8.8	22	6.3
N-α-*t*-Boc-His-3OHKG	18.2	37	14.9

4.5.6.1 Analytical data of Cys-3OHK

L-Cysteinyl-3-hydroxy-DL-kynurenine

$M+H^+$, 329.0802 Da. Calculated for $C_{13}H_{17}N_2O_6S$. LC-MS/MS *m/z* 329.1 ($M+H^+$, 28%), 311.1 ($M+H^+$ - H_2O, 100%), 202.2 (30%); ^{1}H NMR δ (MeOD) 7.39 (1H, m, ArH-6), 6.88 (1H, br dd, *J* 1.9, 7.9, ArH-4), 6.56 (1H, dd, *J* 8.0, 78.1, ArH-5), 4.37 (0.5H, m, SCH_2CH), 4.24 (0.5H, m, SCH_2CH), 3.96 (1H, m, H-2), 3.67 (1H, m, H-3), 3.64 (0.5H, m, SCH_2CH), 3.47 (0.5H, m, SCH_2CH), 3.46 (1H, m, H-3), 3.29 (0.5H, m, SCH_2CH), 3.13 (0.5H, m, SCH_2CH); ^{13}C NMR δ (MeOD) 201.1 (CO-4), 200.9 (CO-4), 176.7 (CO-1), 171.7 ($NH_2CHCOOH$), 147.4 (ArC-3), 143.3 (ArC-2), 123.5 (ArC-6), 119.3 (ArC-1), 119.1 (ArC-4), 117.0 (ArC-5), 55.2 (SCH_2CH), 54.2 (SCH_2CH), 44.9 (C-2), 42.9 (C-3), 42.4 (C-2), 42.1 (C-3), 34.6 (SCH_2), 33.7 (SCH_2); UV/Vis λ_{max} 268 and 368 nm, fluorescence λ_{ex} 340 / λ_{em} 520 nm and λ_{ex} 400 / λ_{em} 470 nm.

4.5.6.2 Analytical data of Lys-3OHK

L-Lysyl-3-hydroxy-DL-kynurenine

$M+H^+$, 354.1674 Da. Calculated for $C_{16}H_{24}N_3O_6$; LC-MS/MS *m/z* 354.1 ($M+H^+$, 28%), 203.0 (100%), 147.1 ($M+H^+$ - 3OHK, 40%), 128.0 (12%), 208.1 ($M+H^+$ - Lys, 10%), 130.2 ($M+H^+$ - Lys – NH_3, 9%); ^{1}H NMR δ (CD_3OD) 7.32 (1H, dd, *J* 1.7, 8.0, ArH-6), 6.91 (1H, dd, *J* 1.8, 8.0, ArH-4), 6.54 (1H, dd, *J* 8.0, 8.1, ArH-5), 4.33 (1H, m, H-2), 3.97 (1H, m, $CHNH_2$), 3.80 (2H, m, H-3), 3.21 (2H, m, $NH\text{-}CH_2$),

1.95 (2H, m, $CH_2CH_2CH_2$), 1.84 (2H, m, $CH_2CH_2CH_2$), 1.55 (2H, m, $CH_2CH_2CH_2$); ^{13}C NMR δ (CD_3OD) 198.2 (CO-4), 171.5 (CO-1), 171.3 ($NH_2CHCOOH$), 145.8 (ArC-3), 142.3 (ArC-2), 121.5 (ArC-6), 117.7 (ArC-4), 116.6 (ArC-1), 115.4 (ArC-5), 57.0 (C-2), 53.1 ($CHNH_2$), 47.2 ($NHCH_2$), 38.4 (C-3), 29.8 (CH_2-CH_2-CH_2), 25.2 (CH_2-CH_2-CH_2), 22.0 (CH_2-CH_2-CH_2); UV/Vis λ_{max} 270 and 372 nm, fluorescence λ_{ex} 345 / λ_{em} 510 nm.

4.5.6.3 Analytical data of His-3OHK

L-Histidyl-3-hydroxy-DL-kynurenine

$M+H^+$, 363.1313 Da. Calculated for $C_{16}H_{19}N_4O_6$; LC-MS/MS *m/z* 363.0 ($M+H^+$, 28%), 156.1 ($M+H^+$ - 3OHK, 100%), 317.1 ($M+H^+$ - HCOOH, 64%), 110.0 ($M+H^+$ - His - $C(O)CH_2CH(NH_2)COOH$, 33%), 208.1 ($M+H^+$ - Lys, 26%), 255.1 (8%), 162.0 ($M+H^+$ - Lys - HCOOH, 6%); ^1H NMR δ (CD_3OD) 8.90 (1H, s, NCHN), 7.57 (1H, s, NCHC), 7.38 (1H, d, *J* 8.1, ArH-6), 6.76 (1H, d, *J* 7.8, ArH-4), 6.45 (1H, dd, *J* 7.9, 8.0, ArH-5), 5.74 (1H, m, H-2), 4.21 (1H, m, CH_2-CH-COOH), 4.02 (1H, m, H-3), 3.94 (1H, m, H-3), 3.36 (1H, m, CH_2-CH-COOH), 3.22 (1H, m, CH_2-CH-COOH); ^{13}C NMR δ (CD_3OD) 198.0 (CO-4), 171.8 (CO-1), 170.9 ($CH_2CHCOOH$), 146.1 (ArC-3), 142.3 (ArC-2), 137.1 (NCHN), 122.0 (ArC-6), 121.1 (NCHC), 117.9 (ArC-4), 117.4 (ArC-1), 115.4 (ArC-5), 59.0 (C-2), 52.9 ($CH_2CHCOOH$), 42.5 (C-3), 27.4 ($CH_2CHCOOH$); λ_{max} 270 and 374 nm.

4.5.6.4 Analytical data of Cys-3OHKG

L-Cystyl-3-hydroxy-DL-kynurenine *O*-β-D-glucoside

$M+H^+$, 491.1330 Da; LC-MS/MS *m/z* 491.1 ($M+H^+$, 28%), 473.0 ($M+H^+$ - H_2O, 100%), 311.0 ($M+H^+$ - H_2O - Glu, 23%), 202.0 (12%); ^1H NMR δ (D_2O) 7.68 (1H, d, *J* 8.1, ArH-6), 7.34 (1H, d, *J* 7.6, ArH-4), 6.79 (1H, dd, *J* 8.0, 8.2, ArH-5), 5.05 (1H, d, *J* 7.3, H-1'), 4.05 (1H, m, CH_2CH), 3.93 (1H, dd, *J* 2.0, 12.9, H-6'), 3.90 (1H, m, H-2), 3.79 (0.5H, m, H-3), 3.78 (0.5H, m, H-3), 3.76 (0.5H, m, H-3), 3.75 (0.5H, m, H-3), 3.70 (1H, dd, *J* 5.3, 12.4, H-6'), 3.68 (1H, m, H-3'), 3.63 (1H, m, H-2'), 3.61 (1H, m, H-5'), 3.58 (1H, m, H-4'), 3.53 (0.5H, m, CH_2CH), 3.51 (0.5H, m, CH_2CH), 3.39 (0.5H, m, CH_2CH), 3.32 (0.5H, m, CH_2CH); ^{13}C NMR δ (D_2O) 201.9 (CO-4), 201.8 (CO-4), 184.0 (CO-1), 175.4 (CO-Cys), 145.2 (ArC-3), 141.1 (ArC-2), 126.9 (ArC-6), 121.7 (ArC-4), 119.5 (ArC-1), 117.1 (ArC-5), 102.4 (C-1'), 77.1 (C-5'), 76.5

(C-2’), 73.8 (C-3’), 70.3 (C-4’), 61.4 (C-6’), 49.0 (CH_2CH), 43.1 (C-2), 42.4 (C-3), 33.7 (CH_2CH); λ_{max} 265 and 364 nm, fluorescence λ_{ex} 340 / λ_{em} 440 nm.

4.5.6.5 Analytical data of Lys-3OHKG

L-Lysyl-3-hydroxy-DL-kynurenine *O*-β-D-glucoside

$M+H^+$, 516.2188 Da; LC-MS/MS *m/z* 516.2 ($M+H^+$, 28%), 370.0 ($M+H^+$ - Lys, 100%), 314.0 ($M+H^+$ - Lys – CH_3COOH, 60%), 208.1 ($M+H^+$ - Lys - Glu, 49%), 152.0 (50%), 147.1 ($M+H^+$ - 3OHKG, 41%), 203.0 (38%), 152.1 (25%), 498.0 ($M+H^+$ - H_2O, 11%), 354.1 ($M+H^+$ - Glu, 9%); ^{1}H NMR δ (D_2O) 7.63 (1H, dd, *J* 1.8, 8.0, ArH-6), 7.35 (1H, dd, *J* 2.0, 8.2, ArH-4), 6.78 (1H, dd, *J* 7.9, 8.2, ArH-5), 5.05 (1H, d, *J* 7.5, H-1’), 4.09 (1H, m, CH_2CH), 3.94 (1H, m, $CHNH_2$), 3.75 (1H, dd, *J* 2.0, 12.8, H-6’), 3.75 (1H, m, H-2), 3.74 (0.5H, m, H-3), 3.74 (0.5H, m, H-3), 3.73 (0.5H, m, H-3), 3.73 (0.5H, m, H-3), 3.64 (1H, dd, *J* 5.3, 12.4, H-6’), 3.63 (1H, m, H-3’), 3.63 (1H, m, H-2’), 3.59 (1H, m, H-5’), 3.58 (1H, m, H-4’), 3.53 (0.5H, m, CH_2CH), 3.52 (0.5H, m, CH_2CH), 3.19 (2H, m, $NH-CH_2$), 1.92 (2H, m, $CH_2CH_2CH_2$), 1.83 (2H, m, $CH_2CH_2CH_2$), 1.52 (2H, m, $CH_2CH_2CH_2$); ^{13}C NMR δ (D_2O) 200.0 (CO-4), 199.9 (CO-4), 174.9 (CO-1), 173.5 ($NH_2CHCOOH$), 145.1 (ArC-3), 142.1 (ArC-2), 126.1 (ArC-6), 121.1 (ArC-4), 118.2 (ArC-1), 116.1 (ArC-5), 101.7 (C-1’), 76.5 (C-5’), 75.9 (C-2’), 73.2 (C-3’), 69.7 (C-4’), 60.8 (C-6’), 58.2 (C-2), 54.8 ($CHNH_2$), 47.3 ($NHCH_2$), 39.1 (C-3), 30.2 (CH_2-CH_2-CH_2), 25.6 (CH_2-CH_2-CH_2), 21.9 (CH_2-CH_2-CH_2);

4.5.6.6 Analytical data of His-3OHKG

L-Histidyl-3-hydroxy-DL-kynurenine *O*-β-D-glucoside

$M+H^+$, 525.1827 Da; LC-MS/MS *m/z* 525.2 ($M+H^+$, 28%), 363.1 ($M+H^+$ - Glu, 100%), 208.1 ($M+H^+$ - His - Glu, 23%), 156.1 ($M+H^+$ - 3OHKG, 21%), 370.0 ($M+H^+$ - His, 10%); ^{1}H NMR δ (D_2O) 8.40 (1H, s, NCHN), 7.49 (1H, s, NCHC), 7.27 (1H, d, *J* 8.4, ArH-6), 7.16 (1H, d, *J* 7.6, ArH-4), 6.61 (1H, dd, *J* 7.7, 8.2, ArH-5), 5.28 (1H, m, H-2), 4.87 (1H, d, *J* 7.2, H-1’), 3.82 (1H, m, CH_2-CH-COOH), 3.80 (1H, dd, *J* 1.9, 12.9, H-6’), 3.79 (1H, m, H-3), 3.62 (1H, m, H-3), 3.60 (1H, dd, *J* 5.4, 12.5, H-6’), 3.44 (1H, m, CH_2-CH-COOH), 3.44 (1H, m, H-3’), 3.37 (1H, m, H-2’), 3.18 (1H, m, H-5’), 3.17 (1H, m, H-4’), 3.02 (1H, m, CH_2-CH-COOH); ^{13}C NMR δ (D_2O) 199.9 (CO-4), 145.1 (CO-1), 141.9 ($CH_2CHCOOH$), 137.2 (ArC-3), 136.7 (ArC-2), 126.1 (NCHN), 121.0 (ArC-6), 120.3 (NCHC), 119.8 (ArC-4), 118.6 (ArC-

1), 116.2 (ArC-5), 101.7 (C-1’), 75.8 (C-5’), 73.1 (C-2’), 69.7 (C-3’), 60.7 (C-4’), 60.1 (C-6’), 54.1 (C-2), 48.2 ($CH_2CHCOOH$), 42.2 (C-3), 27.1 ($CH_2CHCOOH$);

4.5.7 Preparation of proteins from bovine lenses

Fresh bovine eyes (≤ 2 years old) were obtained from Ziems Butcher (Corrimal, NSW, Australia). The lenses were quickly removed from each eyeball and taken out of the lens capsule. The lens on its own was then stored at -20 °C until it was used. Soluble bovine lens proteins (BLP) were then extracted and purified from decapsulated lenses as previously described by Parker *et al.* [18, 200]. The lyophilised lens proteins were also stored at -20 °C until used. I wish to acknowledge that almost all of the bovine lens BLP preparation work was performed by Jasminka Mizdrak.

4.5.8 Modification of BLP with UV filters

BLP (10 mg/mL) were treated with UV filters (2 mg/mL) in oxygen depleted Na_2CO_3/$NaHCO_3$ buffer (50 mM, pH 9.5) or in NaH_2PO_4/Na_2HPO_4 buffer (100 mM, pH 7.4) as described previously [18, 124, 225]. The reaction mixtures were incubated at 37 °C for 48 h (pH 9.5; Kyn, 3OHK, 3OHKG, AHB, 3OHKG-Y, 3OHKG-W, AHAG) or 7 days (pH 7.4; Kyn, 3OHK, 3OHKG, AHB, 3OHKG-Y, 3OHKG-D) in an anaerobic chamber. The mixtures were dialysed at 2°C for 48 h (with 3 buffer changes). The coloured proteins (pale yellow, brown or brown/red) mixtures were then lyophilised and stored under nitrogen at -20 °C until used.

4.5.9 Pronase digestion of BLP

BLP modified with the UV filters (2 mg/mL) were mixed with Pronase in Milli-Q water in a 4:1 ratio and incubated at 40 °C in the dark for 72 h. To minimise oxidation, samples where degassed and de-oxygenated by bubbling with nitrogen prior to digestion. After incubation, the samples were filtered through a 0.22 nm Eppendorf spin-down filter and then stored at 20 °C. Hydrolysis was performed on untreated BLP (as negative control), control BLP, the UV filter modified BLP and Pronase alone (for baseline subtraction).

4.5.10 Acid hydrolysis of BLP

UV filter-treated BLP (2 mg) were hydrolysed in 6 M HCl as reported previously by Korlimbinis *et al*. [124]. Untreated BLP were also hydrolysed and used as a negative control.

4.5.11 Extraction of unbound UV filters from modified BLP

BLP (~2 mg) treated with Kyn, 3OHK, 3OHKG, AHB, 3OHKG-Y, 3OHKG-W, AHAG or 3OHKG-D were dissolved in ice-cold and nitrogen gassed (~20 min) guanidine hydrochloride (6 M, pH 5.5, 2 mg protein/mL), and stirred at room temperature for 30-40 min in the dark. The mixture was poured into the protein concentrator and spun at 4,700 g at 4 °C. The concentrated BLP were twice washed with guanidine hydrochloride (6 M, pH 5.5). The concentrated proteins (~150 μL) and the combined filtrates were then separately lyophilised. The protein samples were digested in 6 M HCl for 12 h, as described above, and analysed by reversed phase HPLC. The dried guanidine hydrochloride filtrates were extracted with CH_3CN (3 x ~10 mL) and dried on a rotary evaporator. The collected solids were redissolved in 0.05% formic acid/water and analysed by HPLC, as previously described for protein acid hydrolysates. The extraction and acid hydrolysis procedure was repeated with unmodified protein used as a negative control.

4.5.12 Stability of UV filters and amino acid adducts

The stability of a known quantity (~0.5 mg) of UV filter (Kyn, 3OHK, 3OHKG, AHB, 3OHKG-Y, 3OHKG-W or AHAG), UV filter amino acid adduct (Cys-Kyn, Cys-3OHK, Cys-3OHKG, Lys-Kyn, Lys-3OHK, Lys-3OHKG, His-Kyn, His-3OHK or His-3OHKG) or a mixture of unmodified BLP (~10 mg) and free UV filter (Kyn, 3OHK, 3OHKG, AHB, 3OHKG-Y, 3OHKG-W, AHAG, Cys-3OHKG, 0.5 mg) were tested under the conditions of 6 M HCl acid hydrolysis, 1% formic acid reflux and incubation at 40 °C in duplicate and analysed by reversed phase LC-MS/MS or HPLC (as described previously) for either protein acid hydrolysates or other breakdown products. The identity of products was confirmed by spiking experiments with authentic standards (where possible) and simply by the LC-MS/MS fragmentation of the product peak. Under 6 M HCl hydrolysis, the recovery of 3OHKG and Cys-3OHKG was determined by quantifying the levels of their de-glucosylated residues (3OHK and Cys-3OHK, respectively). AHB and AHA

were quantified using a standard curve constructed using synthetic standards of AHB and AHA, respectively.

4.5.13 Derivatisation of untreated BLP amino acid composition

The amino acid composition of hydrolysed untreated BLP was quantified using a method by through derivatisation using *o*-phthaldialdehyde (OPA), and then measuring fluorescence after separation on a Shimadzu Nexera UPLC system with a RF-20Axs fluorescence detector (λ_{ex} 340 / λ_{em} 440 nm) [235, 286]. Hydrolysed proteins were diluted to >50 µg/mL in MilliQ water, with a standard curve in the similar concentration range made from 500 µM amino acid standard solution. And prepared in aliquots of 40 µL. With each derivatisation run, 20 µL of activated OPA is injected into that sample, mixed three times and allowed to stand for 1 min. Then 15 µL of OPA/sample in injected into and separated on a Shim-pack XR-ODS (Shimadzu, 100cm x 4.6 mm, 2.2 µm) with column at 30 °C. Total elution is a flow rate of 1.2 mL/min using a gradient system with 5% buffer B (2 min), before a linear increase to 50% buffer B (6 min), held at 50% buffer B (2 min) then increasing to 100% B (5 min), held at 100% buffer B (1 min), decreasing to 5% buffer B (0.5 min) washing with 5% buffer B (3.5 min). Buffer composition, buffer A: pH 5.0 0.1 M sodium acetate 2.5% THF/20% MeOH/water (v/v); buffer B: pH 5.0 0.1 M sodium acetate 2.5% THF/80% MeOH/water (v/v).

CHAPTER 5

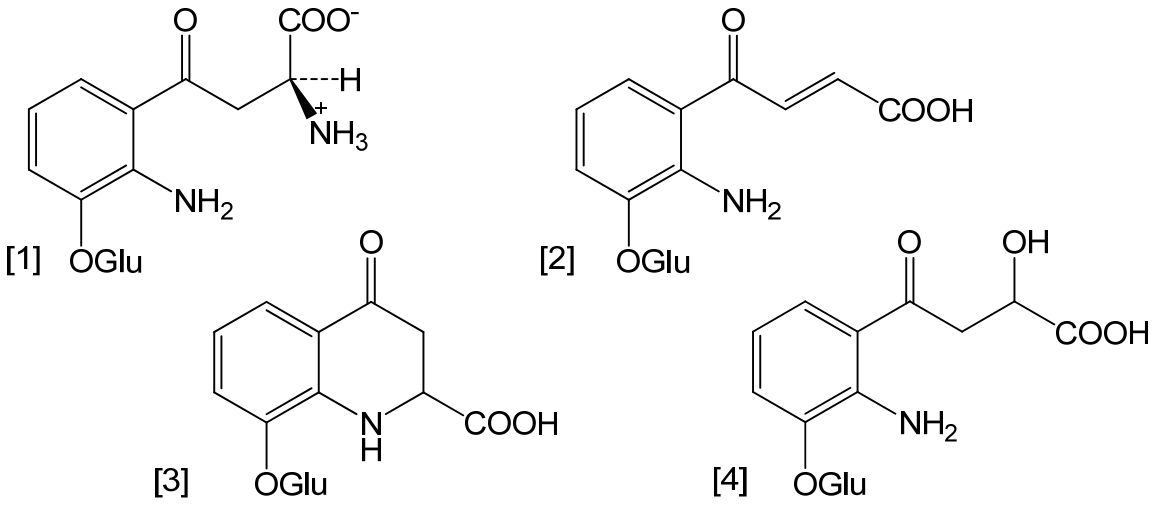
FINAL CONCLUSION AND FUTURE DIRECTIONS

Behind the evolution of the human brain, the eye is probably the most amazing anatomical apparatus to have evolved in the whole Animalia kingdom. As a testament to its success, virtually all higher order animals have eyes, with humans having one of the most evolved forms of the organ. One of the more recent adaptive improvements to the eye is the lens, and though nominally its only function is to refract incoming light, even this relatively simple structure has many complexities. The focus of this thesis has been on one of those variations found between species in the lens, the presence (or absence) of a small class of UV protective compounds called the UV filters. Though these compounds are thought to protect the retina of humans and other long lived animals from UV damage, these compounds are unstable and upon binding to the proteins of the lens become photosensitisers. It is believed that this gradual photo-damage with age is a major factor in the development of age related nuclear cataract character in older persons.

IDENTIFICATION OF 3-HYDROXYKYNURENINE *O*-B-D-GLUCOSIDE DERIVED NOVEL UV FILTERS

Though the protein structure and composition of most mammalian optical lenses are similar, there are some subtle differences. Whilst human lenses share a similar protein homology with other mammals, as a long lived being they have multiple protective UV filters, of which 3OHKG is the most abundant and important [25]. In this oxygenated environment, 3OHKG is known to form other UV filter-like compounds, of which three novel UV filter metabolites: 4-(2-amino-3-hydroxyphenyl)-2-hydroxy-4-oxobutanoic acid *O*-β-D-glucoside, 3-hydroxykynurenine *O*-β-D-glucoside yellow and 2-amino-3-hydroxyacetophenone *O*-β-D-glucoside were identified in the extracts of 24 human lenses, synthesised and quantified. In addition, the presence of deaminated 3-hydroxykynurenine *O*-β-D-glucoside (3OHKG-D) was also confirmed, however due to its low concentration it could not be quantified. Similar to earlier studies, 3OHKG concentrations in the eye decreased with age. In contract, these novel UV filter metabolites increased

in the nucleus of the eye. In the cortex of the lenses, the concentrations of the metabolites remained constant with age. Though only found in pmol/mg quantities, they all (except for 3OHKG-D) have similar UV absorbance characteristics to the major UV filters [219], and were hypothesised to function in an analogous manner to 3OHKG, 3OHK and Kyn.



Scheme 5.1: Structures of identified 3OHKG derived UV filter metabolites. [1] 3OHKG [2] 3OHKG-D [3] 3OHKG-Y, and [4] 3OHKG-W.

UV IRRADIATION OF MODIFIED BOVINE LENS PROTEINS

It is known that 3OHKG, 3OHK and Kyn can deaminate under physiological (pH 7.4), and at an accelerated rate under basic (pH 9.5) conditions [68]. These UV filters can then bind to the Cys residues of BLP, causing photo-damage to the lens proteins upon exposure to UV light, which is believed to be a contributing factor to the development of age related nuclear cataract [50, 124]. It was of particular interest to observe whether any of the newly identified metabolites, in addition to 3OHKG-D, could bind with BLP and induce any changes or photo-damage upon irradiation with UV light. BLP were treated at pH 7.4 or pH 9.5 with 3OHKG, 3OHK, Kyn, AHB, 3OHKG-D, 3OHKG-Y, 3OHKG-W and AHAG. The treated/modified BLP were then irradiated with UV light above 305 nm, with any changes or protein damage being observed upon separation of the BLP *via* SDS-PAGE and silver staining. Evidence of binding and protein photo-damage was only observed in BLP modified with 3OHKG, 3OHK, Kyn and 3OHKG-D. There was evidence of both cross-linking (Cys-Cys binding) and of non-disulphide binding (Tyr-Tyr binding) in the BLP modified with Kyn, 3OHK, 3OHKG and 3OHKG-D. A

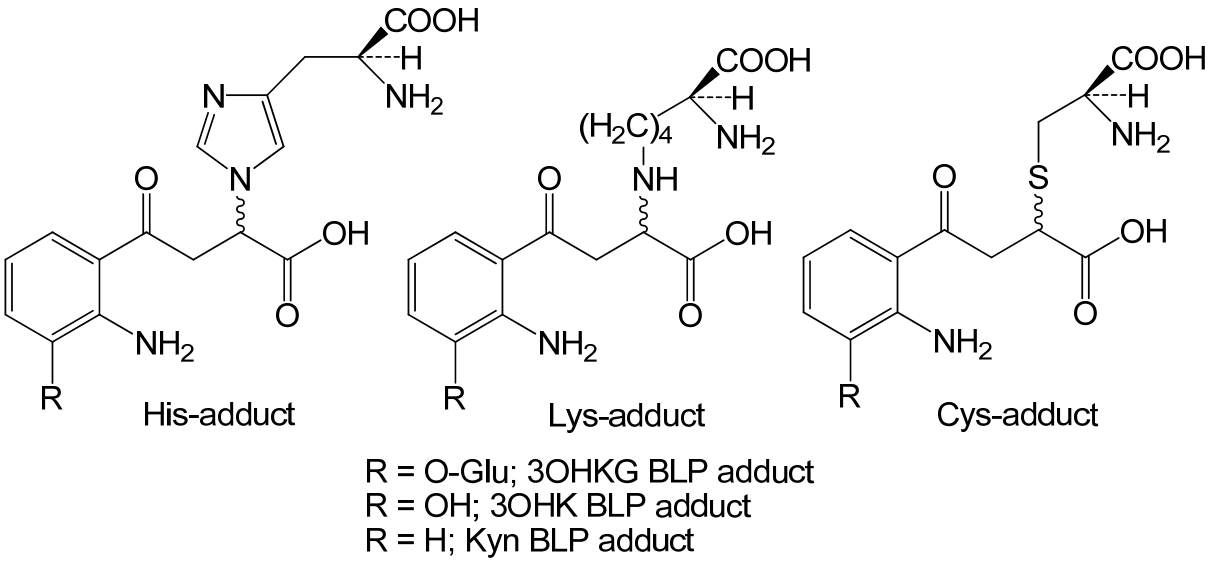
third type of binding, possibly involving histidine or lysine, was also suggested by the results as protein changes were observed where both disulphide and di-Tyr bonds were not possible on likely. There was a pronounced difference in the protein changes observed between bound UV filters with both an *o*-aminophenol and α,β-unsaturated carbonyl moieties (3OHK) and those with only an α,β-unsaturated carbonyl moiety (3OHKG, Kyn, 3OHKG-D) (**Sections 3.2.2.3 - 3.2.2.6**). The gel band formation and separation patterns of 3OHK were different to those of Kyn and 3OHKG. After 120 min of irradiation, 3OHK modified BLP had few visible protein bands under all conditions. Upon bubbling with air, remaining bands were significantly less pronounced. Both changes suggest a possible loss of soluble protein through the aggregation of modified BLP, though the presence of oxygen has a different effect on 3OHK compared to the other major UV filters.

Though all the tested metabolites (AHB, 3OHKG-Y, 3OHKG-W and AHAG) have similar absorption profiles to 3OHKG, covalent binding between BLP and a UV filter occurs mainly *via* the α,β-unsaturated carbonyl moieties, which these metabolites all lack. Without this covalent binding, photo-oxidative damage from UV light irradiation was not visible on the silver stained SDS-PAGE gels. No evidence of binding or protein damage was observed in samples treated with AHB, 3OHKG-Y, 3OHKG-W or AHAG upon irradiation, compared to irradiated untreated BLP *via* SDS-PAGE. Even though AHB could potentially bind to proteins *via* auto-oxidation of the *o*-aminophenol, no visible changes or damage was observed in the SDS-PAGE gels.

ANALYSIS OF LENS PROTEINS MODIFIED WITH KYNURENINE BASED HUMAN LENS UV FILTERS AND METABOLITES

With age, the production of UV filters in the lens decreases and it has been shown that *in vivo*, these UV filters can bind to lens proteins [224]. In addition to Cys [225], it has been hypothesised that deaminated Kyn based UV filters may bind to other amino acids with nucleophilic side-chains like His and Lys. One of the aims of this thesis was to provide a comprehensive analysis of the protein amino acid binding sites of the three UV filters Kyn, 3OHK, 3OHKG and 3OHKG derived metabolites. BLP modified/treated with Kyn, 3OHK, 3OHKG, 3OHKG-D, AHB, 3OHKG-Y, 3OHKG-W or AHAG at both physiological (pH 7.4) and basic (pH 9.5) pHs were each hydrolysed into their individual amino acid residues with Pronase and then tested against synthetic standard through LC-MS/MS analysis. Standards were

synthesised of Cys, His and Lys amino acid adducts, each bound to either Kyn, 3OHK or 3OHKG.



Scheme 5.2: Structures of synthetic amino acid standards

In general, lens proteins modified/treated with 3OHKG, 3OHK, Kyn and AHB at pH 9.5 were found to be coloured and fluorescent, resembling those from aged and age related nuclear cataractous lenses. Colouration and fluorescence was lower at pH 7.4, with AHB treated BLP having negligible colour. All the 3OHKG derived metabolites (3OHKG-Y, 3OHKG-W and AHAG) had negligible impact on the colouration or fluorescence through treatment with BLP and at both pH values.

3OHKG at pH 9.5 was found to react with lens proteins at Cys, His and Lys residues, with the greatest level of binding on the Cys. At pH 7.4 low levels of binding were detected for Cys and His only.

3OHKG-D at pH 7.4 was found to bind to both Cys and His residues at a higher rate than 3OHKG at pH 7.4, with very low binding to Lys. Modification with 3OHKG-D gave a good indication of where binding is likely to occur when not rate limited by the deamination of 3OHKG.

3OHK at pH 9.5 reacted with Cys and His residues to a similar extent, with lower binding to Lys. Interestingly, binding with His was slightly higher than for Cys. At pH 7.4 low binding was observed at the Cys residue, with very low detection at the His and Lys residue.

Kyn at pH 9.5 reacted strongly with Cys residues, but only at very low levels with His and Lys. At pH 7.4, binding was detected with all residues, but at levels close to the detection limit. Kyn amino adducts were generally found to be unstable and this may have had an impact on the detection rate.

3OHKG-Y at pH 7.4 had very low binding at approximately 436 pmol/mg of protein. A similar binding curve was present at pH 9.5, but was obscured by other fragments and could not be quantified. This binding was not expected, as no other metabolites (AHB, 3OHKG-W or AHAG) showed any evidence of covalent binding with BLP.

Generally, the main factor that affects binding between a UV filter/metabolite and a target protein is its readiness to deaminate. A basic pH of 9.5 greatly accelerates the level of deamination (and is the pH used to synthesis a number of metabolites from 3OHKG) however it may also play a part in denaturing the BLP thus allowing reactions to occur with structurally protected residues. Unbound, and non-binding UV filters, had no effect on the level of cross-linking and aggregation in the protein. Hence, for a UV filter to act as a photosensitiser of oxidative damage, results in this study confirm that it must be bound to the target protein, and binding by Kyn, 3OHK, 3OHKG and 3OHKG-D confirms their roles in damaging lens proteins. Otherwise, binding occurs preferably with Cys residues, and with His to a lesser extent. The UV filters react with Lys residues but only in very low amounts. As demonstrated here, these reactions occur faster under basic conditions (pH 9.5) but can likewise occur (though at a slower rate) under physiological conditions (pH 7.4).

FUTURE DIRECTIONS

The nest step in the analysis of the modified BLP would be to identify the structural position of bound UV filters. This could be performed through tryptic digests of the proteins followed by LC-MS/MS analysis. This would also give a fuller picture of the differences between physiological (pH 7.4) and basic (pH 9.5) treatment. The basic pH in UV filter modification is generally believed to increase the reaction rate without necessarily affecting the maximum amount of binding possible (after an extended period of time). Thus it would be interesting to experiment with BLP modification over longer periods of time, and to see what effect the denaturing aspect of high pH has on UV filter binding to BLP. A simple experiment design would be to denature some BLP and then perform UV filter

binding at physiological pH. As a smaller addendum to this research, these same modification experiments could also be performed on human lens proteins (in small scale) to confirm how representative these BLP experiments. A similar set of experiments could be performed, though with some adaptation for the smaller amount of proteins in human lenses.

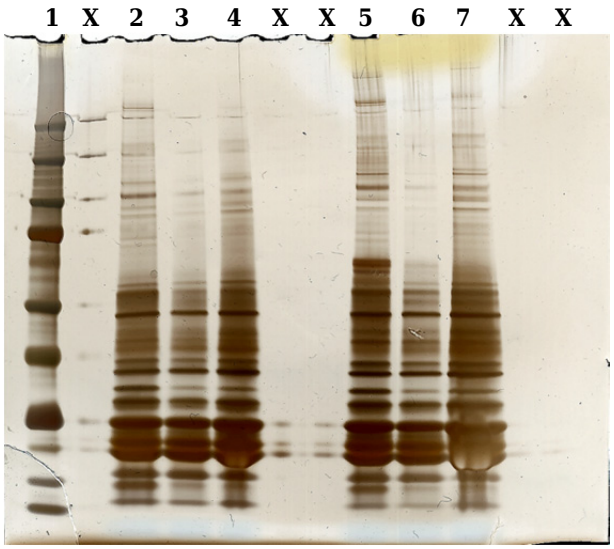
In vivo, both αA- and αB-crystallins have chaperone functions so as to protect the structure of the lens [287]. It would be interesting to see what effect bound UV filters have on the chaperone functions of αA- and αB-crystallins, and to potentially measure any such changes. This could lead into exploring a broader question of what effect UV filter binding has on the crystallins, especially on their structure and visual function. Experiments could also be adapted for the modification of whole (or unprocessed sections) lens. One could then observe what effect UV light induced damage has on the surrounding structure of the lens *in vitro*. Likewise, other cross-linking reactions may occur between the proteins, of which intramolecular binding between 5-cysteinyl-DOPA residues (generated from DOPA quinone and cysteine) upon UV irradiation is a potential candidate for investigation.

Lastly, though bovine lens proteins are a close analogue of human lens proteins, analysis of normal and cataract human lens would provide valuable information on how representative the BLP binding actually is. Some sequencing and binding data has been obtained and published in the past (**Table 4.8**) [82, 224], with some minor comparisons being made in this thesis (**Section 4.3**). With the increase in LC-MS/MS sensitivity, a greater picture of binding could be obtained particularly with the observation of both His and Lys binding (like observed in these BLP models). Through the information gathered in these individual experiments, eventually a testable *in vivo* or *in vitro* model of ARN cataract may be developed.

CHAPTER 6

APPENDIX

A. (3.2.2.2.4 Untreated BLP)



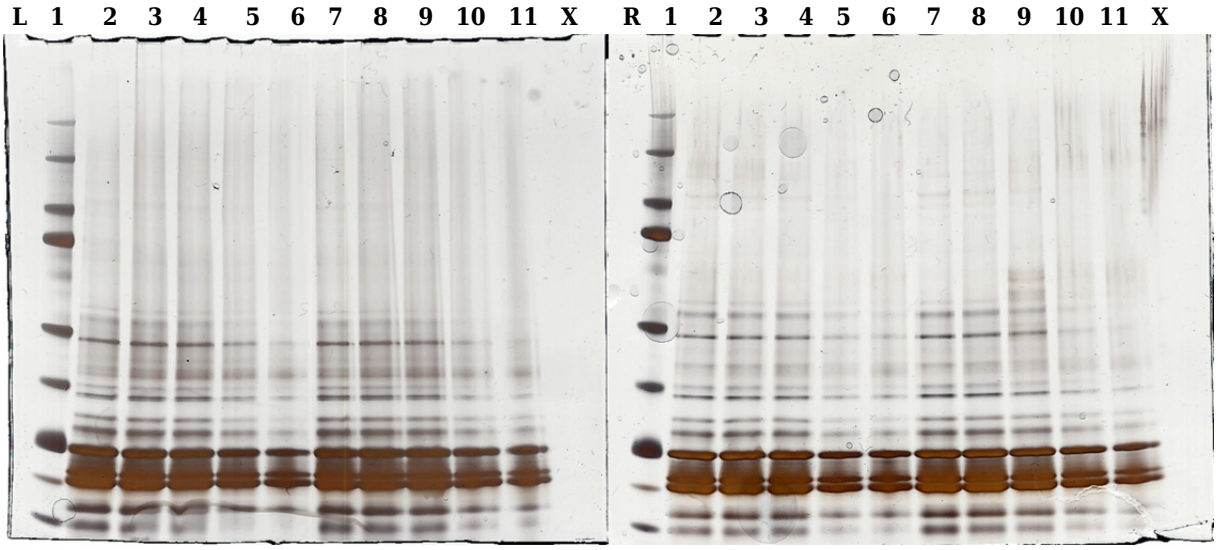
Untreated BLP negative controls run on a Bis-Tris gel with MOPS running buffer under [2-4] normal and [5-7] reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).

Lanes: Molecular weight marker [1] 2 µl

Normal BLP [2] 0 min [3] 60 min [4] 120 min

Reduced BLP [5] 0 min [6] 60 min [7] 120 min

B. (3.2.2.3 Kyn)

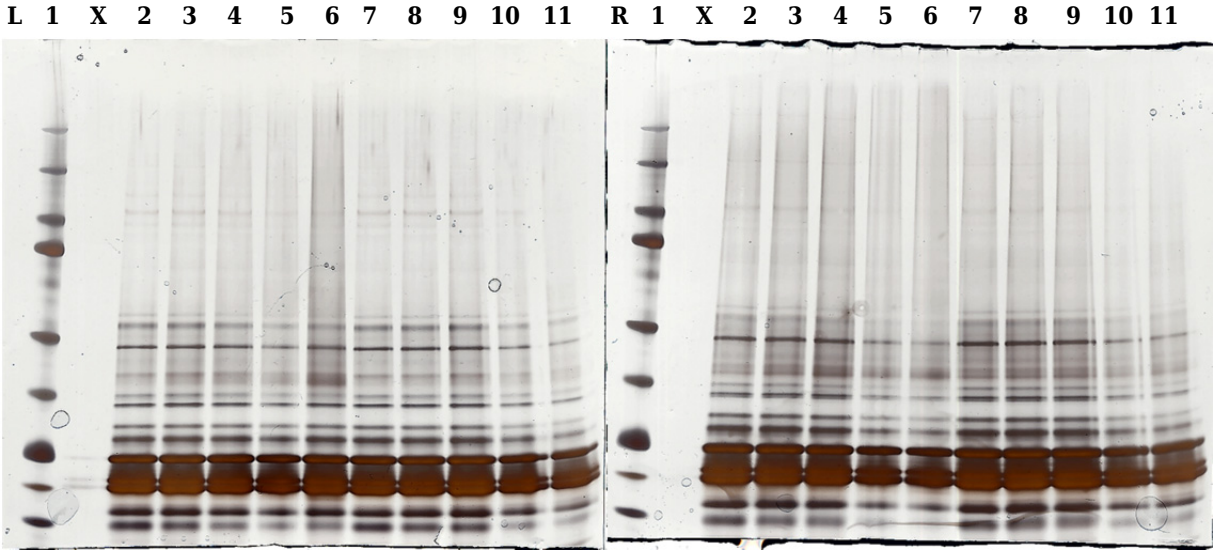


Kyn pH 7.4 BLP run on a Bis-Tris gel with MOPS running buffer under (left) normal and (right) reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).

Lanes: Molecular weight marker [1] 2 µl

N_2 Bubbled Kyn BLP [2] 0 min [3] 15 min [4] 30 min [5] 60 min [6] 120 min

Air Open Kyn BLP [7] 0 min [8] 15 min [9] 30 min [10] 60 min [11] 120 min

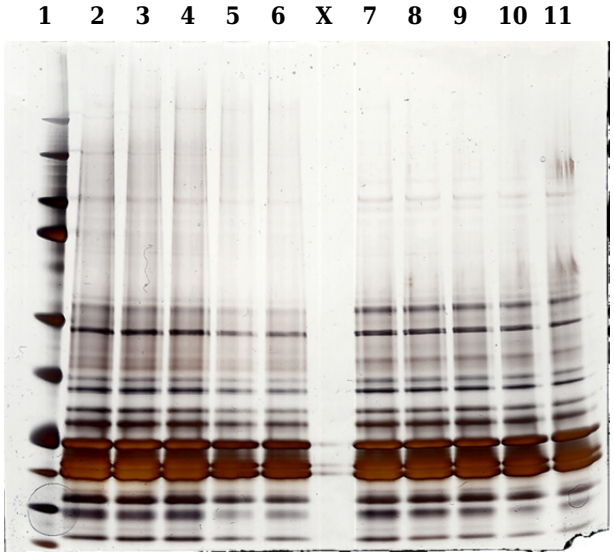


Kyn pH 7.4 BLP run on a Bis-Tris gel with MOPS running buffer under (left) normal and (right) reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).

Lanes: Molecular weight marker [1] 2 µl

Air Bubbled Kyn BLP [2] 0 min [3] 15 min [4] 30 min [5] 60 min [6] 120 min

N_2 Sealed Kyn BLP [7] 0 min [8] 15 min [9] 30 min [10] 60 min [11] 120 min



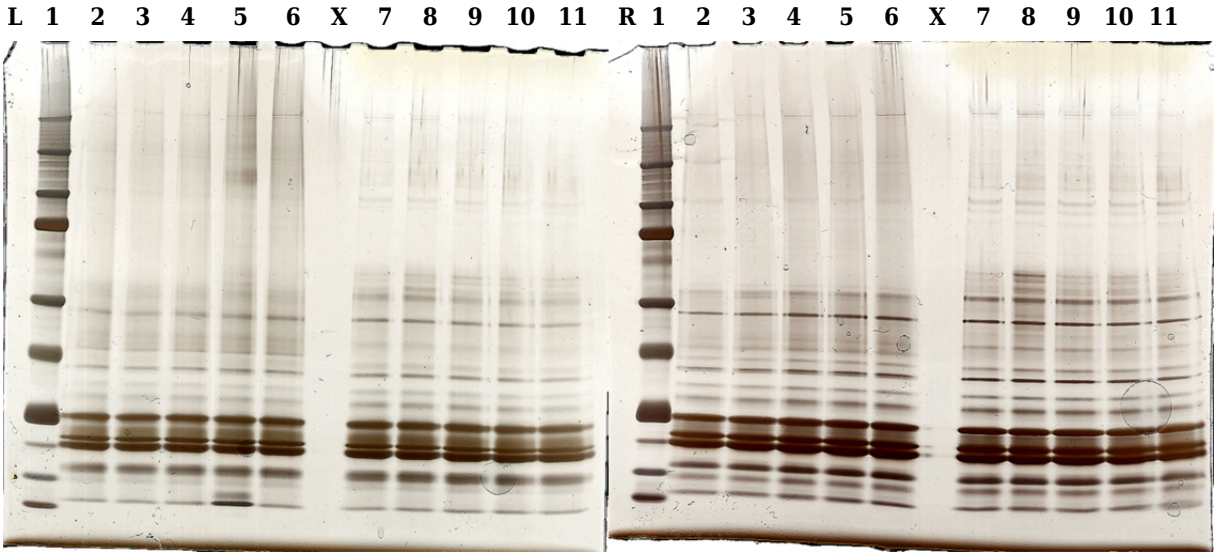
Kyn pH 7.4 BLP negative controls run on a Bis-Tris gel with MOPS running buffer under [2-6] normal and [7-11] reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).

Lanes: Molecular weight marker [1] 2 µl

Normal Kyn BLP [2] 0 min [3] 15 min [4] 30 min [5] 60 min [6] 120 min

Reduced Kyn BLP [7] 0 min [8] 15 min [9] 30 min [10] 60 min [11] 120 min

C. (3.2.2.3 / 3.2.2.4 Kyn and 3OHK)



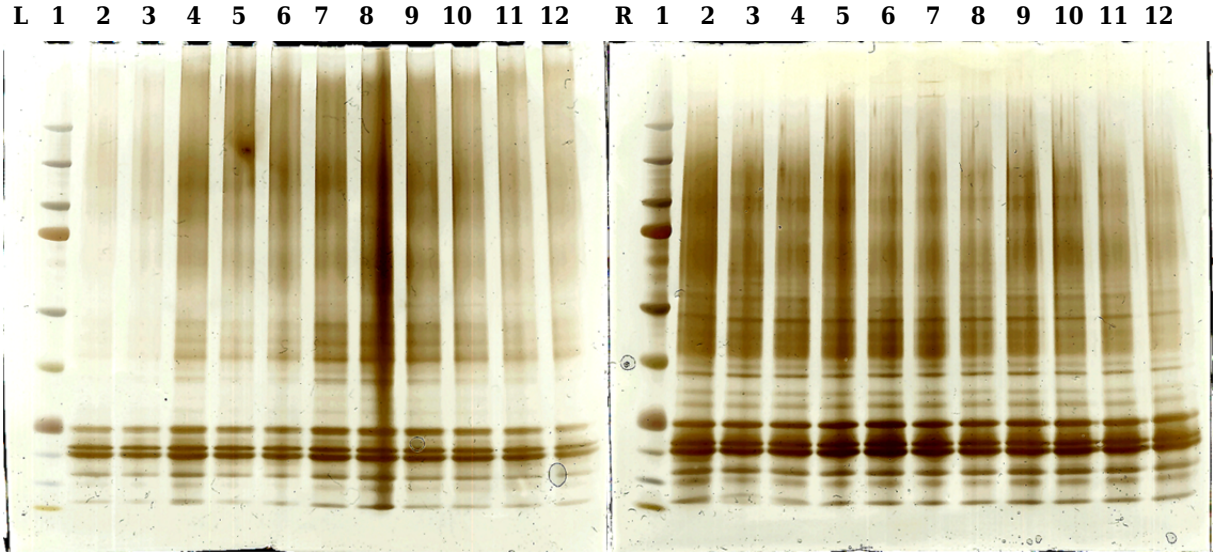
pH 9.5 BLP negative controls run on a Bis-Tris gel with MOPS running buffer under (left) normal and (right) reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).

Lanes: Molecular weight marker [1] 2 µl

Kyn pH 9.5 BLP [2] 0 min [3] 15 min [4] 30 min [5] 60 min [6] 120 min

3OHK pH 9.5 BLP [7] 0 min [8] 15 min [9] 30 min [10] 60 min [11] 120 min

D. (3.2.2.4 3OHK)



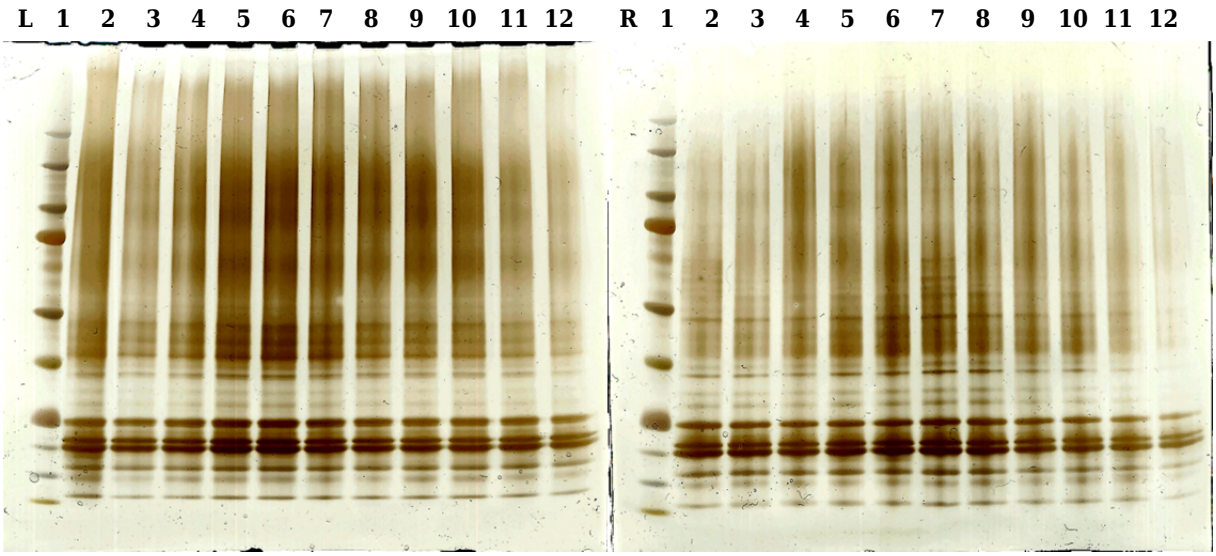
3OHK pH 7.4 BLP run on a Bis-Tris gel with MOPS running buffer under (left) normal and (right) reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).

Lanes: Molecular weight marker [1] 2 µl

Air Bubbled 3OHK [2] 0 min [3] 15 min [4] 30 min [5] 60 min [6] 120 min

Air Open 3OHK [7] 0 min [8] 15 min [9] 30 min [10] 60 min [11] 120 min

N_2 Sealed 3OHK [12] 120 min



3OHK pH 7.4 BLP run on a Bis-Tris gel with MOPS running buffer under (left) normal and (right) reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).

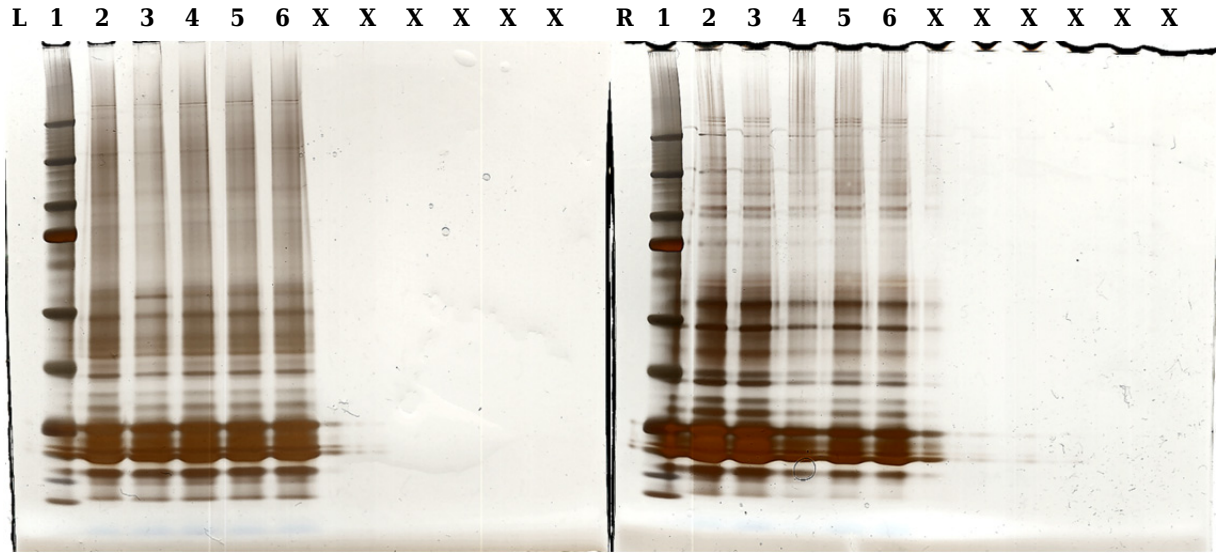
Lanes: Molecular weight marker [1] 2 µl

N_2 Bubbled 3OHK [3] 0 min [4] 15 min [5] 30 min [6] 60 min [7] 120 min

Control 3OHK [8] 0 min [9] 15 min [10] 30 min [11] 60 min [12] 120 min

N_2 Sealed 3OHK [2] 120 min

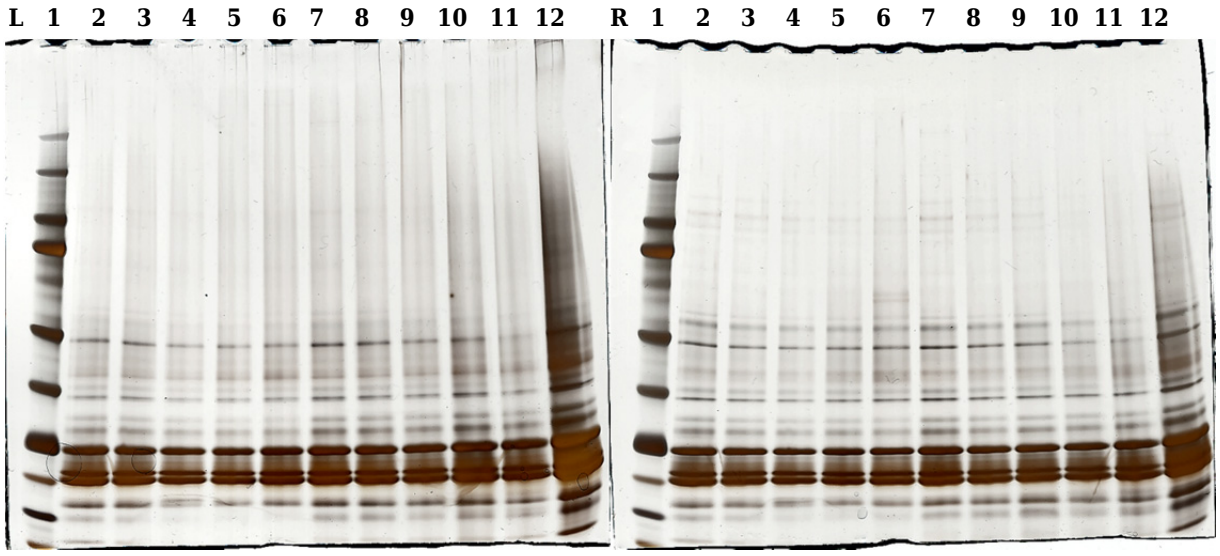
E. (3.2.2.5 3OHKG)



3OHKG pH 9.5 BLP negative controls run on a Bis-Tris gel with MOPS running buffer under (left) normal and (right) reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).

Lanes: Molecular weight marker [1] 2 µl

3OHKG BLP [2] 0 min [3] 15 min [4] 30 min [5] 60 min [6] 120 min



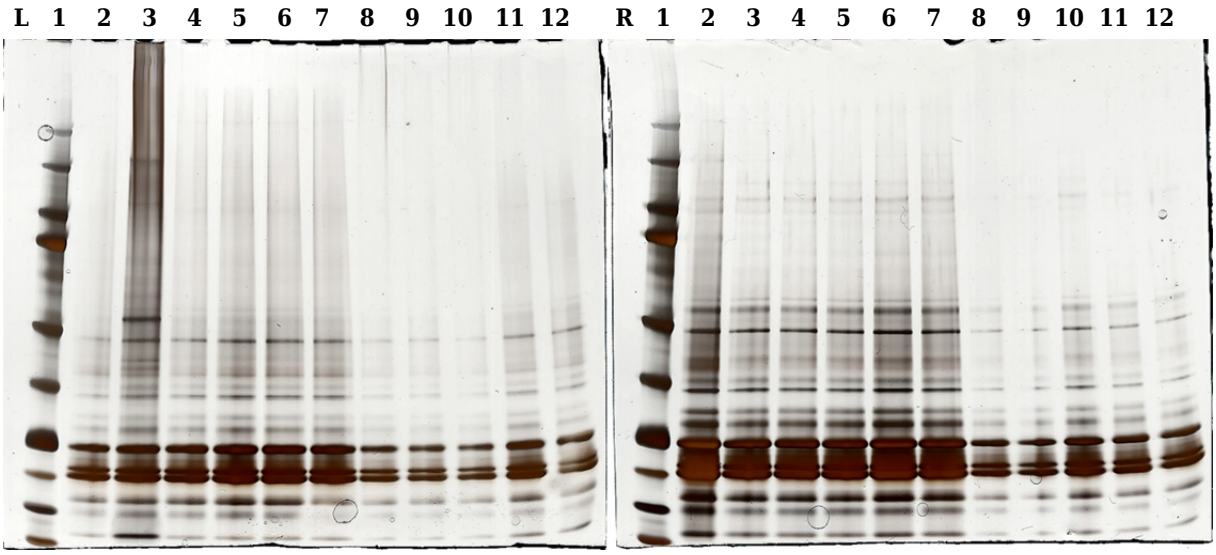
3OHKG pH 7.4 BLP modified samples run on a Bis-Tris gel with MOPS running buffer under (left) normal and (right) reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).

Lanes: Molecular weight marker [1] 2 µl

Air Bubbled [2] 0 min [3] 15 min [4] 30 min [5] 60 min [6] 120 min

Air Open [7] 0 min [8] 15 min [9] 30 min [10] 60 min [11] 120 min

N_2 Sealed [12] 120 min



3OHKG pH 7.4 BLP modified samples run on a Bis-Tris gel with MOPS running buffer under (left) normal and (right) reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).

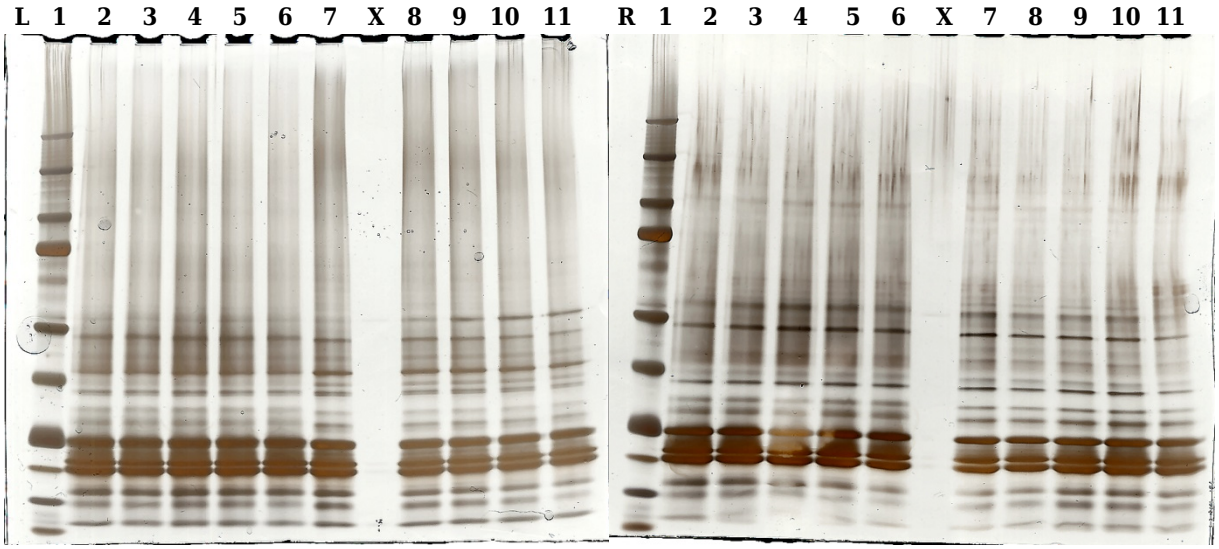
Lanes: Molecular weight marker [1] 2 µl

N_2 Bubbled [2] 0 min [4] 15 min [5] 30 min [6] 60 min [7] 120 min

Control [8] 0 min [9] 15 min [10] 30 min [11] 60 min [12] 120 min

N_2 Sealed (8 µl) [3] right [2] left 120 min

F. (3.2.2.6.1 / 3.2.2.6.2 3OHKG-D and 3OHKG-W)



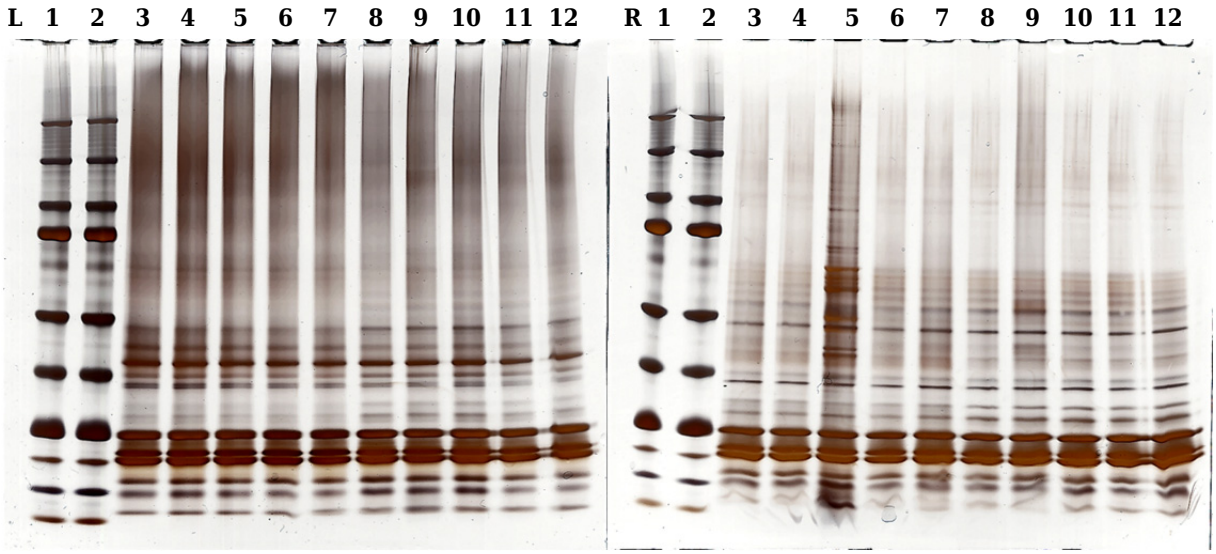
3OHKG-D pH 7.4 and 3OHKG-W pH 9.5 modified BLP negative controls run on a Bis-Tris gel with MOPS running buffer under (left) normal and (right) reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).

Lanes: Molecular weight marker [1] 2 µl

3OHKG-D [2] 0 min [3] 15 min [4] 30 min [5] 60 min [6] 120 min

3OHKG-W [7] 0 min [8] 15 min [9] 30 min [10] 60 min [11] 120 min

G. (3.2.2.6.3 / 3.2.2.6.4 AHB and AHAG)



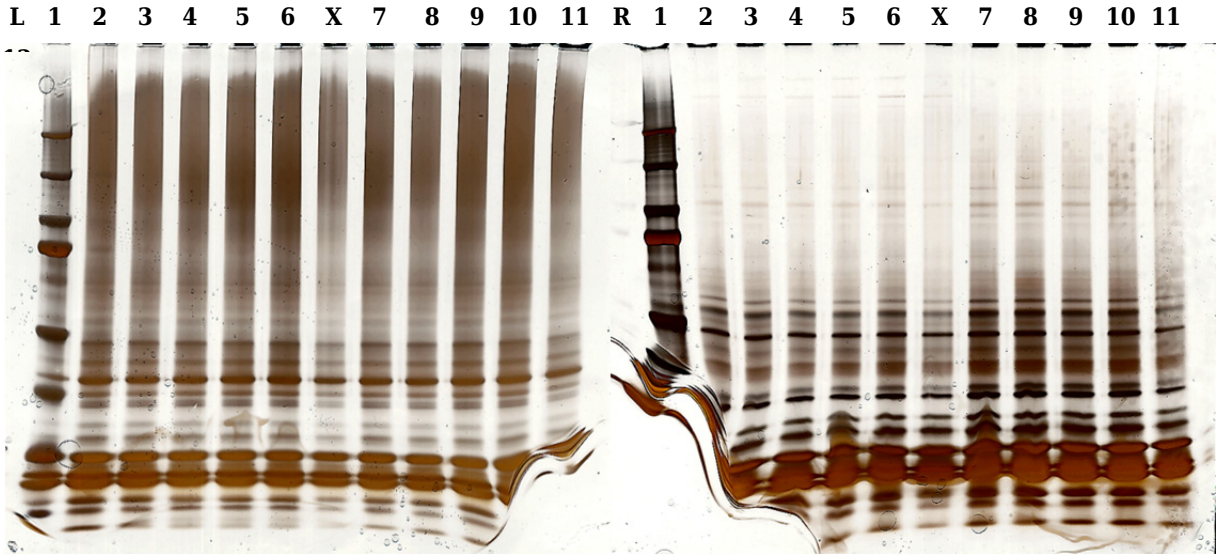
AHB pH 9.5 and AHAG pH 9.5 modified BLP negative controls run on a Bis-Tris gel with MOPS running buffer under (left) normal and (right) reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).

Lanes: Molecular weight marker [1] 2 µl [2] 2 µl

AHB [3] 0 min [4] 15 min [5] 30 min [6] 60 min [7] 120 min

AHAG [8] 0 min [9] 15 min [10] 30 min [11] 60 min [12] 120 min

H. (3.2.2.6.5 3OHKG-Y)

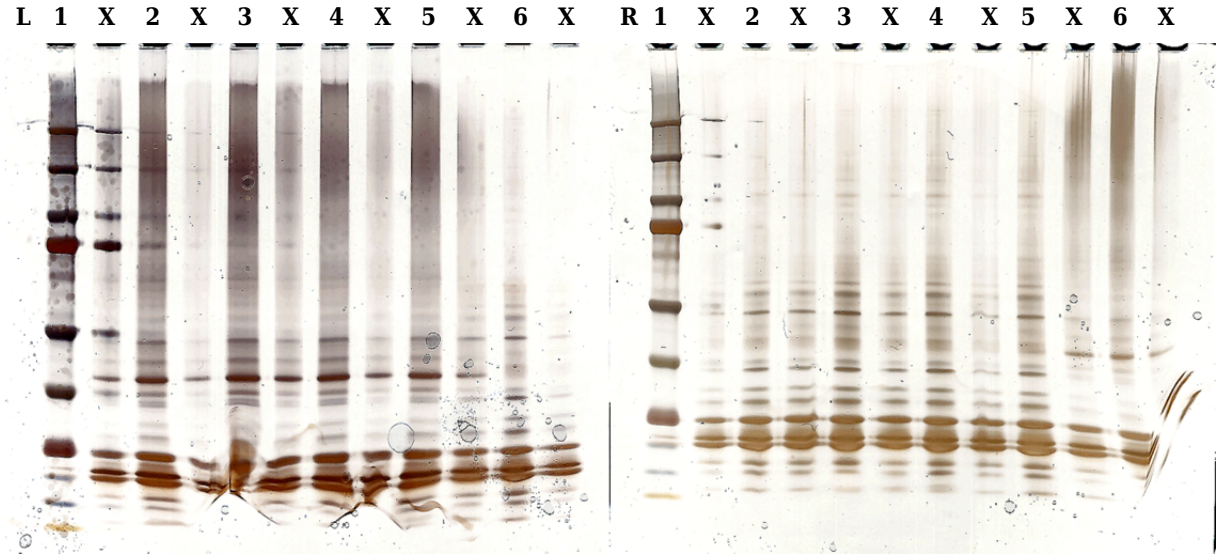


3OHKG-Y pH 9.5 modified BLP irradiated sampled run on a Bis-Tris gel with MOPS running buffer under (left) normal and (right) reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).

Lanes: Molecular weight marker [1] 2 µl

Air Open [2] 0 min [3] 15 min [4] 30 min [5] 60 min [6] 120 min

Air Bubbled [7] 0 min [8] 15 min [9] 30 min [10] 60 min [11] 120 min



3OHKG-Y pH 9.5 negative control BLP irradiated sampled run on a Bis-Tris gel with MOPS running buffer under (left) normal and (right) reducing conditions. Lane X was run empty. All wells were injected with 16 µl of sample (2 µl for molecular weight markers).

Lanes: Molecular weight marker [1] 2 µl

Control [2] 0 min [3] 15 min [4] 30 min [5] 60 min [6] 120 min

I. (4.2.1.3 Amino acid composition)

Amino acid composition of modified/treated BLP *via* Pronase digestion Meaning of abbreviations are given below, last two digits signify pH of modification or treatment. Control BLP were treated with buffer of stated pH for same period as modified BLP. 3K74-2 is a second analysis of 3K74 to observe decomposition whilst waiting for HPLC analysis (12 h delay).

C - Control	**Kyn**	**3K – 3OHK**	**G – 3OHKG**
AHB	**Y – 3OHKG-Y**	**D – 3OHKG-D**	**W – 3OHKG-W**
AHAG	**74 – pH 7.4**	**95 – pH 9.5**	

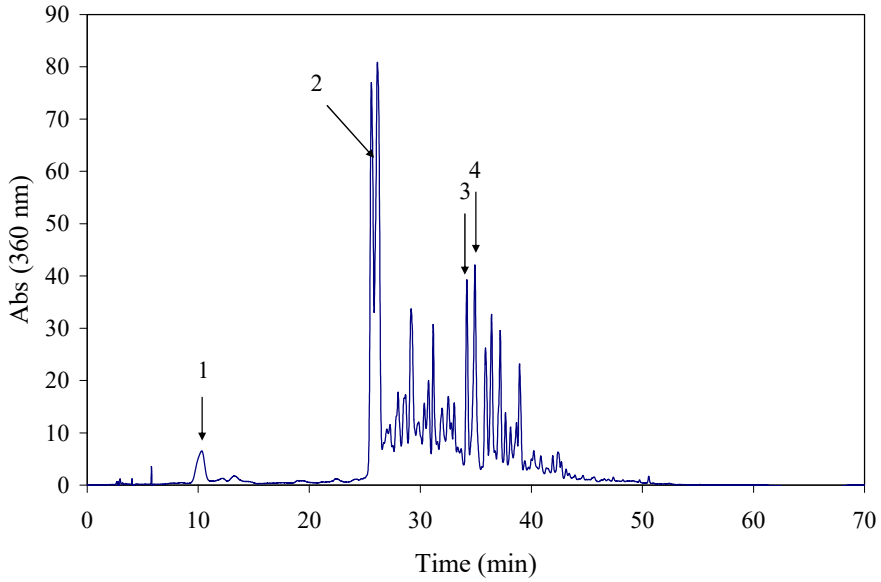
Acid	C74	Kyn74	3K74	G74	AHB74	Y74	D74	3K74-2
Asp	3.2%	3.2%	4.3%	5.3%	4.9%	5.1%	4.9%	3.6%
Glu	7.2%	7.4%	6.9%	8.0%	7.9%	8.0%	7.9%	7.0%
His	4.4%	4.0%	4.5%	5.1%	5.0%	5.1%	5.1%	4.5%
Ser	9.6%	9.5%	9.9%	11.3%	10.1%	11.3%	11.5%	9.5%
Arg	12.5%	12.3%	12.3%	13.9%	13.3%	13.9%	14.2%	12.5%
Gly	1.4%	1.4%	0.8%	1.4%	0.8%	1.5%	1.4%	0.8%
Thr	1.5%	1.3%	1.2%	1.5%	1.3%	1.6%	1.5%	1.2%
Ala	3.3%	3.5%	3.2%	3.2%	3.0%	3.3%	3.2%	3.1%
Tyr	7.4%	8.7%	8.6%	8.2%	8.5%	8.3%	8.4%	9.8%
Trp	3.2%	3.1%	2.5%	2.6%	2.7%	2.6%	2.4%	2.5%
Met	8.0%	7.5%	7.7%	7.1%	7.5%	7.1%	6.6%	7.0%
Val	5.9%	5.8%	5.9%	4.9%	5.2%	4.9%	4.9%	5.5%
Phe	8.6%	8.4%	8.9%	7.6%	8.5%	7.7%	7.9%	9.3%
Ile	8.0%	8.0%	7.8%	6.4%	7.2%	6.4%	6.6%	7.6%
Leu	10.3%	10.3%	10.0%	8.1%	8.7%	8.0%	8.1%	10.0%
Lys	5.6%	5.5%	5.5%	5.4%	5.5%	5.4%	5.4%	5.9%
µg	1376.3	1112.5	1104.8	1242.9	872.8	1363.3	1006.0	1151.2
µg/mL	688.2	556.3	552.4	621.4	436.4	681.7	503.0	575.6

C - Control	**Kyn**	**3K – 3OHK**	**G – 3OHKG**
AHB	**Y – 3OHKG-Y**	**D – 3OHKG-D**	**W – 3OHKG-W**
AHAG	**74 – pH 7.4**	**95 – pH 9.5**	

Acid	C95	K95	3K95	G95	AHB95	Y95	W95	AHAG95
Asp	1.2%	2.0%	2.6%	2.5%	3.0%	2.7%	3.0%	2.8%
Glu	3.9%	4.0%	5.4%	5.4%	5.3%	6.2%	6.2%	6.1%
His	3.4%	2.5%	3.4%	3.5%	3.4%	4.0%	4.0%	3.9%
Ser	5.9%	6.6%	7.4%	7.2%	6.7%	7.8%	8.0%	7.9%
Arg	10.5%	10.8%	11.2%	11.3%	10.8%	11.7%	11.8%	11.8%
Gly	0.3%	0.5%	0.0%	0.2%	0.0%	0.2%	0.3%	0.4%
Thr	1.0%	1.0%	0.7%	0.9%	0.6%	0.9%	1.0%	1.0%
Ala	2.1%	2.5%	1.7%	2.1%	1.5%	2.2%	2.4%	2.4%
Tyr	11.4%	10.9%	11.7%	11.4%	12.0%	10.6%	10.1%	10.7%
Trp	3.4%	3.1%	2.8%	3.3%	3.3%	3.3%	3.2%	3.2%
Met	9.7%	9.7%	7.8%	9.1%	9.1%	9.0%	8.9%	8.9%
Val	5.8%	5.8%	5.6%	5.4%	5.3%	5.6%	5.7%	5.6%
Phe	11.7%	11.2%	11.9%	11.2%	12.3%	10.5%	10.3%	10.3%
Ile	10.2%	10.0%	9.6%	9.0%	9.4%	8.8%	8.5%	8.5%
Leu	14.0%	13.9%	13.2%	12.1%	13.1%	10.9%	10.8%	11.0%
Lys	5.4%	5.2%	5.0%	5.5%	4.5%	5.7%	5.7%	5.8%
µg	1047.0	1131.3	763.7	1024.7	770.7	858.5	979.4	1008.9
µg/mL	523.5	565.6	381.8	512.3	385.4	429.3	489.7	504.4

J. (4.2.1.3.4 3OHK)

(A)



(B)

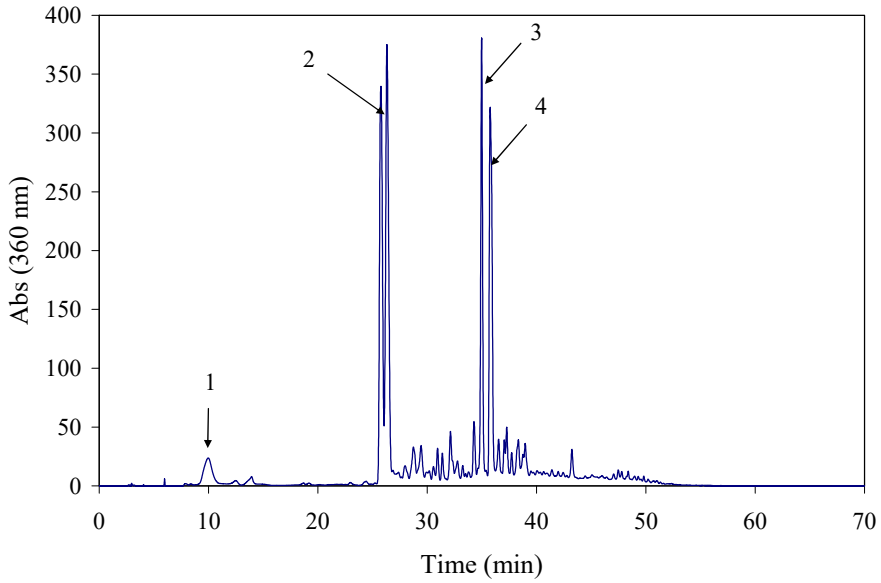


Figure 4.4: RP-HPLC profiles of acid-hydrolysed 3OHKG-treated BLP pH 7.4, 14 days at 37 °C and (B) pH 9.5, 48 h at 37 °C. Marked peaks are (1) Lys-3OHK; (2) diastereomers of Cys-3OHK; Peaks at (3) 35 min and (4) 36 min were not identified (unpublished results). Data and diagram courtesy of Jasminka Mizdrak.

CHAPTER 7

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SUPPLEMENTARY

JOURNAL ARTICLE

Detection, Quantification, and Total Synthesis of Novel 3-Hydroxykynurenine Glucoside–Derived Metabolites Present in Human Lenses

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Purpose. 3-Hydroxykynurenine *O*-β-D-glucoside (3OHKG) protects the lens from UV damage, and novel related species may act analogously. The aim of this study was to detect, quantify, and elucidate the structures of novel 3-hydroxykynurenine glucoside–derived metabolites present in the human lens.

Methods. Compounds were detected and quantified by liquid chromatography with tandem mass spectrometry (LC-MS/MS) in 24 human lenses of different ages, of which 22 were normal and two had cataract. Structures of these were confirmed through total synthesis.

Results. 3OHKG concentrations decreased with age in the lens nuclei, whereas the levels of three novel species, 4-(2-amino-3-hydroxyphenyl)-2-hydroxy-4-oxobutanoic acid *O*-β-D-glucoside (3OHKG-W), 3-hydroxykynurenine *O*-β-D-glucoside yellow (3OHKG-Y), and 2-amino-3-hydroxyacetophenone *O*-β-D-glucoside (AHAG), increased, though to different extents. In contrast, the concentrations present in the cortex of the lens remained constant with age.

Conclusions. Three novel 3OHKG-derived metabolites have been detected in extracts from human lenses.

Keywords: UV filters, tryptophan, photooxidation, age related nuclear cataract

Kynurenine-based compounds derived from tryptophan, which have strong optical absorbance bands in the 300- to 400-nm wavelength range, are present in the human lens and protect this structure from UV-induced damage by acting as physical quenchers of excited state species generated by incident radiation.[1] These compounds, which are collectively known as UV filters and occur predominantly in primates, include the abundant species 3-hydroxykynurenine *O*-β-D-glucoside (3OHKG), 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-β-D-glucoside (AHBG), kynurenine, and 3-hydroxykynurenine.[2,3] 3OHKG, 3-hydroxykynurenine, and kynurenine can deaminate at physiological pH to form reactive α,β-unsaturated carbonyls.[4] The deaminated form of 3OHKG (3OHKG-D) appears to be a precursor for other UV filters including glutathione and cysteine adducts and the reduced compound AHBG.[5,6] The deaminated forms of kynurenine and 3-hydroxykynurenine have been reported to form glutathione adducts and reduced compounds that are present at pmol/mg protein levels in the human lens.[7] It has been proposed that these UV filter compounds when bound to lens proteins contribute to protein oxidation and cross-linking, as well as the lens discoloration observed in age-related nuclear (ARN) cataractous lenses.[8]

Herein we describe the identification and quantification of three novel lens metabolites: 4-(2-amino-3-hydroxyphenyl)-2-hydroxy-4-oxobutanoic acid *O*-β-D-glucoside (3OHKG-W), 3-hydroxykynurenine *O*-β-D-glucoside yellow (3OHKG-Y), and 2-amino-3-hydroxyacetophenone *O*-β-D-glucoside (AHAG). 3OHKG-W and 3OHKG-Y are proposed to derive from deaminated 3OHKG and AHAG from cleavage of 3OHKG.

Methods

Materials

Twenty-four human lenses aged from 18 to 84 years were obtained from the Sydney Eye Bank after ethical approval (University of Sydney Human Research Ethics Committee #7292). The nucleus and cortex of each lens was extracted separately, first with absolute ethanol and then 80% ethanol, as described previously.[9] All chemicals used were of analytical reagent grade or higher. Non-HPLC grade organic solvents were distilled prior to use. Trifluoroacetic acid (TFA, >99%) and formic acid (>98%) were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). 3OHKG was synthesized as described previously.[10] Milli-Q water (Millipore, North Ryde, NSW, Australia) was used to prepare all solutions. Thin-layer chromatography (TLC) used reversed phase silica gel 60 RP-18 F254 plates (Merck, Darmstadt, Germany) and a mobile phase of *n*-butanol/acetic acid/water (12:3:5, vol/vol/vol). Plates were sprayed with ninhydrin and visualized using 254- and 365-nm UV light.

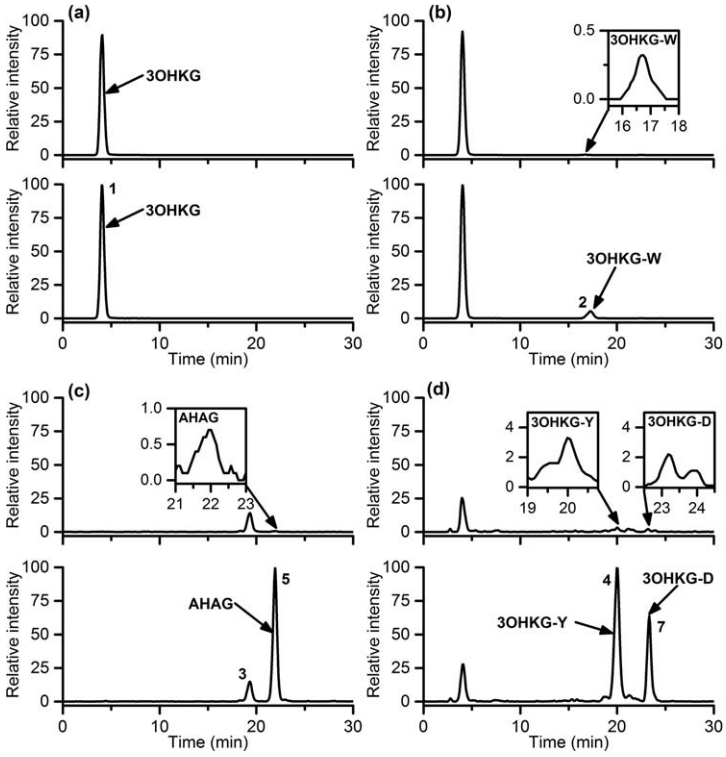


Figure 1. LC-MS chromatograms of novel UV filter metabolites (**a**) 3OHKG, *m/z* 387.1, (**b**) 3OHKG-W, *m/z* 388.1, (**c**) AHAG, *m/z* 314.1, and (**d**) 3OHKG-Y and 3OHKG-D, both *m/z* 370.1, in lens samples that are unspiked (*above*) or spiked with synthetic standards (*below*). Peak numbers correlate with text and Table 1. *Insets* show expanded peaks of interest in unspiked samples.

Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H, ^{13}C, correlation spectroscopy (COSY), HSQC (^{1}H–^{13}C heteronuclear single quantum correlation), and HMBC (^{1}H–^{13}C heteronuclear multiple bond correlation) data were obtained using a Bruker Avance 400 spectrometer (^{1}H, 400 MHz; ^{13}C, 100 MHz; Bruker Biosciences, Preston, VIC, Australia) at 25°C. Compounds were dissolved in D_2O or CD_3OD, with undeuterated residual compounds used as reference. Resonances are quoted in parts per million (ppm), and coupling constants (*J*) are given in Hertz.

Liquid Chromatography–Mass Spectrometry (LC-MS)

Lens extracts (see above) were centrifuged through a 10-kDa cutoff filter to remove high molecular mass compounds, lyophilized, and resuspended in 50 µL 1% (vol/vol) acetonitrile/water. Samples were separated using a Thermo Fischer Scientific (North Ryde, NSW, Australia) Surveyor HPLC system with a Phenomenex (Lane Cove, NSW, Australia) Synergi 4 µm Fusion-RP 80 column (150 × 2.0 mm × 80 Å; 30°C) coupled to a Thermo Fischer Scientific LCQ Deca XP Max ion trap with electrospray ionization (ESI) in positive ion mode. Samples were eluted at a flow rate of 0.2 mL/min using a gradient system with 100% buffer A (5 minutes) before a linear increase to 40% buffer B over 40 minutes, then increasing to 95% B over 2 minutes, washing with 95% B for 1 minute before returning to 100% A over 2 minutes, and re-equilibration with 100% A for 14 minutes (buffer A: 0.05% [vol/vol] TFA in water; buffer B: 100% acetonitrile). Eluted compounds were detected by UV absorbance at 360 nm, and MS selected reaction monitoring (SRM) of expected ion fragments (Table 1). Mass spectrometer conditions were electrospray needle voltage 4.5 kV, sheath gas flow rate 42, sweep gas flow rate 24, and a capillary temperature of 275°C. The sheath and sweep gases were N_2, while the collision gas was helium. High resolution mass spectrometry (HRMS/MS) data were obtained on a Thermo Fischer Scientific LTQ FT Ultra Hybrid Mass Spectrometer (Mark Raftery, University of New South Wales, Sydney, Australia).

UV-Vis Absorbance Spectroscopy

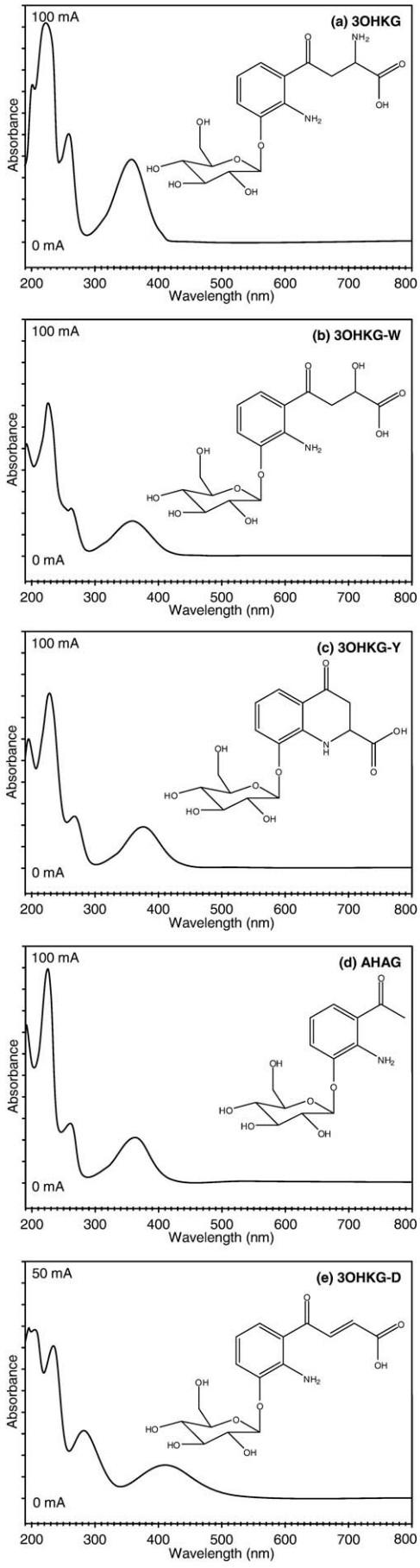
A Shimadzu (Rydalmere, NSW, Australia) SPD-M20A Photodiode array detector was used to record ultraviolet-visible (UV-Vis) spectra.

Synthesis and Characterization of 3OHKG-Y, 3OHKG-W, and 3OHKG-D

3OHKG-Y, 3OHKG-W, and 3OHKG-D were synthesized from 3OHKG using a modified method of Tokuyama et al.[11] 3OHKG (100 mg, 0.26 mmol) was dissolved in $NaHCO_3$ (15 mL, 0.60 M, pH 8.25, sparged with argon), then heated to reflux for 4 hours

Table 1. LC-MS Retention Times, *m/z* M+H$^+$, and *m/z* M+H$^+$ of Fragment Ion Following Loss of Glucose

Peak	Expected Compound	Retention, min	*m/z* M+H$^+$	*m/z* M+H$^+$ -Glucoside
1	3OHKG	4.1	387.1	225.1
2	3OHKG-W	16.7	388.1	226.0
3	Unknown 3	19.3	314.1	152.3
4	3OHKG-Y	20.1	370.1	208.0
5	AHAG	21.9	314.1	152.1
6	AHBG	22.6	372.1	210.0
7	3OHKG-D	23.2	370.1	208.0



under argon in the dark. Reaction progress was monitored by TLC (R_f 0.89, reversed phase [RP], 10% acetonitrile). The solution was acidified to ~pH 6 with 1 M HCl and then lyophilized. Salts were removed using a Waters (Rydalmere, NSW, Australia) C18 RP Sep-Pak column using water/0.05% TFA, followed by 5% acetonitrile/0.05% TFA. Samples were purified by preparative reversed phase HPLC (250 × 21.2 mm × 80 Å Phenomenex RP column) using gradient elution (flow rate 8.0 mL/min) starting at 0% B (10 minutes), 0-40% B (80 minutes), 40-95% (4 minutes), 95% (2 minutes), 95-0% (4 minutes), 0% (30 minutes), with buffers A and B as stated above. During preparative scale HPLC separation, 3OHKG-W eluted as two close adjoining peaks (diastereomers, ~1:1), while 3OHKG-Y eluted as a shouldered peak. ^{1}H and ^{13}C NMR data of the purified 3OHKG-Y confirmed the presence of two diastereomers (1:1). ^{1}H NMR data for 3OHKG-D determined that the *E* isomer was synthesized.

Analytical data (HRMS/MS, ^{1}H and ^{13}C NMR, UV-Vis) for 3OHKG-Y, 3OHKG-W, and 3OHKG-D are reported as Supplementary Material.

Synthesis of AHAG

AHAG was synthesized as described previously,[12] and analytical data are provided as Supplementary Material.

Extraction Efficiency

Method 1 (Wood and Truscott Method). Bovine lens tissue, 100 to 200 mg, was mixed with an equivalent mass of 500 μg/mL aqueous solutions of each of 3OHKG, 3OHKG-W, 3OHKG-Y, and AHAG standards. The mixtures were homogenized and extracted (2 × 150 μL absolute ethanol), then centrifuged, filtered, lyophilized, resuspended into 150 μL 1% (vol/vol) acetonitrile/water, and analyzed by RP LC-MS as described above, with quantification based on UV-Vis absorption of the eluted compounds. All tests were performed in triplicate.[13]

Method 2 (Inoue and Satoh Method). Bovine lens tissue (100–200 mg) was mixed with an equivalent mass of 500 μg/mL aqueous 3OHKG standard. The samples were then extracted with 5% KOH in 80% ethanol/water solution, with aliquots removed at 0, 8, 24, 32, and 48 hours. The aliquots were then centrifuged to remove proteins, neutralized with 1 M HCl, and analyzed by RP LC-MS as described above.[14]

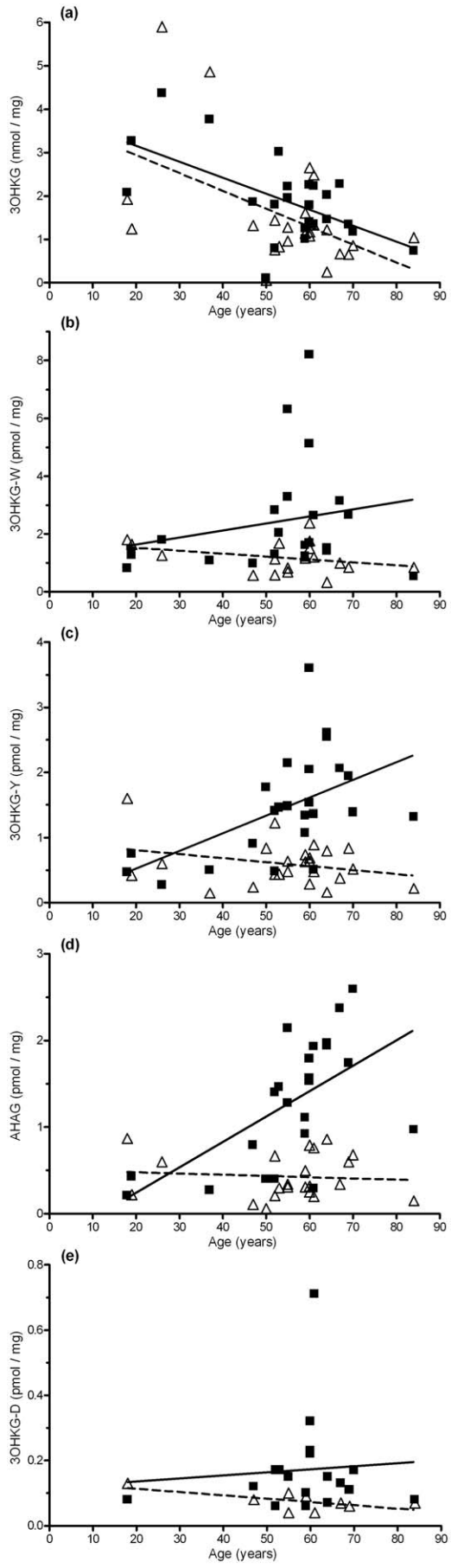
Results

Twenty-four human lenses from donors aged 18 to 84 years were obtained from the Sydney Eye Bank. Twenty-two of these donors had normal lenses (with four being a second lens from the same donor), and two donors had ARN cataract. Each lens was separated into nucleus and cortex, and the UV filters were extracted as described previously.[13]

Mass Spectrometric (LC-MS) Analysis of Lens Extracts

The lens extracts were centrifuged to remove high molecular mass compounds, lyophilized, and resuspended in acetonitrile/water before analysis. Materials present in the extracts were separated and analyzed by RP LC-MS/MS (ESI+) with data for the nuclear and cortex regions of the normal and cataractous lenses examined separately (Fig. 1, Table 1; structures in Figs.

Figure 2. UV-Vis profiles of synthesized standards.



2, 4). ESI analysis in positive mode confirmed the presence of 3OHKG (*m/z* 387.1, peak 1; Fig. 1a), along with protonated molecular ions of *m/z* 388.1 (peak 2; Fig. 1b), *m/z* 314.1 (peak 3; Fig. 1c), *m/z* 370.1 (peak 4; Fig. 1d), *m/z* 314.1 (peak 5; Fig. 1c), *m/z* 372.1 (peak 6; not shown), and *m/z* 370.1 (peak 7; Fig. 1d). These peaks also showed fragment ions consistent with the loss of a glucose residue (162.0 Da).

Peak 1 (3OHKG). Loss of the glucose moiety from 3OHKG (*m/z* 387.1) produced a fragment ion with *m/z* 225.1 and a stronger *m/z* 208.0 fragment due to further loss of ammonia (17.0 Da). The main fragment ion observed at *m/z* 370.1 corresponds to the mass of 3OHKG-Y and 3OHKG-D, which are formed by loss of ammonia (17.0 Da) from 3OHKG. Two minor fragment ions with *m/z* 314.1 (AHAG) and *m/z* 388.1 (3OHKG-W) were also observed.

Peak 2 (3OHKG-W). Peak 2 exhibited a protonated molecular ion of 1 Da greater mass than 3OHKG (*m/z* 388.1), which is consistent with the β-hydroxy analogue of 3OHKG (3OHKG-W). A *m/z* 226.0 fragment ion eventuates upon loss of the glucoside, with a weaker-intensity *m/z* 208.0 fragment ion due to a further loss of the water.

Peaks 3 and 5 (Unknown 3 and AHAG). Two peaks with *m/z* 314.1 (peaks 3 and 5) were observed, each having a mass consistent with the cleavage of the 3OHKG side chain at carbon 3 via a reverse Aldol reaction. Both peaks also displayed a fragment ion with *m/z* 152.3. Spiking of the samples with authentic AHAG confirmed that AHAG coeluted at peak 5. The species corresponding to peak 3 (Unknown 3) gave a stronger signal for a *m/z* 152.3 fragment ion than AHAG, but displayed no UV absorption at 360 nm. Thus, peak 3 is believed to correspond to either a contaminant or another small molecule present in the human lens that does not act as a UV filter compound.

Peaks 4 and 7 (3OHKG-Y and 3OHKG-D). The observation of two species with *m/z* 370.1 (peaks 4 and 7) is consistent with the presence of the deaminated 3OHKG-D species. 3OHKG-D (assigned to peak 7 through spiking experiments) can isomerize through a ring-closing Michael addition, resulting in peak 4 *m/z* 370.1 (3OHKG-Y). Both compounds gave a fragment ion with *m/z* 208.0.

3OHKG-Y was differentiated from 3OHKG-D as it coeluted with a compound having *m/z* 324.1, consistent with thermal loss of formic acid (46.0 Da) from 3OHKG-Y in the heated source of the mass spectrometer. In contrast, 3OHKG-D does not readily lose its carboxylic acid and hence does not exhibit a coeluting species corresponding to this thermal decomposition.

Peak 6 (AHBG). Peak 6 (not shown) had a mass (*m/z* 372.1) and UV profile consistent with the structure of the known UV filter AHBG, with a fragment ion of *m/z* 210.0 due to the loss of the glucoside.

Total Synthesis of Novel UV Filter Metabolites

As the quantities of the lens UV filter metabolites were too low for unambiguous structure elucidation by NMR, the proposed compounds 3OHKG-W, 3OHKG-Y, and AHAG were synthesized for comparison with the lens-derived compounds (Fig. 2). The structures of the synthesized compounds were confirmed by NMR, HRMS, and UV-Vis spectroscopy and were consistent with the above LC-MS structural assignments. Although UV-Vis spectra could not be obtained for these compounds from the

FIGURE 3. Correlation plots of the concentration of novel metabolites in the nucleus (■, *solid line*) and cortex (Δ, *dashed line*) of human lenses with age of the donor.

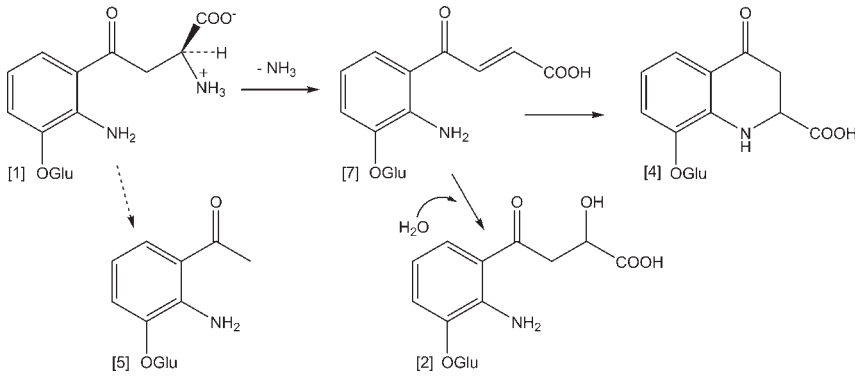


Figure 4. Proposed mode of formation of 3OHKG-W [2], 3OHKG-Y [4], AHAG [5], and 3OHKG-D [7] from 3OHKG [1]. Compound numbers correlate with peak numbers, text, and Table 1.

lens samples, due to their low concentrations, such data were obtained for synthetic 3OHKG-W, 3OHKG-Y, and AHAG, with each of these having significant absorption maxima in the 300- to 420-nm region. The presence of 3OHKG-D in cataract human lenses has been reported previously.[15]

Quantification of UV Filter Metabolites in Human Lenses

The three new lens metabolites (3OHKG-Y, 3OHKG-W, and AHAG) and two known metabolites (3OHKG and 3OHKG-D) were quantified using LC-MS/MS detection.

The compounds were detected by SRM of the loss of the glucoside ($[M+H]^+ \rightarrow [M\text{-glucoside}+H]^+$), and the peak intensities were compared to standard curves generated from the synthetic samples.

Normal Lenses. All of the new metabolites were found in normal lenses, with 3OHKG-Y, 3OHKG-W, and AHAG being present at concentrations in the pmol/mg of dry protein range (Table 2). The concentrations of these compounds were correlated with the age of the lens (Fig. 3). In the normal nucleus, 3OHKG decreased in concentration with age of the lens in a statistically significant manner (Table 2). The concentration of 3OHKG approximately halved in the nucleus of lenses aged from 18 to 84 years, consistent with previous reports.[8,16] The other compounds examined increased significantly in concentration with lens age, with the greatest increase occurring with AHAG and 3OHKG-Y. 3OHKG-W showed a trend toward an increase with age, but this was not statistically significant. 3OHKG-D was detected in low concentrations in some but not all lenses, with no statistically significant changes in concentration detected.

In the normal lens cortex samples, the concentrations of most of the tested compounds did not change in a statistically significant manner. 3OHKG was an exception that showed a decrease with age, though these levels varied markedly, particularly in the younger lens samples.

Cataract Lenses. Two cataract lenses were also examined; larger numbers of intact cataract lenses were unavailable due to changes in clinical management and practice. These cataract lenses were found to have similar concentrations of the various compounds in both the nucleus and the cortex. Low levels of 3OHKG were detected in the lens nuclei; these values were 32% of the average 3OHKG concentration of normal lenses (2.01 nmol/mg normal lens protein versus 0.64 nmol/mg cataract lens protein), while the 3OHKG concentrations detected in cataract cortex were 28% of those detected in normal lens cortex (1.63 nmol/mg normal lens protein versus 0.46 nmol/mg cataract lens protein). 3OHKG-Y and AHAG concentrations in cataract lenses were similar to those of normal lenses. For both cataract lenses, the concentration of 3OHKG-W was close to the detection limit and therefore not quantified. 3OHKG-D was observed only at a low level in one of the cataract lenses.

Extraction Efficiency

The levels of the UV filters and UV filter-derived compounds reported here are dependent on the efficient extraction of these metabolites from the lens samples. This was therefore assessed by two independent methods using bovine lens tissue extracted with either ethanol (method 1) or 5% KOH in 80% ethanol/water (method 2).

Method 1 (Wood and Truscott Method[13]). Extraction efficiencies for 3OHKG, AHAG, 3OHKG-Y, and 3OHKG-W were determined; data for 3OHKG-D were not obtained as this proved to be too unstable for quantification. Reversed phase LC-MS data for 3OHKG did not show any evidence for breakdown and conversion to other UV filters (such as AHAG), and an extraction efficiency of 57 ± 4% (mean ± standard deviation) was determined. For the other species, the recovery efficiencies were AHAG, 79 ± 15%; 3OHKG-Y, 68 ± 14%; and 3OHKG-W, 41 ± 12%. These data indicate that the detection of these species in the lens samples is not a result of artifacts arising from the extraction method. All data presented (except for 3OHKG-D) in Figure 3 have been corrected with the corresponding average extraction efficiency.

Method 2 (Inoue and Satoh Method[14]). Under the basic conditions of this extraction method, the 3OHKG concentration decreased and that of AHAG increased, over time. After 48 hours, ~10% of the starting 3OHKG was still detectable by RP LC-MS, with AHAG the only other detectable product. These data indicate that 3OHKG can be converted to AHAG under basic conditions but that this is a slow process.

Discussion

Three novel lens metabolites, 3OHKG-W, 3OHKG-Y, and AHAG, were identified and quantified in human lenses varying in age from 18 to 84 years. The synthetic compounds had high-resolution masses and fragmentation patterns consistent with the structures postulated for the materials identified in the lens extracts. The UV-Vis spectra determined for the synthetic compounds were similar to those for known UV filter compounds.[15]

In the nucleus all three metabolites increased in quantity with age, with AHAG showing a 6.5-fold increase between ages ~20 and ~68 (0.42–2.71 pmol/mg lens protein). 3OHKG-Y showed a 3.3-fold increase over the same age period (0.69–2.26

Table 2. Concentrations of Novel and Known Lens Metabolites in Human Lens Nucleus and Cortex Extracts, and Correlation of Data With Age

Compound	Concentration Range	Pearson Coefficient	*P* (2-Tailed)	Significance
Nucleus				
3OHKG	0.09–4.16 nmol/mg	−0.5960	0.0021	Yes
3OHKG-W	0.36–5.60 pmol/mg	0.2059	0.3705	No
3OHKG-Y	0.31–4.08 pmol/mg	0.5336	0.0072	Yes
AHAG	0.28–3.41 pmol/mg	0.5997	0.0025	Yes
3OHKG-D	0.06–0.71 pmol/mg	0.0806	0.7505	No
Cortex				
3OHKG	0.05–5.61 nmol/mg	−0.4867	0.0159	Yes
3OHKG-W	0.23–1.63 pmol/mg	−0.3233	0.1778	No
3OHKG-Y	0.18–1.81 pmol/mg	−0.2843	0.1758	No
AHAG	0.08–1.14 pmol/mg	−0.0886	0.6965	No
3OHKG-D	0.04–0.13 pmol/mg	−0.6777	0.0654	No

pmol/mg lens protein), and 3OHKG-W increased 2.8-fold (0.71–1.98 pmol/mg lens protein). The only compound that decreased in concentration over the same age period was 3OHKG (2.54–1.72 nmol/mg protein). This decrease in 3OHKG concentration levels is consistent with other reports.[17] 3OHKG-Y and 3OHKG-W are postulated to arise from a common intermediate, 3OHKG-D ([7] in Fig. 4). Both 3OHKG-Y and 3OHKG-W were found to increase in concentration with age, though 3OHKG-W is found at a higher average concentration in young lenses. In contrast, 3OHKG-Y was present at higher levels in old lenses (>57 years of age). These differences with age were not observed in the lens cortex, where the concentrations of 3OHKG-W, 3OHKG-Y, and AHAG decreased moderately with age. Overall, concentrations of each compound in the cortex were, in general, 2- to 3-fold lower than in the nucleus, and these levels did not vary dramatically with age, with the concentrations remaining at the levels detected in young lenses.

AHAG is postulated to arise from a reverse Aldol reaction of 3OHKG, via cleavage of the amino acid side chain between the second and third carbon atoms.[18] AHAG has been reported previously as a UV filter present in cataract lenses; however, the detection of this species may be due, at least in part, to the basic extraction conditions used (5% KOH in 80% ethanol), which accelerate side-chain cleavage in 3OHKG. Thus the extraction method used previously[14] results in diminishing levels of 3OHKG over time, with almost complete conversion to AHAG after 48 hours. In the current study we provide data consistent with the presence of AHAG in normal and cataract lenses, as the extraction method employed did not give rise to any detectable artifactual generation of AHAG from 3OHKG. In contrast to the other novel lens metabolites reported here, AHAG appears to be relatively stable; nevertheless, the concentrations detected in the lens were low, likely due to the deamination of 3OHKG as a more favored reaction than the reverse Aldol reaction that yields AHAG. 3OHKG-D was observed in only some of the normal lens samples, both in the nucleus and cortex, and only in very low quantities in one of the cataract lenses tested. No significant changes in the concentration of 3OHKG-D over time were detected in the normal lenses. This is consistent with the observation that 3OHKG-D is highly reactive, thereby precluding its accumulation. Likewise, in having an absorbance maxima at 409 nm, 3OHKG-D mainly absorbs blue light. This may be another factor contributing to its greater instability.

While 3OHKG-W, 3OHKG-Y, and AHAG all have absorption maxima in the 300- to 420-nm region, they are present in low concentrations compared to the known lens UV filters. Additionally, along with 3OHKG-D being unstable, there is evidence that 3OHKG-Y is photochemically active, with Zelentsova et al.[19] showing that the structurally similar kynurenine yellow is reactive upon UV irradiation. Thus, 3OHKG-W, 3OHKG-Y, and AHAG most likely do not function as significant UV filter compounds in the lens, but are intermediate products of 3OHKG decomposition.

In conclusion, three novel lens metabolites, 3OHKG-Y, 3OHKG-W, and AHAG, have been identified and quantified in 24 human lenses. They are proposed to be derived from the major UV filter 3OHKG. These compounds were present at pmol/mg dry lens protein concentrations and at lower levels than 3OHKG (which is present at nmol/mg lens protein).[17] Although these metabolites are present at low concentrations, the data obtained are consistent with their presence in both normal and cataractous lenses. The concentrations of these compounds may reflect their reactivity when compared to other species, with this potentially resulting in additional products and/or binding to lens proteins. In the nucleus, 3OHKG-Y and AHAG increased in concentration with age, and higher levels were detected in cataract lenses, though additional lens analyses are required to confirm the latter observation. These metabolites, with the exception of 3OHKG-D, have UV absorbance profiles similar to those of the abundant UV filter compound 3OHKG and other known UV filters.[13] The findings of these novel 3OHKG-derived lens metabolites extends our understanding of the chemistry of the human lens, especially with regard to the major human lens UV filter compound, 3OHKG, and of how the concentrations of these novel species alter with both subject age and the presence of cataract.

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