BIOPHYSICAL AND BIOCHEMICAL STUDIES ON CATARACT OF THE EYE LENS

Thesis submitted for the degree of
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By

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Dedicated to my beloved parents

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DECLARATION

The research work embodied in this thesis entitled, **"Biophysical and Biochemical studies on cataract of the eye lens**", has been carried out by me at the L. V. Prasad Eye Institute, Hyderabad, under the guidance of Profs. D. Balasubramanian and T. Suryanarayana. I hereby declare that this work is original and has not been submitted in part or full for any other degree or diploma of any other university.

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CERTIFICATE

This is to certify that this thesis entitled, **"Biophysical and Biochemical Studies on cataract of the eye lens",** submitted by Ms. Geetha Thiagarajan for the degree of **Doctor of Philosophy** to the University of Hyderabad is based on the work carried out by her at the L. V. Prasad Eye Institute, Hyderabad, under our supervision. This work has not been submitted for any diploma or degree of any other University or Institution.

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CONTENTS

Chapter 1: INTRODUCTION 1

Chapter 2: ROLE OF XANTHURENIC ACID 8-O-β-D-GLUCOSIDE IN THE LENS 16

2.1.0. Materials and Methods 22
2.1.1. Chemical synthesis of XA8OG and 3-HKG 22
2.1.2. Fluorescence measurements 22
2.1.3. Singlet oxygen generation assay 23
2.1.4. Superoxide generation assay 23
2.1.5. Electrophoresis (SDS/PAGE) 24
2.1.6. XA8OG and Apoptosis 24
2.2.0. Results and Discussion 25

Chapter 3: REACTION OF PEROXYNITRITE WITH LENS CRYSTALLINS 29

3.1.0. Materials and Methods 32
3.1.1. Purification of lens proteins 32
3.1.2. Preparation of peroxynitrite 32
3.1.3. Peroxynitrite modification of test proteins 32
3.1.4. SDS/PAGE 33
3.1.5. Estimation of nitrotyrosine and nitrotryptophan 33
3.1.6. Spectral studies 33
3.1.7. Functional studies - assay for protein aggregation 34
3.1.8. Trypsin digestion of human αA-crystallin 35
3.1.9. HPLC of tryptic digest of αA-crystallin 35
3.2.0. Results and Discussion 37

Chapter 4: ROLE OF ANTIOXIDANTS IN DELAYING ONSET OF CATARACT 48

4.1.0. Materials and Methods 57
4.1.1. Preparation of extracts 57
4.1.2. ABTS antioxidant assay 57
4.1.3. Protein oxidation 58
4.1.4. Single cell gel electrophoresis 59
4.1.5. Assay of cytoprotective ability 60
4.1.6. Phase separation model of cataract (cold cataract) 61
4.2.0. Results and Discussion 62

Chapter 5: CLONING AND EXPRESSION OF MUTANT PA3/A1-CRYSTALLIN IN *E. COLI* 69

5.1.0. Materials and Methods 75
5.1.1. Cloning of mutant βA3/A1-crystallin cDNA 76
5.1.2. Overexpression of mutant βA3/A1-crystallin 76
5.1.3. Extraction of mutant βA3/A1-crystallin 77
5.1.4. Solubilization of mutant (3A3/A1-crystallin from inclusion bodies 78
5.1.5. Folding of mutant βA3/A1-crystallin 79
5.1.6. Purification of mutant βA3/A1-crystallin 79
5.2.0. Results and Discussion 80

SUMMARY **86**

REFERENCES **89**

PUBLICATIONS 125

ABBREVIATIONS

λ:	Wavelength of light, in nm
μM :	Micromolar
μm:	Micrometer
ε_M:	Molar extinction coefficient
μs:	Microsecond
$^1O_2$:	Singlet oxygen
3-HK:	3-Hydroxykynurenine
3-HKG:	3-Hydroxykynurenine glucoside
ABTS:	Azinobis-(3-ethylbenzothiazoline)-6-sulfonic acid
AGE:	Advanced glycation end products
AHBG:	4-(2-amino 3-hydroxyphenyl)-4-oxobutanoic acid O-ß -D-glucoside
ANS:	8-anilinonaphthalene 1-sulfonic acid
AR:	Aldose reductase
AREDS:	Age-related Eye Disease study
BPTI :	Bovine pancreatic trypsin inhibitor
CCRG:	Co-operative Cataract Research Group
CD:	Circular dichroism
cDNA:	Complementary DNA
cM:	centimorgan
DAPI:	4'-6-Diamidino-2-phenylindole
DEAE:	Diethylaminoethyl
DMEM:	Dulbecco's modified eagle's medium
DNase I:	Deoxyribonuclease I
DTT:	Dithiothreitol
EDTA:	Ethylenediaminetetraaceticacid
FCS:	Fetal calf serum
GBE:	*Ginkgo biloba extract*
GPx:	Glutathione peroxidase
GR:	Glutathione Reductase
GSH:	Glutathione

HEPES:	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HGQ:	4-(hydroxy 3-glycine)-quinoline
HLE:	Human lens epithelial cell line, SRA 01/04
HSF:	Heat shock factor
Hsp:	Heat shock protein
IPTG:	Isopropyl-β-D-thiogalactopyranoside
kcal/m:	kilocalories per mole
kcal:	Kilocalorie
kDa:	Kilodalton
KUA:	Kynurenic acid
Ky:	Kynurenine
LB:	Luria-Bertani broth
LOCS:	Lens Opacities Case-control Study
MALDI/MS:	Matrix assisted laser desorption ionisation / mass Spectrometry
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
mW:	Milliwatt
NFK:	N-formylkynurenine
NMR:	Nuclear magnetic resonance
ns:	Nanosecond
$O_2^{-\bullet}$:	Superoxide anion radical
OD:	Optical density
OXA:	Oxoxanthurenic acid
PAGE:	Polyacrylamide gel electrophoresis
PBS:	Phosphate buffered saline
PMSF:	Phenylmethylsulfonylfluoride
PON:	Peroxynitrite
ps:	Picosecond
QA:	Quinaldic acid
RF:	Riboflavin
RNase A:	Bovine pancreatic ribonuclease A
RNO:	N, N-dimethyl-4-nitrosoaniline
RP-HPLC:	Reverse-phase high performance liquid chromatography

SDS:	Sodium dodecylsulphate
SIRC:	Rabbit corneal keratocyte cell line
SOD:	Superoxide dismutase
Tc:	Phase transition temperature
TFA:	Trifluoroacetic acid
Tris:	Tris(hydroxymethyl)aminomethane
WSE:	Withania somnifera extract
XA:	Xanthurenic acid
XA80G:	Xanthurenic acid 8-O-β-D-glucoside

Chapter 1

1.0.0. INTRODUCTION

1.0.1. THE EYE LENS

The lens is a transparent, highly refractive structure located between the pupillary portion of the iris and the vitreous. It is held in place by zonular fibers that extend vertically across either side from the lens to the ciliary body, and by the vitreous posteriorly. The lens is enclosed by a thick non-cellular, optically clear basement membrane called 'capsule' to which the zonular fibers are attached. Figure 1.1 illustrates the structure of the mammalian eye.

The lens continues to increase in volume, weight and size throughout life. The dimensions of the lens at birth are about 3.5 mm anterior to posterior with an equatorial diameter of about 6.5 mm. It increases to 5 x 9 mm in the adult eye. The weight grows from 65 mg at birth to 130 mg at one year of age and slowly rises thereafter to about 250 mg at 90 years (Duke-Elder and Cook, 1963; Duke-Elder, 1969). The lens is also known to thicken by 0.023 mm/year.

The function of the lens is to transmit light and focus the image onto the retina. An important function of the lens is termed accommodation. This refers to the property of the lens to rapidly change its shape and thus the refractive index, so as to change the focal length in a manner that aids the falling of the image on the

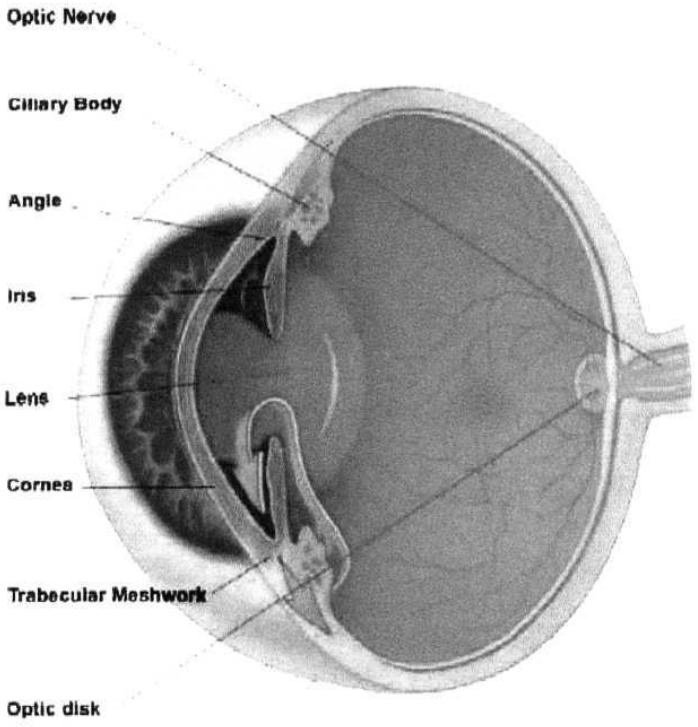


Figure 1.1. Structure of the eye

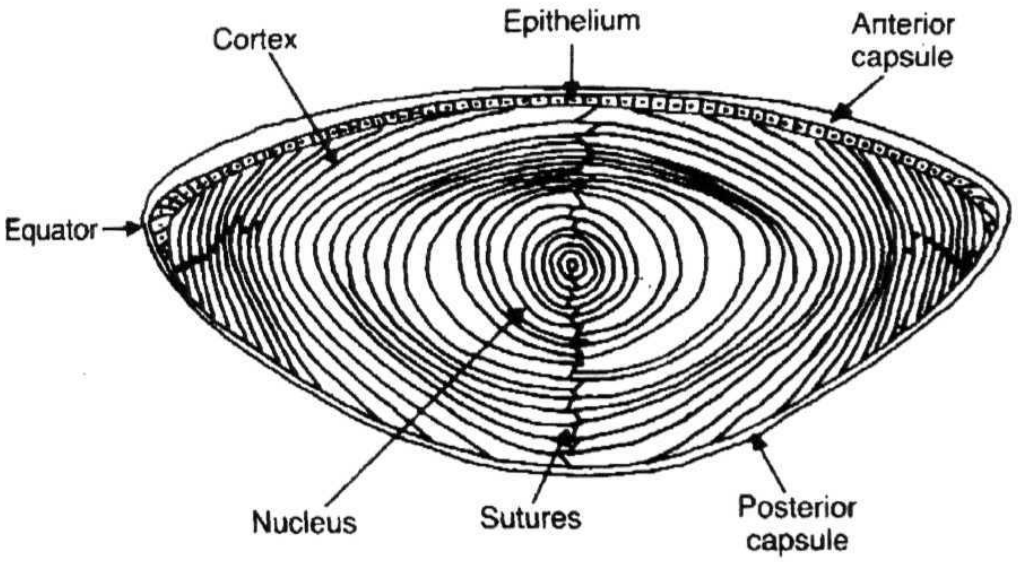


Figure 1.2. Diagramatic section through lens
Reproduced from *Cataract: Biochemistry, epidemiology and pharmacology,* by Harding (1991).

retina. The lens is thus a plastic (deformable) and an elastic (regain its original prolate ellipsoidal form once the pressure is removed) body. The lens contains a single layer of actively dividing epithelial cells, which lies immediately behind the anterior capsule. These epithelial cells divide and move towards the equator where they elongate and form fiber cells. To remain scatter-free, the lens lacks blood vessels, lymphatics and nerves. During the process of differentiation, these epithelial cells lose organelles like mitochondria, ribosomes and nuclei thereby eliminating any structural interference with transmission of light. The fiber cells extend from the front of the lens curving round to the back of the lens to meet in a region called the lens sutures (Fig. 1.2).

As the lens grows and the epithelial cells continue to divide and differentiate, previously formed fibers get progressively more internalized. Since the lens never sheds its cells, the oldest fibers are always found towards the center of the lens (called nucleus) and younger, newly formed fibers toward the periphery (called cortex). As the protein turnover in the lens is negligible, the adult lens contains a complete historical record of the changes that have occurred during growth and development. The lens is essentially a gel with high concentration of soluble proteins, with the nucleus having approximately 500 mg/ml of protein, while the cortex contains lower amounts (150-200 mg/ml) (van Heyningen, 1977). It is this gel nature that makes the lens both plastic and elastic.

1.0.2. LENS PROTEINS - CRYSTALLINS

The lens contains about 35% by weight structural proteins, of which crystallins constitute the bulk of water-soluble proteins of the lens. There are three main classes of crystallins in the mammalian lens, a-, β-, and γ-crystallins. α-Crystallin is an oligomer of about 800 kDa with an average monomeric molecular weight of about 20 kDa (van der Ouderaa *et al.,* 1974a; van der Ouderaa *et al.,* 1974b). a-Crystallin includes 2 subunits, αA and αB, which are two different gene products. The molecular weight of the A chain is 19.5 kDa and of the B chain 22.5 kDa (Spector *et al.,* 1971). β-Crystallin also exists as a multimer with a molecular weight between 40 and 200 kDa. γ-Crystallin is a monomer with a molecular weight of 20 kDa. y-Crystallin is expressed more during early development and is therefore concentrated towards the center/nucleus of the lens while a- and β-crystallins are present in higher ratios towards the cortex of the lens.

In the bovine lens, the three crystallins are represented in the overall ratio of 40 (a): 40 ((3): 20 (y) (van Kamp and Hoenders, 1973). These crystallins exist within the fiber cells in a specific arrangement characterized by short-range order (Delaye and Tardieu, 1983). The secondary structure of the polypeptide chains of all three crystallins consists almost exclusively of the anti-parallel [3-sheet conformation (Horwitz, 1976; Horwitz *et al.,* 1977; Chiou *et al.,* 1979).

Since there is a large difference in the sizes of the three different classes of crystallins, they are easily separated by gel filtration on materials like Sephadex G-200 or BioGel-A 1.5 m. a-Crystallin elutes first followed by β-crystallin and then γ-crystallin. Both α- and β-crystallins elute in the form of two species, α_L-crystallin / β_L-crystallin (comprises mainly dimers and trimers) α_H-crystallin / β_H-crystallin (contains hexamers and higher order multimers). Figure 1.3 shows the elution profile of bovine lens crystallins on Sephadex G-200.

The [3- and y-crystallins possess a high degree of homology between themselves, and both these proteins are included in what is called the p-y superfamily of proteins (Driessen *et al.,* 1981; Blundell *et al.,* 1981). All the p- and y-crystallin polypeptides fold into two similar domains and each domain folds into two similar Greek-key motifs. In β-crystallin genes the motifs are encoded in separate exons while in y-crystallins, exons code for whole domains (Lubsen *et al.,* 1998). Each Greek-key motif consists of around 40 amino acid residues that fold into four consecutive anti-parallel beta strands (a, b, c and d) and bears a sequence signature that includes structurally important conserved aromatics at positions 5 and 11, glycine at position 13 and serine at position 34, numbered relative to the first motif of γB-crystallin (Blundell *et al.,* 1981; Wistow *et al.,* 1983; Wistow *et al.,* 1985; Wistow, 1995; Clout *et al.,*

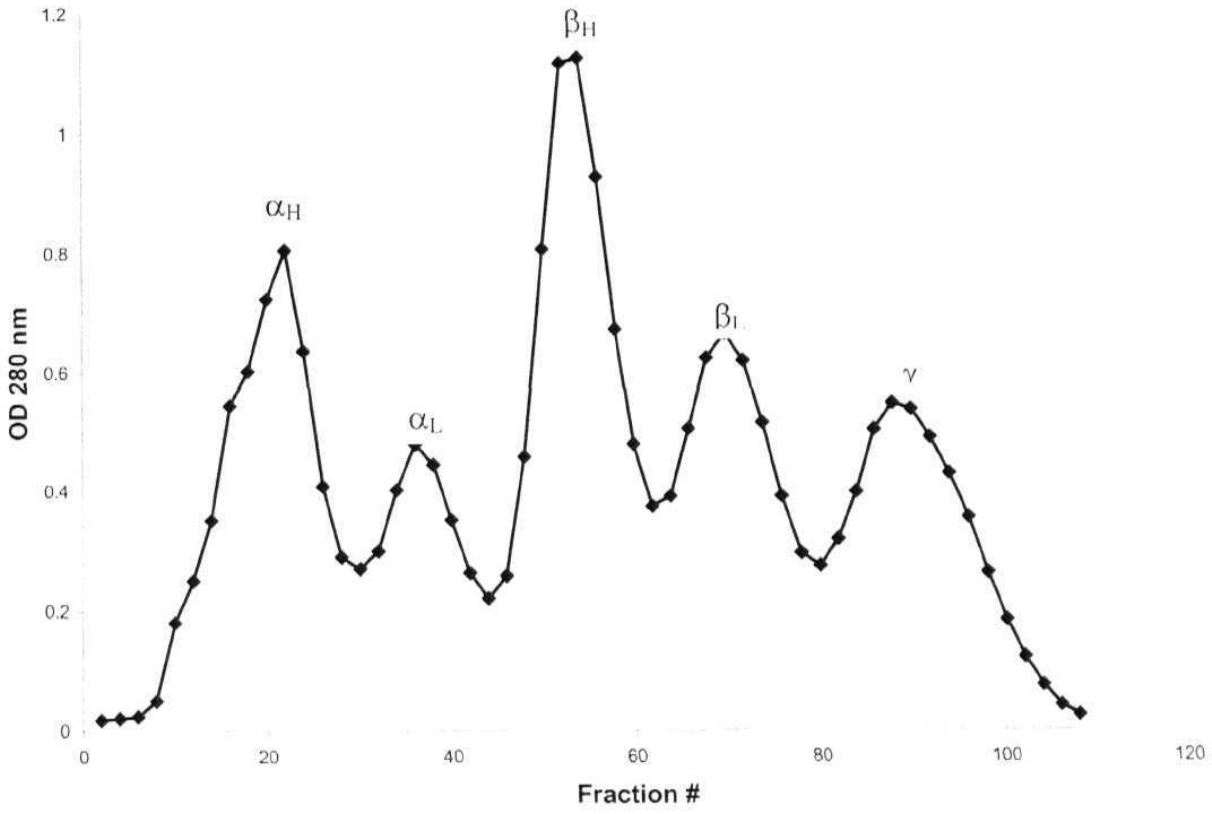


Figure 1.3. Elution profile of bovine lens crystallins from Sephadex G-200

2001; Jaenicke and Slingsby, 2001). Two consecutive Greek-key motifs are intercalated in a symmetrical way about an approximate dyad to form a wedge-shaped p-sheet sandwich filled with hydrophobic side chains (Fig. 1.4).

α-Crystallin, which is the most abundant of lens proteins, shows structural and functional similarities to the group of small heat shock proteins (sHSP) (Ingolia and Craig, 1982). Like HSPs, α-crystallin also displays chaperone-like activity and forms stable complexes with a wide range of chemically or thermally denatured proteins (Horwitz, 1992; Merck *et al.,* 1993; Farahbakhsh *et al.,* 1995; Rao *et al.,* 1995). a-Crystallin is also expressed in non-lenticular tissues like heart, skeletal muscles, brain and kidneys (Bhat and Nagineni, 1989; Iwaki *et al.,* 1989). The p- and y-crystallins act as calcium-binding proteins thereby maintaining calcium homeostasis in the lens (Sharma *et al.,* 1989; Sharma and Balasubramanian, 1996; Rajini *et al.,* 2001).

1.0.2.1. Taxon-specific crystallins

A remarkable phenomenon called 'recruitment' is seen in the lens of many vertebrates (Piatigorsky and Wistow, 1989). Protein sequence comparisons reveal that a number of taxon-specific crystallins are actually enzymes, which have been recruited in the lens to play a structural role. δ-Crystallin, found in the lenses of birds and reptiles, is a modified form of the enzyme

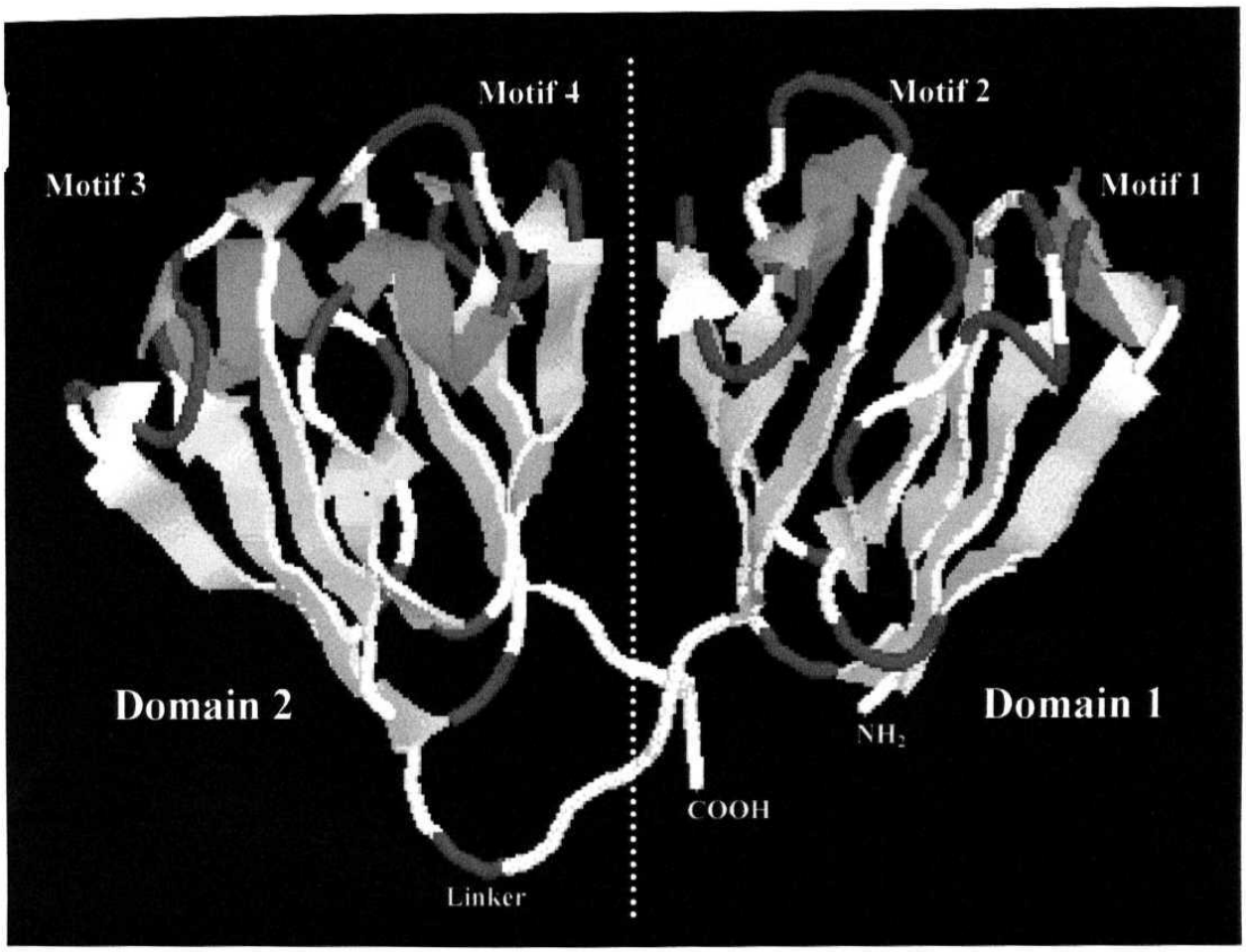


Figure 1.4. The three-dimensional structure of yB-crystallin showing two domains of similar structure connected by the linker (Blundell *et al.*, 1981).

argininosuccinate lyase (Beacham *et al.,* 1984; O'Brien *et al.,* 1986; Piatigorsky *et al.,* 1988). The amphibian ε-crystallin is closely similar to vertebrate lactate dehydrogenases (Wistow and Piatigorsky, 1987). The τ-crystallin found in duck and turtle lenses is similar to α-enolase, while ξ-crystallin of guinea pigs, camel and cattle is related to alcohol dehydrogenase (Borras *et al.,* 1989), and is found to have quinone oxidoreductase activity (Rao *et al.,* 1992). The most abundant mammalian taxon-specific crystallin, elephant r|-crystallin, is similar to cytoplasmic aldehyde dehydrogenase (Wistow and Kim, 1991). ρ-Crystallin found in the lenses of frogs belongs to an aldo-keto reductase superfamily (Bohren *et al.,* 1989). λ-Crystallin found in rabbits and hares, is distantly related to hydroxyacyl- and hydroxybutyryl-CoA dehydrogenase (Mulders *et al.,* 1988). However, the activities of both p- and λ-crystallin have not yet been confirmed (Mulders *et al.,* 1988; Carper *et al.,* 1989).

1.0.3. PHYSICAL BASIS FOR LENS TRANSPARENCY

In most types of senile cataracts (subcapsular cortical, supranuclear cortical and nuclear) the physical basis for opacification is increased light scattering which is caused by variations in the refractive index within the lens. Local changes in the distribution of proteins within the lens are the major cause for fluctuations in refractive index during the early stages of cataract.

That increased light scattering forms the physical basis for reduction in lens transparency in cataracts was first discussed in detail by Trokel (1962). Philipson (1969), studied lens changes in relation to increased light scattering in X-ray and galactose cataracts in rats and found, using a quantitative microradiographic technique, that local fluctuations in the protein concentration of the lens were to be found in the same location as the opacities. As refractive index is a linear function of protein concentration it could be shown that refractive interfaces were created which were responsible for the greater part of the scattering of light in these experimental cataracts.

When the protein matrix of the nuclear whitish cataract was studied under high magnification, dense aggregates of cell matrix from about 50-100 nm in diameter could be seen (Philipson, 1973). In transparent lenses, these large and dense aggregates were not seen. Here, spherical particles with a diameter of approximately 14 nm, probably mainly α-crystallin molecules, dispersed and formed a relatively loose network. This arrangement conforms to the low light scattering in the normal lens. The influence of large macromolecules made from aggregated lens protein molecules has also been theoretically worked out by Benedek (1971).

1.0.4. CATARACT

In simple terms, cataract is defined as an opacification of the lens. Protein aggregation resulting in high molecular weight aggregates capable of scattering light leads to cataract formation (Benedek, 1971). An increase in water-insoluble proteins due to aggregation has been observed in most cases of cataract development (Harding, 1972; Spector, 1985). The etiology of cataract is multiple, arising due to genetic, traumatic, metabolic (secondary to diabetes, arthritis) factors, or could be drug-induced (steroids), light-mediated, dietary (malnutrition), infection-related (diarrhea), and also oxidative stress and other age-related factors.

1.0.4.1. Types of Cataract

Cataracts are classified based on etiology, degree and location of opacification and whether congenital or acquired. Based on the location of the opacity, cataracts are classified as nuclear (central opacity), cortical (peripheral opacity) and posterior subcapsular.

(i) Senile cataract: Age-related or senile cataract contributes to about 90% of all cataracts. Though the apparent cause of this type of cataract is still not very clear, cumulative oxidative stress over time appears to be a major factor in the development of age-related cataracts. In addition, diabetes,

glaucoma, and the use of certain medications are some of the underlying factors that aggravate its development. Based on the precise location of the opacity, senile cataracts are mainly of three types; nuclear, cortical and posterior subcapsular.

(ii) Traumatic cataract: Blunt injury to the eye may lead to lens opacities which could be either rapid or delayed. These injuries may be penetrating, causing direct damage to the lens or concussion injuries leading to pigmentation on the lens capsule (vossius rings) due to the iris imprinting of the anterior lens capsule.

(iii) Metabolic/Diabetic cataract: The most thoroughly studied of all experimental cataracts is diabetic cataract (Datiles, 1992) which involves the enzyme aldose reductase (AR) (Stevens, 1998). The excess glucose is converted to sorbitol by the action of AR and the accumulation of sorbitol in the lens imposes an osmotic stress on the lens leading to water uptake, fiber disruption and opacity (van Heyningen, 1959).

(iv) Toxic cataract: Drug-induced or prolonged use of corticosteroids results in subcapsular cataract, but the exact relationship between steroid usage and cataract is not clear. McGhee and coworkers (2002) observed the development of posterior subcapsular cataract as early as 4 months following topical corticosteroid use, which could be due to the covalent

binding of steroids to lens proteins with subsequent oxidation. α-Crystallin has been identified as the principal soluble glucocorticoid-binding protein in the lens (Jobling *et al.,* 2001).

(v) Congenital cataract: Congenital cataracts are responsible for a major proportion of childhood blindness. This form of cataract either presents as an independent entity or is associated with syndromes (Francois, 1982) and could be inherited in an autosomal dominant, autosomal recessive or X-linked manner. Mutations in many lens proteins, including crystallins have been reported to lead to inherited cataract. Several mouse models of congenital cataract have been developed which serve as appropriate models for corresponding human cataracts (Graw, 1999).

Secondary cataracts arise as a result of primary ocular diseases like anterior uveitis, Wagner's & Stickler's syndrome and high myopia.

1.0.4.2. Factors influencing cataractogenesis /Risk factors

(i) Disease: Diabetes has been associated with cataract for many years and is considered to be a major risk factor (Klein *et al.*, 1985) and it accounts for more than one tenth of cataracts in developed countries alone (Stevens, 1998).

Other diseases like cardiovascular disease (Klein *et al.,* 1998), renal disease and diarrhea (van Heyningen and Harding, 1988) have also been related to the development of cataract. The direct relationship however, remains obscure and the mechanism is thought to proceed mostly through corticosteroid therapy.

(ii) UV radiation / Sunlight: UV radiation is also considered to be an important factor in cataractogenesis through generation of protein crosslinks and fluorescent compounds (Lerman, 1980; Lerman, 1985; Balasubramanian, 2000). Light of wavelength greater than 300 nm can cause photo-oxidation of certain specific amino acid residues in lens proteins resulting in cataract (Datiles, 1992; Balasubramanian, 2000).

(iii) Nutrition: Epidemiological data indicate that elevated plasma levels of specific nutrients like carotenoids, ascorbate, tocopherol and taurine which act as antioxidants are associated with diminished or delayed onset of cataract (Taylor, 1989; Taylor *et al.,* 1995). Analysis of free amino acid content of aqueous humor in subjects with senile cataracts has shown that the concentration of various amino acids was quite low suggesting that amino acid deficiency can lead to cataractogenesis (Bunce *et al.,* 1972; Zarina *et al.,* 1987).

The chain of events occuring to nuclear proteins during cataract are the following - (i) shape changes: the molecules unfold with increased reactivity of thiol and possibly other groups, (ii) soluble protein polymers form, and (iii) a completely insoluble dark brown protein aggregate develops.

Electrophoresis revealed that during cataract formation there is a gradual loss of the proteins of low molecular weight. Using the more powerful analytical method of 2D electrophoresis (paper in the first dimension followed by starch-gel electrophoresis at 90° to the initial direction), Maisel & Goodman (1965) compared embryonic, normal adult and cataractous human lenses. In mature cataract, the γ-crystallins were found to be missing. The loss of γ-crystallin appears to support the idea of Testa *et al.* (1968) that these proteins are lost as a consequence of oxidation and subsequent insolubilization. Recently, Lampi *et al.* (2002) have produced a two-dimensional electrophoresis-based proteome map of rat lens crystallins, which will help in future analysis of age-related and cataract-specific modifications in rat crystallins. The proteomics of mouse lens has been well studied in an age-dependent manner by Ueda *et al.* (2002) where they have identified various modifications to lens crystallins which could explain the insolubilization.

1.0.5. SCOPE OF THIS STUDY

During the development of the lens, the terminally differentiated fiber cells do not possess the necessary machinery for active metabolism. Due to this low turnover in the lens, any damage occurring to the constituents accumulate and interfere with transmission and refraction of light. Since the eye is continually exposed to light, photic and oxidative stress play an important role in the pathology of cataract.

Oxidative modifications to the lens include:

(i) Formation of protein crosslinks - through intermolecular covalent links, (ii) Modification of ionic side chains of proteins leading to precipitation, and (iii) Generation of new protein-associated/free chromophores/fluorophores.

Protein oxidation can be brought about either by direct photo-oxidation or by reactive oxygen species (ROS) like the hydroxyl radical ("OH), singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet-}$), and hydrogen peroxide (H_2O_2). ROS can be generated either photodynamically (Zigler and Goosey, 1981) or in dark by reaction with metal ions (Zigler *et al.,* 1985; Garland *et al.,* 1986). More recently, the role of reactive oxygen/nitrogen species, particularly peroxynitrite ($OONO^-$) and nitrite (NO_2^-), in covalently damaging lens proteins has been recognized (Gracy *et al.,* 1999; Paik and Dillon, 2000).

The focus of this thesis is to study

(i) The role of fluorophores/chromophores that accumulate in the lens with age

(ii) The reaction of a typical oxidant, peroxynitrite, with α-crystallin: structure-function correlation of oxidized a-crystallin

(iii) Evaluating the antioxidant activity of two plant extracts, namely *Ginkgo biloba* and *Withania somnifera,* in relation to their cataracto-static abilities.

(iv) Cloning and expression of a mutant crystallin associated with cataract, specifically to study the structural consequence of a splice site mutation in the βA3/A1-crystallin gene in autosomal dominant zonular cataract.

The lens accumulates certain tryptophan derivatives (members of the kynurenine family) which show absorption maxima in the UV-A region. These compounds can either remain inert in the lens and act as UV filters, or can generate reactive oxygen species which bring about a spate of oxidation reactions with both proteins and nucleic acids. We have studied the role of one such novel fluorophore called Xanthurenic acid 8-O-β-D-glucoside in the lens, which is found in high amounts in brunescent cataractous lenses, whose function in the lens has so far remained elusive.

We have also studied the reaction of lens proteins with the potent oxidizing and nitrating agent, peroxynitrite, so as to see the effect of oxidation on protein structure/stability and function. We have looked particularly into the structural and functional alteration in α-crystallin due to its reaction with peroxynitrite, and find that though its structure is affected its chaperone-like function is not.

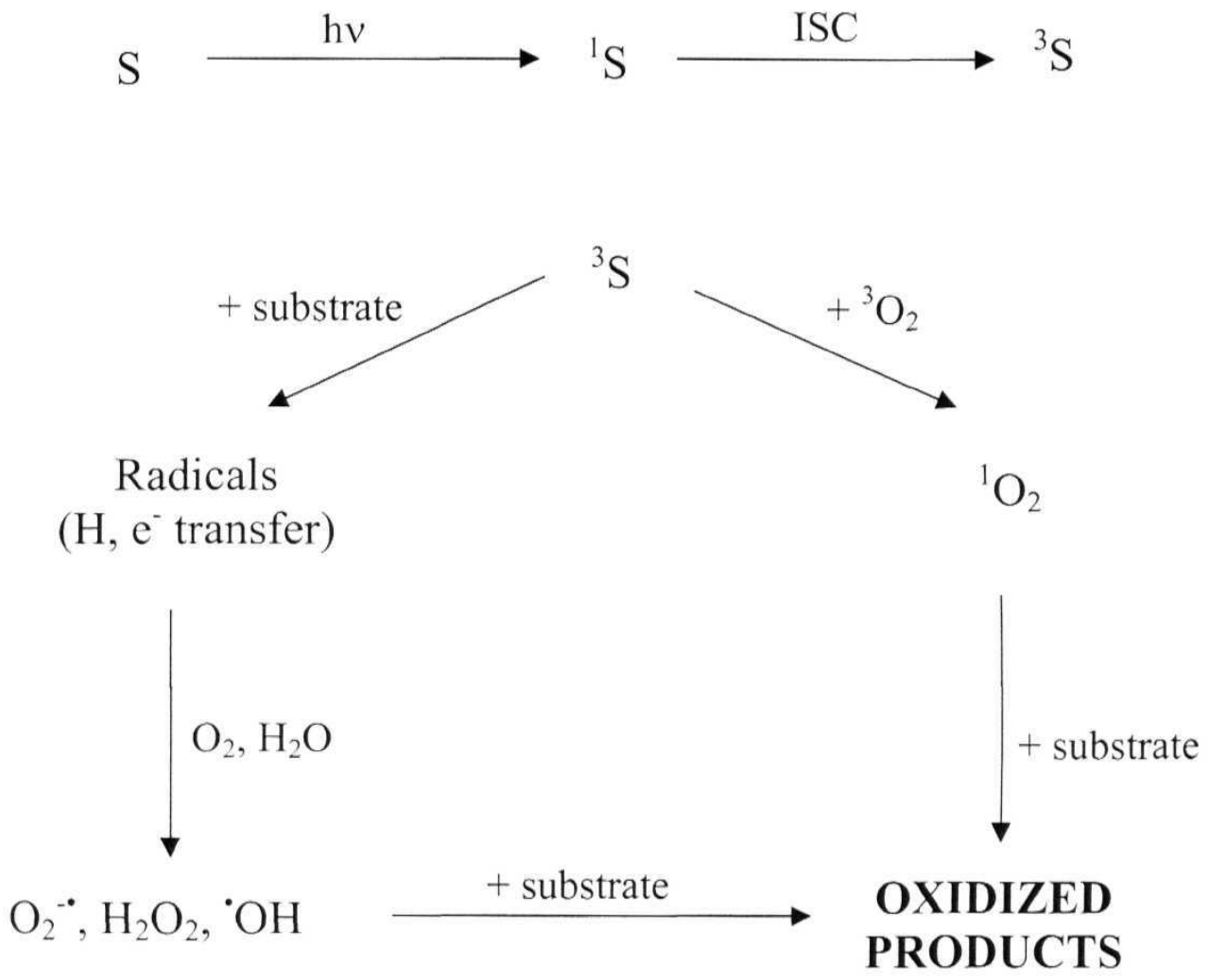
Turning our attention from the mechanisms involved in the loss of lens transparency due to oxidation, we have looked into the potential use of dietary antioxidants to retard the onset/progression of age-related cataract. We have studied the extracts from two plants, namely *Ginkgo biloba* (from Chinese herbal medicine) and *Withania somnifera* (from Indian *Ayurveda),* with respect to their antioxidant, cytoprotective and anti-cataract properties. We find both extracts to be efficient antioxidants with cytoprotective effect *in vitro.*

Finally, we have addressed the effect of a splice-site mutation on the structure of pA3/A1-crystallin; this mutation is known to cause autosomal dominant zonular cataract (Kannabiran *et al.*, 1998). The mutant protein when cloned and overexpressed in *E.coli* was found to aggregate at a higher rate than wild-type PA3/A1-crystallin.

Chapter 2

2.0.0. ROLE OF XANTHURENIC ACID 8-0-p-D-GLUCOSIDE IN THE LENS

Proteins comprise a major portion of the dry weight of the lens and are therefore potential targets for photo-oxidation. Two major types of processes can occur with proteins. The first of these involves direct photo-oxidation arising from the absorption of UV radiation by the protein, or bound chromophore groups, thereby generating excited states (singlet or triplet) or radicals via photo-ionisation. The second major process involves indirect reaction on the protein through photodynamic action of sensitizer molecules. Upon absorption of light, these molecules are sent to the singlet excited state, from where they move to the long-lived triplet excited state by intersystem crossing. In what is termed the type I process, the triplet excited state energy is transferred to a substrate molecule (e.g. a protein) through H atom or electron transfer, and also produces reactive oxygen species such as superoxide ($O_2^{-\bullet}$), H_2O_2 and the hydroxyl radical ($^{\bullet}OH$). In the type II process, the sensitizer molecule interacts in its triplet excited state with ground state (triplet) molecular oxygen and excites the latter (by about 25 kcal/m or so) into the highly reactive and short-lived ($\leq$ 1 μs) singlet oxygen molecule. The singlet oxygen reacts with a neighbouring substrate (e.g. protein) and oxidizes it. Scheme 2.1 summarizes the photodynamic process where S represents the sensitizer molecule. Type I chemistry is favoured by substances that are readily oxidized (e.g. phenols, amines) or reduced (e.g. quinones), while type II or singlet oxygen reactions occur more commonly with olefins, dienes,



Scheme 2.1. Photodynamic action of a sensitizer (S) molecule.

^1S = singlet state of sensitizer
^3S = triplet state of sensitizer
ISC = Inter-system crossing

aromatics and heterocycles. Some sensitizers promote the type II or singlet oxygen pathway (e.g. Rose Bengal) while others (e.g. riboflavin) operate by both pathways. We discuss here a particular system - the brunescent cataractous lens, where UV photo-oxidation plays a role in the etiology of the disease, and tryptophan-derived metabolites act as UV filters.

2.0.1. FUNCTION OF THE EYE LENS

The eye is constantly exposed to sunlight, including UV-B (295 - 320 nm), UV-A (320 - 400 nm) and visible (400 - 700 nm) radiation. The primary functions of the lens is focusing light on the retina, and preventing optical radiation between 295 and 400 nm from reaching the retina. The filtering action of the cornea helps to cut out most of the light in the UV-B region, so that what essentially reaches the lens are the UV-A and visible components. Certain low molecular weight fluorescent compounds found in the lens, termed 'UV filters' absorb most of the incident light between 295 and 400 nm (Wood and Truscott, 1993; Truscott *et al.*, 1994; Wood and Truscott, 1994) thereby protecting the lens and retina from UV-induced photodamage, and aiding visual acuity by decreasing chromatic aberration (Truscott *et al.,* 1994).

2.0.2. CHROMOPHORES/FLUOROPHORES OF THE LENS

An age-dependent increase in lens coloration is observed due to the accumulation of compounds that show characteristic fluorescence in

the visible-UV-A region. This increase in lens color is seen both in diabetic as well as non-diabetic lenses. In the former, lens coloration is largely due to the Maillard reaction between the sugar and amino group of amino acids, and accumulation of AGEs. In non-diabetic lenses, the presence of low molecular weight chromophores/fluorophores is thought to be responsible for the increase in lens coloration.

Collectively called as non-tryptophan fluorophores, these compounds, largely formed from tryptophan via the kynurenine pathway, include kynurenine (Ky), N-formylkynurenine (NFK), 3-hydroxykynurenine (3-HK) and its O-β-D-glucoside (3-HKG), and 4-(2-amino 3-hydroxyphenyl)-4-oxobutanoic acid O-β-D-glucoside (AHBG), quinolines like quinaldic acid (QA), kynurenic acid (KA), xanthurenic acid (XA), oxoxanthurenic acid (OXA) and 4-(hydroxy 3-glycine)-quinoline (HGQ), and β-carbolines (Pirie, 1968; van Heyningen, 1971; Luthra *et al.,* 1994; Malina and Martin, 1996; Bova *et al.,* 1999; Pari *et al.*, 2000; Balasubramanian, 2000). Some of these occur in the free form in the lens, while others are protein-bound.

2.0.3. ROLE OF LENS CHROMOPHORES

It is relevant to study the roles of lenticular compounds in relation to the function of the lens. Some of them (Ky, 3-HK, 3-HKG and AHBG) appear to act as UV-A filters, protecting the vitreous and the retina from light-induced damage, while some others (NFK, KA) have been seen to display photodynamic action, absorbing light in the UV-A region and

generating reactive oxygen species from their excited electronic states (Dillon *et al.,* 1990; Murali Krishna *et al.,* 1991). These endogenous photosensitizers are thus potentially harmful, since oxidative stress is recognized to be a major factor in age-related cataract, retinal dysfunction and macular degeneration. Some of these (3-HK and a β-carboline) display antioxidant properties, being able to quench oxyradicals and inhibit oxidative cross-linking of test proteins in solution (Pari *et al.,* 2000).

2.0.4. BRUNESCENT CATARACT

According to the CCRG (Co-operative Cataract Research Group) system of classification (Chylack *et al.,* 1983), cataractous lenses are graded based on the intensity of color as Grade I (Yellow), Grade II (Dark Yellow), Grade III (Brown) and Grade IV (Dark Brown/Brunescent). Figure 2.1 shows a typical brunescent cataractous lens extracted from a 65-year-old non-diabetic male. In lenses with a nuclear brunescent type of opacity, both light absorption by the brown material and the scattering of light contribute to the loss of transparency. Considerable experimental evidence has been presented showing that the brown color of the lens proteins could result from the binding of coloured quinones to the amino and thiol groups of lens proteins with a consequent lowering of the solubility in water (Pirie, 1968; Zigman, 1971).

The main characteristic aspect of nuclear brunescent cataract appears to be a mechanism of protein insolubilization occurring in the lens nucleus; both Pirie's (1968) observation that an appreciable amount of the

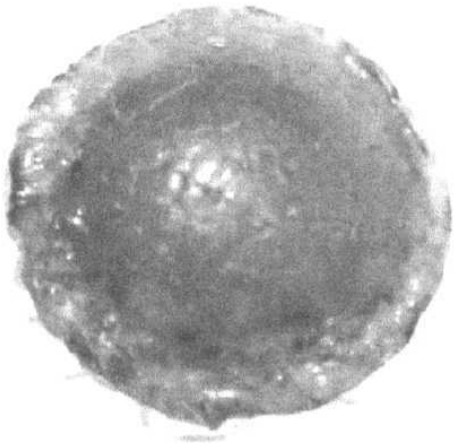


Figure 2.1. A Brunescent cataractous lens from a 65-year-old non-diabetic male

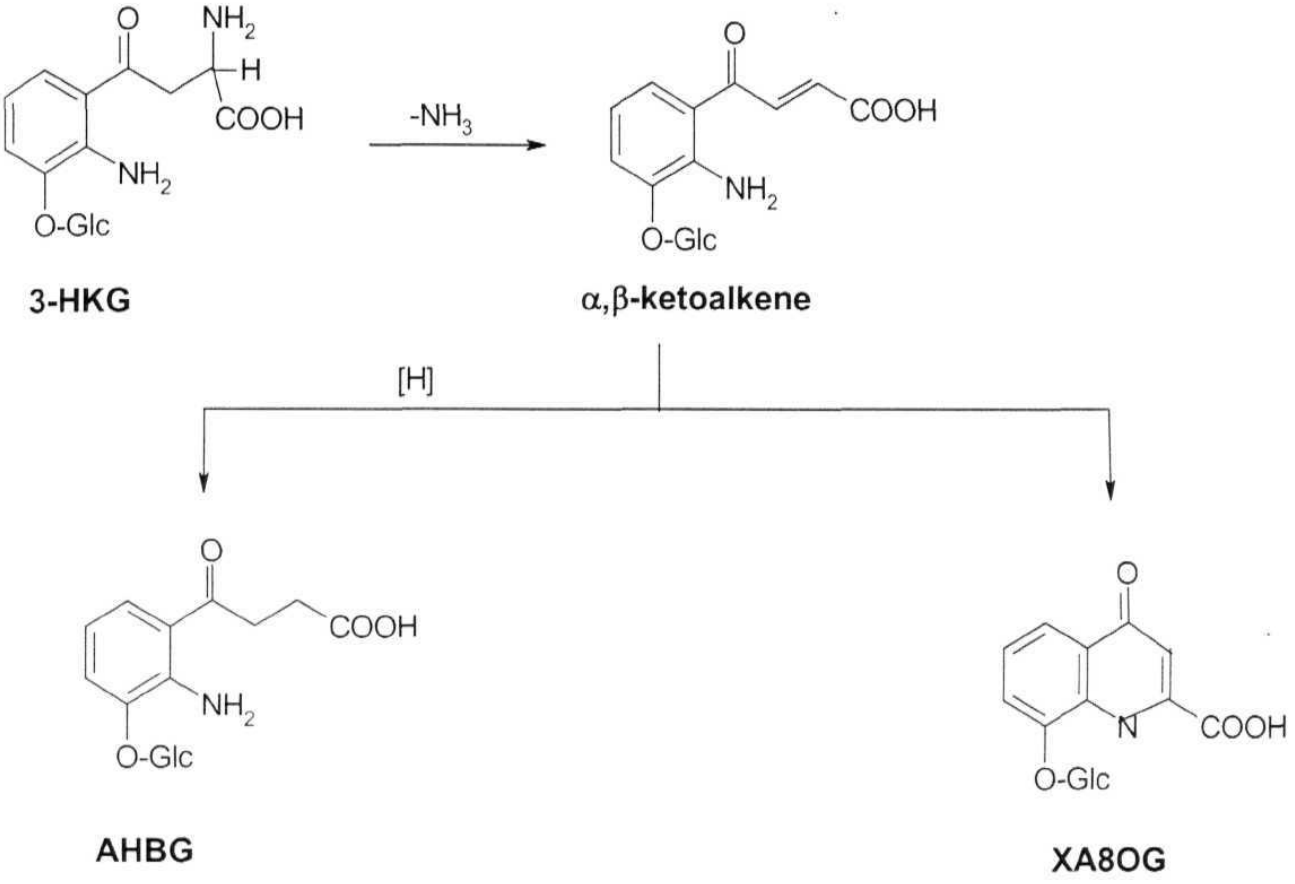
water-insoluble protein fraction of these lenses may not be solubilized by 6M guanidine hydrochloride or 50 mM DTT, and Buckingham's (1972) data suggesting the possible importance in this insolubilization of extensive crosslinkage of a non-disulfide nature between lens proteins. Loss of transparency in this case therefore depends both on scattering of light by these insoluble macromolecular complexes and by light absorption by the brown proteins of these lenses.

An increase in non-tryptophan fluorescence has been observed in brunescent cataractous lenses. Bessems and Hoenders (1987) were the first ones to detect a soluble, low molecular weight, non-protein-bound, fluorescent constituent in non-diabetic human lenses, whose levels increase as the brunescence of the lens intensifies. When intact young human, baboon and rhesus monkey lenses were subjected to near-UV irradiation under identical conditions and the fluorophore buildup monitored, a loss of 3-HKG as a result of irradiation was apparent in all lenses which correlated with the buildup of a blue fluorophore, suggesting that the latter may be a photoproduct of 3-HKG (Ellozy *et al.,* 1994). It was later shown that levels of this particular fluorophore, called Fl-Glc, increased with time (Shirao *et al.,* 2001). This fluorophore was subsequently identified as xanthurenic acid 8-O-β-D-glucoside (XA8OG) (Ando *et al.*, 2000).

XA8OG can be formed either from 3-HKG by the initial deamination of 3-HKG, followed by ring closure to make the 4-oxoquinoline product or from XA by glycosylation. However, the former pathway appears to be

more favoured which can occur either enzymatically by the action of kynurenine aminotransferase (KAT) or through the non-enzymatic degradation of 3-HKG. It has been suggested that AHBG, which is also formed by a similar deamination reaction of 3-HKG followed by hydrogenation is a UV-A protectant filter (Bova *et al.*, 1999) (Scheme 2.2).

In this study, we have investigated the role of xanthurenic acid 8-O-β-D-glucoside (XA80G) in the lens. The Photophysical and photochemical properties of XA8OG and the issue of whether it is a putative fluorophore for age-related cataractogenesis have been addressed. In order to examine the role UV light and UV filters play in the modification of lens proteins and formation of age-related cataract, we studied the effect of UV light on proteins in the absence and presence of UV filters. In addition, as exposure to UV light often leads to production of singlet oxygen, superoxide and hydroxyl radicals, the ability of these compounds to generate reactive oxygen species was also examined.



Scheme 2.2. Suggested pathway for the formation of XA80G
(Shirao *et al.,* 2001).

2.1.0. MATERIALS AND METHODS

2.1.1. CHEMICAL SYNTHESIS OF XA8OG AND 3-HKG

Xanthurenic acid 8-O-β-D-glucoside and 3-hydroxykynurenine-O-p-D-glucoside were synthesized from XA and 3-HK (Sigma/Aldrich Chemical Co), as starting material respectively, and D-glucose, using the procedure described by Real and Ferre (1988, 1990). Their identities were confirmed by NMR and mass Spectrometry. We are grateful to Dr. B. Jayaraman of the Department of Organic Chemistry, Indian Institute of Science, Bangalore for help with the syntheses and purification of 3-HKG and XA8OG.

2.1.2. FLUORESCENCE MEASUREMENTS

Steady state fluorescence measurements were done using a Hitachi F-2500 spectrofluorimeter. Fluorescence quantum yields were estimated using the procedure of Parker and Rees (1960)

$$Q_x = Q_S(F_x/F_S)(A_S/A_x)$$

where subscripts s and x refer to the reference standard and the sample, respectively, F is the wave-number-integrated area of the corrected emission spectrum, and A is the absorbance at excitation wavelength. The standard used was N-acetyl-L-tryptophanamide (Q_s = 0.14) (Szabo and Rayner, 1980). Fluorescence lifetimes were measured using an IBH model 5000U single photon counting spectrofluorimeter. We thank Dr. A.

Samantha and Mr. Satyen Saha of the School of Chemistry, University of Hyderabad for the fluorescence lifetime measurements.

2.1.3. SINGLET OXYGEN GENERATION ASSAY

The method of bleaching, or reduction in the absorption band at 440 nm, of N, N-dimethyl-4-nitrosoaniline (abbreviated as RNO) by singlet oxygen, devised by Kraljic and El Mohsni (1978) was used to monitor $^1O_2$ production. The candidate sensitizer molecule (100 μM) was exposed to light for increasing periods of time (usually 0 to 30 min) at its absorption wavelength in the presence of imidazole and RNO in 50 mM phosphate buffer, pH 7.4. The wavelengths used for excitation for each sensitizer is shown in Table 2.1. The $^1O_2$ generated by photoexcitation of the molecule reacts with imidazole to form a transannular peroxide which bleaches RNO. This is monitored spectrophotometrically by following the drop in the absorption at 440 nm, as a function of time.

2.1.4. SUPEROXIDE GENERATION ASSAY

The ferricytochrome *c* reduction method of McCord and Fridovich (1969), which monitors the change in the absorption intensity at 550 nm, was used to assay the superoxide ($O_2^{\bullet -}$) generating abilities of the lens pigments. The rates of cytochrome *c* reduction were followed with time as air- saturated solutions of the test compounds (100 μM each, in 50 mM phosphate buffer pH 7.4, containing 20 μM ferricytochrome *c)* were illuminated with light at their absorption maxima for 0 to 30 minutes. While

TABLE 2.1. Wavelength of excitation used for each sensitizer

SENSITIZER	ABSORPTION WAVELENGTH
3-HK	371 nm
3-HKG	360 nm
XA	342 nm
XA8OG	338 nm

studying the effect of electron donors on the production of superoxide, 1mM EDTA was added to the reaction mix and then the drop in absorbance was read at 550 nm, as a function of time.

2.1.5. ELECTROPHORESIS

The candidate protein, bovine pancreatic ribonuclease A (RNase A -1 mg/ml) was incubated with 100 μM of test compound and irradiated at the absorption maximum of each test compound for 60 and 120 min, in the sample compartment of a Hitachi spectrofluorimeter (F-2500). The photodynamic reactions of the test compounds with RNase A was monitored by following the products electrophoretically on a polyacrylamide gel (12%) in the presence of sodium dodecylsulfate and β-mercaptoethanol and the bands in the gel visualized by silver staining.

2.1.6. APOPTOSIS IN HUMAN LENS EPITHELIAL CELLS BY XA8OG

The human lens epithelial cell line, SRA01/04 was a kind gift from Prof. Venkat N. Reddy of the University of Michigan. These cells were cultured on cover slips placed in a six-well tissue culture plate, in DMEM with 10% FCS. When cells were 70% confluent, the test agent (30 μM) was added and incubated further for 72 h. The cells, adhered to the cover slips, were then washed in PBS thrice and fixed in cold methanol:acetic acid (3:1) overnight at 4°C. Cells were then thoroughly washed in PBS and stained with DAPI (300 μg/ml) and nuclei were observed using a fluorescence microscope.

2.2.0. RESULTS AND DISCUSSION

The human lens is clear at the time of birth until the age of about 5 years. After that the lens progressively yellows largely due to the presence of 3-HK and its further products, and the exclusively primate lens pigment 3-HKG. While most of the UV filters absorb UV light and protect the lens against UV induced damage (Ky, 3-HK, 3-HKG), at least some of them do form reactive intermediates in the presence of UV light and can modify lens proteins (NFK, XA). Infant lenses have been shown to contain highest levels of 3-HKG and this decreases in both the nucleus and cortex with age (Bando *et al.,* 1981). Young lenses (second decade) contain about 400 nmol/g of 3-HKG in the nucleus and cortex, while older lenses (eighth decade) have about 100 nmol/g (Bova *et al.,* 2001). An increase in other fluorescent compounds like XA, XA8OG and AHBG has been seen in aged lenses concomitant with the decrease in 3-HKG levels.

Figure 2.2 shows the absorption and fluorescence spectrum of XA8OG, with the absorption maximum at 338 nm and a broad fluorescence emission around 440 nm.

Table 2.2 shows the fluorescence parameters of 3-HK, 3-HKG, XA and XA8OG that were measured by us in the laboratory. Note that 3-HK and 3-HKG both have very short emission lifetimes and quantum yields. The quantum yield of XA8OG is comparatively large (Φ= 0.22), about three orders of magnitude greater than XA itself. The fluorescence patterns of 3-HKG, XA and XA8OG are complex, displaying two-component emission

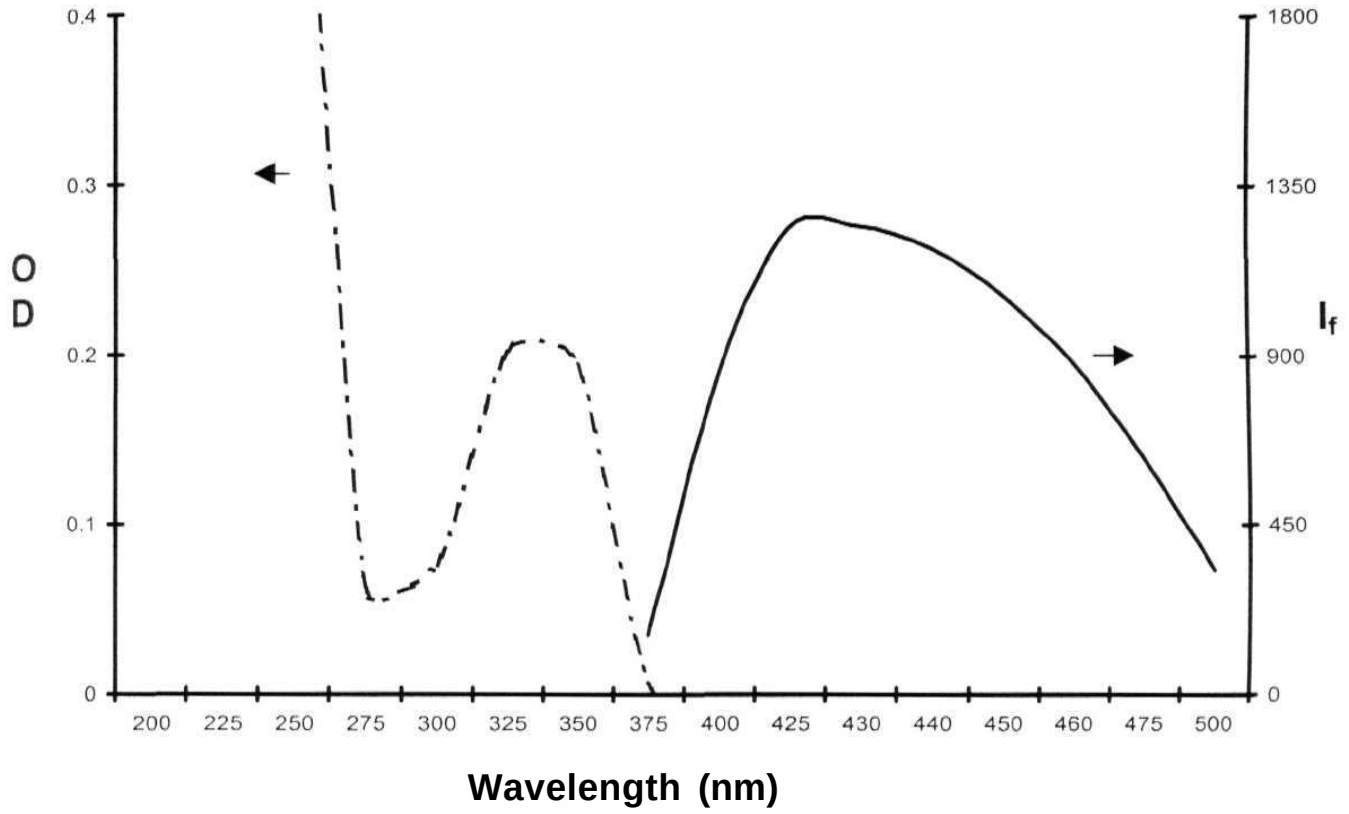


Figure 2.2. Absorption and Fluorescence spectra of XA8OG

Table 2.2. Fluorescence parameters of some lenticular pigments

Compound	Emission lifetimes		Quantum yield
	τ_1	τ_2	φ
3-HK	< 0.5 ns	–	1.2×10^{-4}
3-HKG	0.62 ns(94%)	7.7 ns (6%)	3.1×10^{-4}
XA	0.77 ns(79%)	6.0 ns (21%)	2.9×10^{-4}
XA8OG	12.3 ns(77%)	1.1 ns (23%)	2.2×10^{-1}

modes. The lifetime of XA8OG is significantly longer (12 ns) than the longest lifetime of XA (6 ns). As has been argued earlier (Dillon *et al.,* 1990; Murali Krishna *et al.,* 1991), very short lifetimes, of the kind exhibited by 3-HK and 3-HKG, disfavor inter-system crossing to the triplet state and subsequent sensitization or photochemistry to occur. On the other hand, XA displays a two-component emission mode, with one lifetime around 6 ns, thus allowing such processes to occur in this molecule. We note that XA8OG displays a far longer singlet excited state lifetime, allowing enough time for intersystem crossing to the triplet state and subsequent reactions from this state. Photochemical studies on 3-HK and 3-HKG have indicated that these compounds are inefficient sensitizers of lens proteins. Fluorescence properties of 3-HKG showed it to contain a fast deactivation pathway (ps), which would be expected to have minimal photochemical effect on the integrity of the lens. Further, Photophysical studies on 3-HK indicated that it has an even faster fluorescent lifetime (less than 10 ps) with a much lower quantum yield of fluorescence (0.001 vs 0.03 for 3-HKG). This lifetime has been argued to be too short to produce a transient that could induce photosensitized oxidation reactions in the lens (Dillon *et al.,* 1990).

2.2.1. GENERATION OF SINGLET OXYGEN AND SUPEROXIDE RADICALS

Figure 2.3 compares the relative abilities of several lenticular pigments (100 μM solutions in water) to generate singlet oxygen ($^1O_2$) with that of riboflavin (RF), a known photosensitizer and generator of $^1O_2$. This

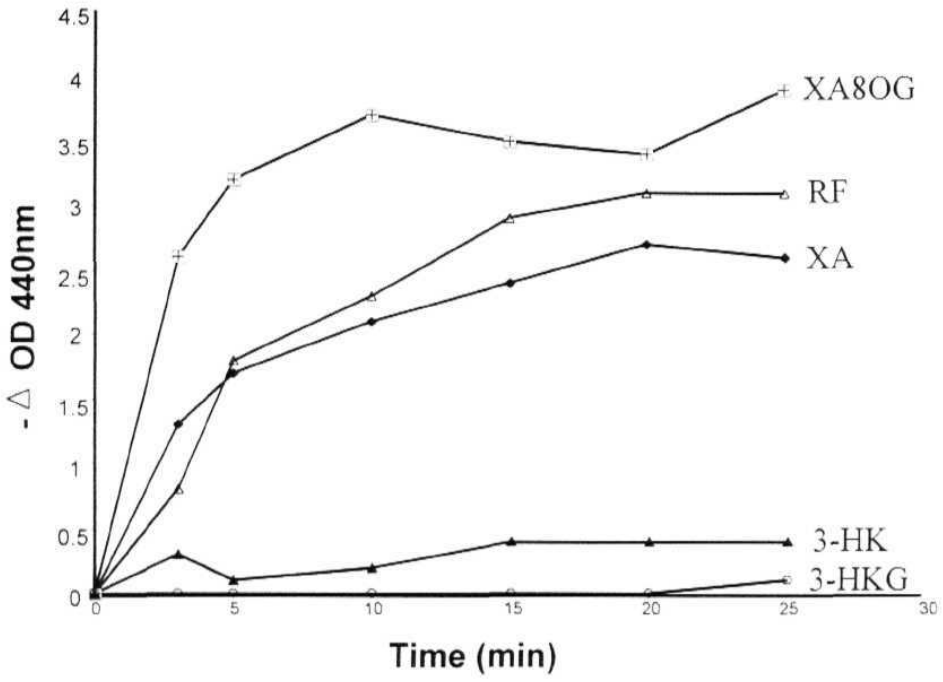


Figure 2.3. Singlet oxygen generation by the lens pigments measured by the RNO bleaching assay

ability is seen to vary in the order XA8OG > RF > XA » 3-HK~ 3-HKG. The quantum yield of $^1O_2$ production of RF was earlier estimated to be about 0.49 (Murali Krishna *et al.,* 1991), while that of XA has been estimated to be 0.17 by Roberts *et al.* (2000). Figure 2.4 compares the photodynamic abilities of these compounds to generate superoxide anion radical ($O_2^{-\bullet}$). XA leads the rest in this property, while interestingly enough, its glucoside XA8OG is not as efficient. It appears that the covalent addition of glucose, with its multiple -OH groups, reduces the efficiency of radical production. Such a role for glycosylation has been suspected earlier (Wei *et al.,* 1999). In an attempt to increase the superoxide-producing ability of XA8OG, we also tried adding electron donors such as EDTA, since added electron donors are known to enhance this ability (Murali Krishna *et al.,* 1991) but this had no perceptible effect in this instance. Both 3-HK and 3-HKG were seen to be inactive.

2.2.2. XA8OG-INDUCED PHOTO-OXIDATION OF PROTEINS

We next tested the abilities of XA, XA8OG, 3-HK and 3-HKG to impart oxidative damage to a test protein in solution. When RNase A was irradiated in the presence of 100 μM of the test compound, for 2h in the UV-A (at wavelengths corresponding to the absorption bands of the additives, where the protein does not absorb), high molecular weight aggregates of the protein were seen on SDS-PAGE only in the presence of XA and XA8OG. Protein irradiated in the absence of sensitizers did not show any additional bands on the gel. Figure 2.5 shows that both XA8OG and XA photo-oxidize the protein, while 3-HK and 3-HKG do not. Even

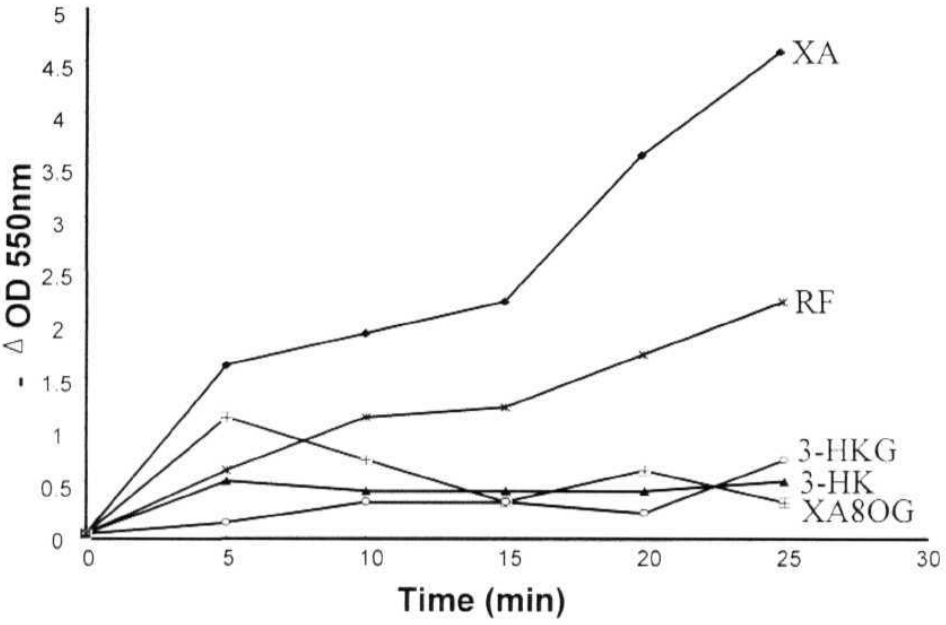


Figure 2.4. Superoxide radical generation by the lens pigments measured by the ferricytochrome c reduction method

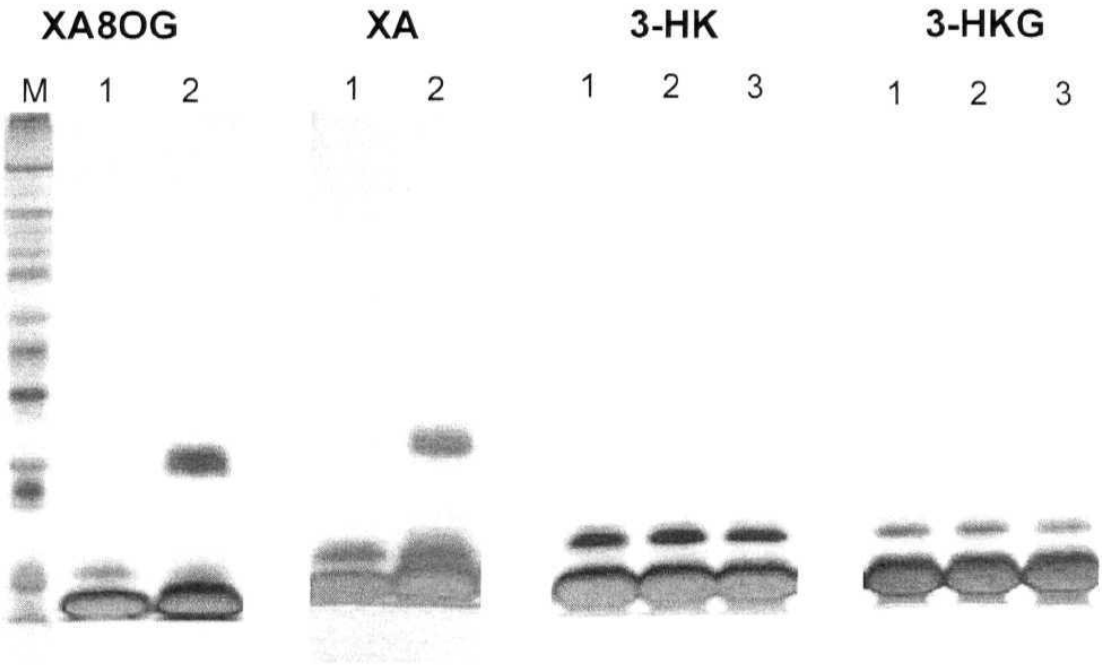


Figure 2.5. SDS/PAGE of RNase A (1 mg/ml) (Lane 1) with 100 μM sensitizer (Lane 2) and 500 μM sensitizer (Lane 3) M: Molecular weight marker

higher concentrations (500 μM) of 3-HK and 3-HKG did not induce any photodamage. That XA acts as a photodynamic agent, causing oxidative damage to lens proteins, has been reported by Malina and Martin (1996) and by Roberts *et al.* (2000, 2001). Both XA and its glucoside photo-oxidize protein constituents of the lens.

2.2.3. XA8OG INDUCED APOPTOSIS

Malina *et al.* (2002) reported that XA induces calpain Ip82 and caspases, causing apoptotic cell death in cultured human lens epithelial cells. Since oxidative stress is known to cause apoptotic cell death, we looked at the behavior of cells when cultured in the presence of XA8OG. Like XA, we find XA8OG also to cause cell death by apoptosis, as shown in Figure 2.6.

Though the endogenous conversion of 3-HKG to AHBG could be benign, its conversion to XA and XA8OG can result in photo-oxidative stress to the lens in the UV-A region, which the lens continuously receives, absorbs and transmits. Bearing in mind its ability to efficiently generate singlet oxygen species, to covalently crosslink proteins and to induce apoptosis in cells, XA8OG appears to be an endogenous chromophore in the lens that can act as a cataractogenic agent. We conclude that the age-dependent depletion of 3-HKG and concomitant generation and accumulation of XA and XA8OG enhance oxidative stress to the lens.

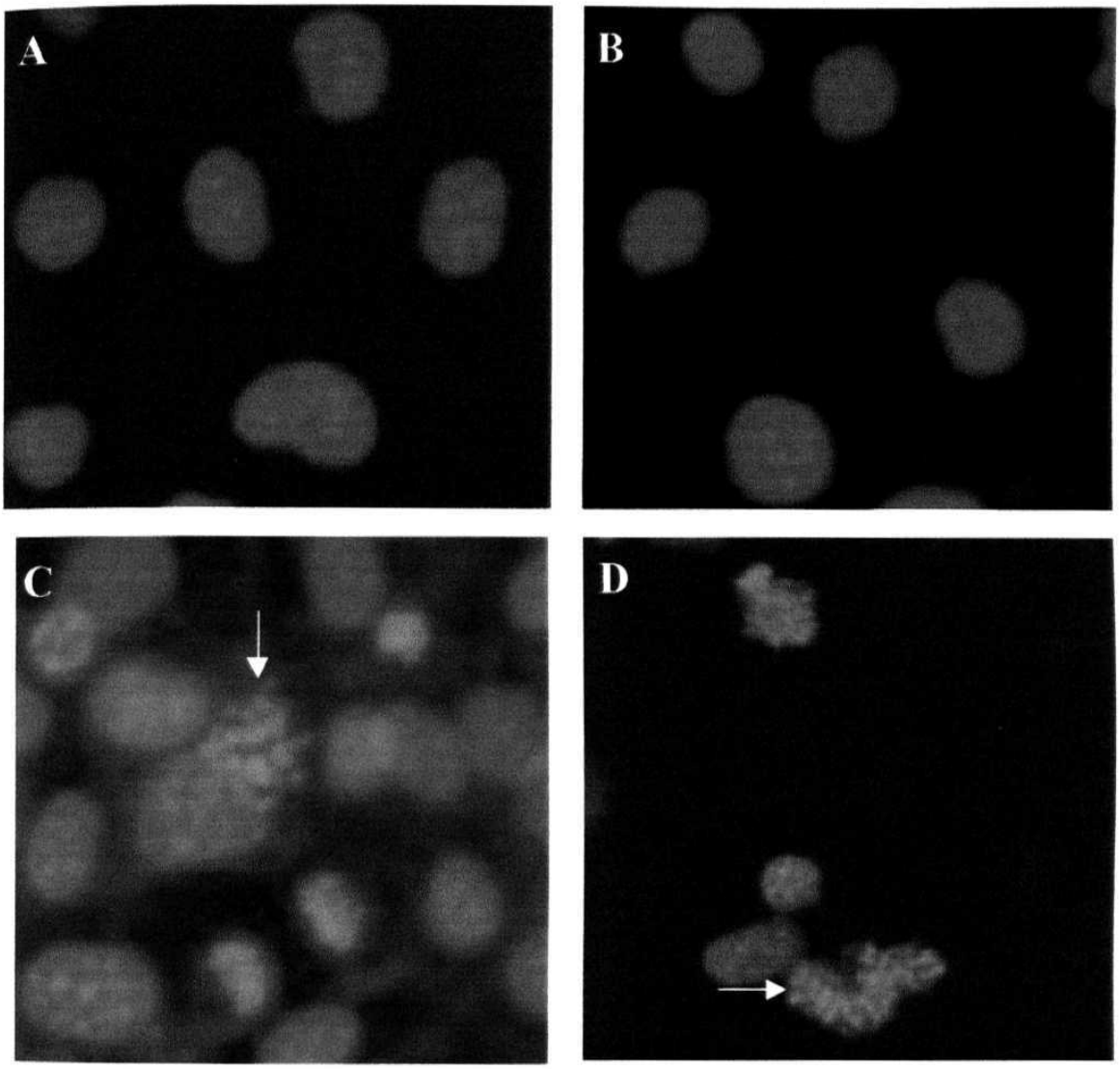


Figure 2.6. Induction of apoptosis in human lens epithelial cells (SRA 01/04) by lens pigments, stained with DAPI.

A = 3-HK, B = 3-HKG, C = XA, D = XA8OG, 30 μM each

Chapter 3

3.0.0. REACTION OF PEROXYNITRITE WITH LENS CRYSTALLINS

Peroxynitrite (ONOO") is a highly potent generator of reactive oxygen and nitrogen species. It is formed by the reaction of two free radicals, namely the superoxide anion ($O_2^{\bullet -}$) and nitric oxide ("NO). As seen from its structure, it is an isomer of the nitrate anion (NO_3^-), but about 36 kcal/m higher in energy. It is an able one-electron oxidant, with an E_{redox} value of 1.4 V at pH 7.0. It readily disproportionates into two potent oxidants, namely $^{\bullet}NO_2$ and $^{\bullet}OH$, or the nitrate anion as:

$$O_2^{\bullet -} + {}^{\bullet}NO \rightarrow {}^{-}ONOO \ (+ H^+) \leftrightharpoons HOONO \rightarrow HNO_3 + {}^{\bullet}OH + {}^{\bullet}NO_2 \quad \ldots (1)$$

The types and yield of radicals produced by peroxynitrite are dependent on the pH of the medium; the yield of the hydroxyl and NO2 radicals is about 30% at pH 6.3, declining at higher pH. Under physiological conditions, it reacts with carbon dioxide to produce a transient adduct that decomposes to yield 67% nitrate and 33% carbonate ($CO_3^{\bullet -}$) and $^{\bullet}NO_2$ radicals, as:

$$ONOO^- + CO_2 \rightarrow (ONOOCOO^-) \rightarrow CO_3^{\bullet -} + {}^{\bullet}NO \quad \ldots (2)$$

Peroxynitrite (abbreviated as PON) is a source of potent free radicals that can oxidize lipids, thiols, amino acids and biopolymers (Hodges *et al.,* 2000). The reaction of peroxynitrite with proteins yields specific products that depend on the presence or absence of physiological

concentrations of bicarbonate. Oxidative reaction products in proteins primarily involve the residues cys, met, trp and tyr (Pryor and Squadrito, 1995). The hallmarks of peroxynitrite-mediated attack on proteins is the formation of 6-nitrotryptophan, 3-nitrotyrosine and 3,3'- dityrosine. Nitrotyrosines have been identified in various pathological conditions and diseases such as atherosclerosis (Moriel and Abdalla, 1997; Leeuwenbergh *et al.,* 1997), Alzheimer's and related syndromes (Hensley *et al.,* 1998; Ischiropoulos, 1998), neurological disorders including amyotrophic lateral sclerosis (Estevez *et al.,* 1999), and some others. Likewise, dityrosine has also been identified as a marker of pathology in a variety of instances such as amyloid fibril formation (Galeazzi *et al.,* 1999), Parkinson's disease (Pennathur *et al.,* 1999), atherosclerotic plaque formation (Leeuwenbergh *et al.,* 1997), cataract of the eye lens (McNamara and Augusteyn, 1980; Wells-Knecht *et al.,* 1993), and oxidative stress such as during exercise (Leeuwenbergh *et al.,* 1999).

Peroxynitrite promotes oxidative damage and is implicated in the pathophysiology of various diseases that involve accelerated rates of nitric oxide and superoxide formation. We focus attention here on one aspect of the oxidative stress imposed by peroxynitrite in the eye. Peroxynitrite is produced endogenously in various tissues of the eye, leading to pathological conditions such as inflammation, uveitis, corneal damage, glaucoma, retinopathy and cataracts (Wu *et al.,* 1997; Allen *et al.,* 1998; Shimizu *et al.,* 1999; Read *et al.,* 2000; Chiou, 2001). One group of proteins that is significantly affected by oxidative stress are the crystallins. We have looked at the reaction of peroxynitrite with a major protein of this

class, namely a-crystallin, *in vitro.* Apart from its structural role in the lens cortex, since a-crystallin is also believed to act in a chaperone-like fashion, inhibiting the aggregation and precipitation of other lens proteins (Horwitz, 1992), we have looked at how the structural and functional properties of α-crystallin are altered upon reaction with peroxynitrite.

3.1.0. MATERIALS AND METHODS

3.1.1. PURIFICATION OF LENS PROTEINS

Bovine or human lenses were homogenised in 0.1 M Tris buffer, pH 7.4, containing 0.5 M NaCl, 1 mM EDTA and 0.1% NaN_3. The three classes of crystallins, a-, β-, and γ-crystallin, were separated from the supernatant by gel filtration chromatography on BioGel-A 1.5 m (1.8 x 0.025m) (Bloemendal, 1981). Each of the crystallins was dialysed against water and stored in the lyophilised form.

3.1.2. PREPARATION OF PEROXYNITRITE (PON)

Peroxynitrite was obtained as described by Koppenol *et al.* (1996), by reacting ice cold solutions of $NaNO_2$ (0.6 M) with H_2O_2 (0.7 M) in an acidic medium (0.6 M HCl), followed by rapid quenching in NaOH (1.5 M). The reaction mixture was frozen at -20°C overnight, and the ONOO" concentrated in the upper yellow layer was collected and its concentration measured at 302nm using a molar extinction coefficient (ε_M) of 1670 M^{-1} cm^{-1}.

3.1.3. PEROXYNITRITE MODIFICATION OF TEST PROTEINS

To the test proteins (1 mg/ml) in 50 mM sodium phosphate buffer, pH 7.4, peroxynitrite (1 mM final concentration in 1.2 M NaOH) was added either in the presence or absence of 10 mM HCO3" with vigorous mixing.

The mixture was incubated at room temperature for 15 min before any further analysis. For control samples, the reaction mix was without peroxynitrite.

3.1.4. SDS/PAGE

After peroxynitrite modification of test proteins, they were analysed by polyacrylamide gel electrophoresis (PAGE) (Hoefer Mighty Small system) according to the Laemmli method (1970), on 12% acrylamide gels with added sodium dodecyl Sulfate (SDS), and stained with silver.

3.1.5. ESTIMATION OF NITROTYROSINE AND NITROTRYPTOPHAN

Nitrated tryptophan, formed upon peroxynitrite modification of crystallins was estimated by measuring the absorbance of 6-nitrotryptophan at 400 nm (ε_M = 5200 $M^{-1}cm^{-1}$) (Alvarez *et al.,* 1996). With tyrosine, 3-nitrotyrosine was estimated in the presence of 0.5-1 N NaOH (4:1 v/v, final pH 11-11.5), at 428 nm (ε_M = 4200 M^{-1} cm^{-1}) (van der Vliet *et al.,* 1995). Dityrosine emission was monitored by fluorescence spectroscopy using an excitation wavelength of 320 nm and emission near 400 nm; its quenching was monitored with 0.5 M borate/boric acid buffer, pH 8.7 (Malencik and Anderson, 1991). Both excitation and emission bandpasses were set at 2.5 nm.

3.1.6. SPECTRAL STUDIES

(i) *Fluorescence spectra:* Intrinsic fluorescence spectra due to tyr and trp were recorded on a Hitachi F-2500 fluorescence spectrophotometer. Excitation wavelengths used for tyr and trp were 275 nm and 290 nm respectively, with a bandpass of 2.5 nm. Fluorescence of the extrinsic probe 8-anilinonaphthalene 1-sulfonic acid (ANS) was monitored using an excitation wavelength of 375 nm and emission in the 500 nm region.

(ii) *Circular Dichroism (CD) Spectra:* Far-UV (250-190 nm) and near-UV (320-250 nm) CD spectra were recorded using a JASCO J-715 spectropolarimeter. The protein concentration was 1.0 mg/ml in 50 mM sodium phosphate buffer, pH 7.4. The near and far UV-CD spectra were recorded using 1-cm and 0.02-cm path length cells, respectively.

3.1.7. FUNCTIONAL STUDIES - ASSAY FOR PROTEIN AGGREGATION

The chaperone-like activity of unmodified and PON-modified α-crystallin was studied using the target protein aggregation assay (Raman *et al.,* 1995).

(i) *DTT-induced aggregation of insulin:* The isothermal aggregation of insulin (0.4 mg/ml in 10 mM phosphate buffer, pH 7.4, containing 100 mM NaCl) was initiated by the addition of 25 μl of 1 M dithiothreitol (DTT) to 1.2 ml of the protein at 37°C. The extent of

aggregation was measured as a function of time by monitoring the scattering at 465 nm. The ratio of a-crystallin : insulin used was 1:2.

(ii) *Thermal aggregation of β_L-crystallin*\ The temperature-dependent self-aggregation of β_L-crystallin was monitored in 10 mM phosphate buffer, pH 7.4, containing 100 mM NaCl at 60°C. The buffer containing unmodified or PON-modified a-crystallin was preincubated at 60°C for 10 minutes prior to the addition of β_L-crystallin to make a final concentration of 0.2 mg/ml β_L-crystallin. The ratio of a-crystallin : β_L-crystallin used was 1 :1, and the aggregation was monitored by measuring light scattering at 465 nm.

3.1.8. TRYPSIN DIGESTION OF HUMAN αA-CRYSTALLIN

Human αA-crystallin, which was cloned and overexpressed in *E.coli,* was a kind gift of Dr. Mohan Rao of the Center for Cellular and Molecular Biology, Hyderabad. The protein was incubated with trypsin in 50 mM Tris.HCl buffer, pH 7.4 at 37°C for 25 min. The ratio of αA-crystallin : trypsin was 100:1. The action of trypsin was stopped by the addition of 10 μg/ml bovine pancreatic trypsin inhibitor (BPTI), and incubating at 37°C for 30 min.

3.1.9. HPLC OF TRYPTIC DIGEST OF αA-CRYSTALLIN

The total tryptic digest of aA-crystallin was loaded and individual peptides were separated by RP-HPLC on a Zorbax C18 column (9.4 x 250 mm),

using 0.1 % TFA for 55 min followed by a linear gradient of 0.4 % per min of acetonitrile in water. Detection was carried out at 214 nm. Injections were repeated to collect sufficient volume of each fraction which was then lyophilised and modified with PON as done earlier.

3.2.0. RESULTS AND DISCUSSION

3.2.1. FLUORESCENCE SPECTRA OF LENS CRYSTALLINS AFTER PEROXYNITRITE MODIFICATION

Figure 3.1 depicts the fluorescence spectra of all three classes of crystallins showing a decrease in emission intensity upon modification with PON. This decrease was further enhanced by bicarbonate. A similar pattern was obtained with two other test proteins, bovine pancreatic RNase A and melittin.

3.2.2. FORMATION OF NITRO-TYR, NITRO-TRP AND DITYROSINE

Earlier studies have shown that the reaction of peroxynitrite with trp and tyr leads to the formation of nitro-trp and nitro-tyr respectively, apart from dityrosine. As can be seen from Fig. 3.2.1, the amounts of nitro-tyr and nitro-trp increases with concentration of PON and further by the presence of bicarbonate. That the relative yields of nitrated products depends on the concentration of PON and the presence of dissolved CO_2 has also been reported in earlier studies (Alvarez *et al.,* 1999; Pfeiffer *et al.,* 2000). Among the crystallins, γ-crystallin was seen to produce the most number of nitrated products, as shown in Fig. 3.2.2.

Exciting at 320 nm and monitoring the emission around 400 nm identified the formation of dityrosine. The band near 400 nm was quenched in the presence of 0.5 M borate/boric acid, which is a specific

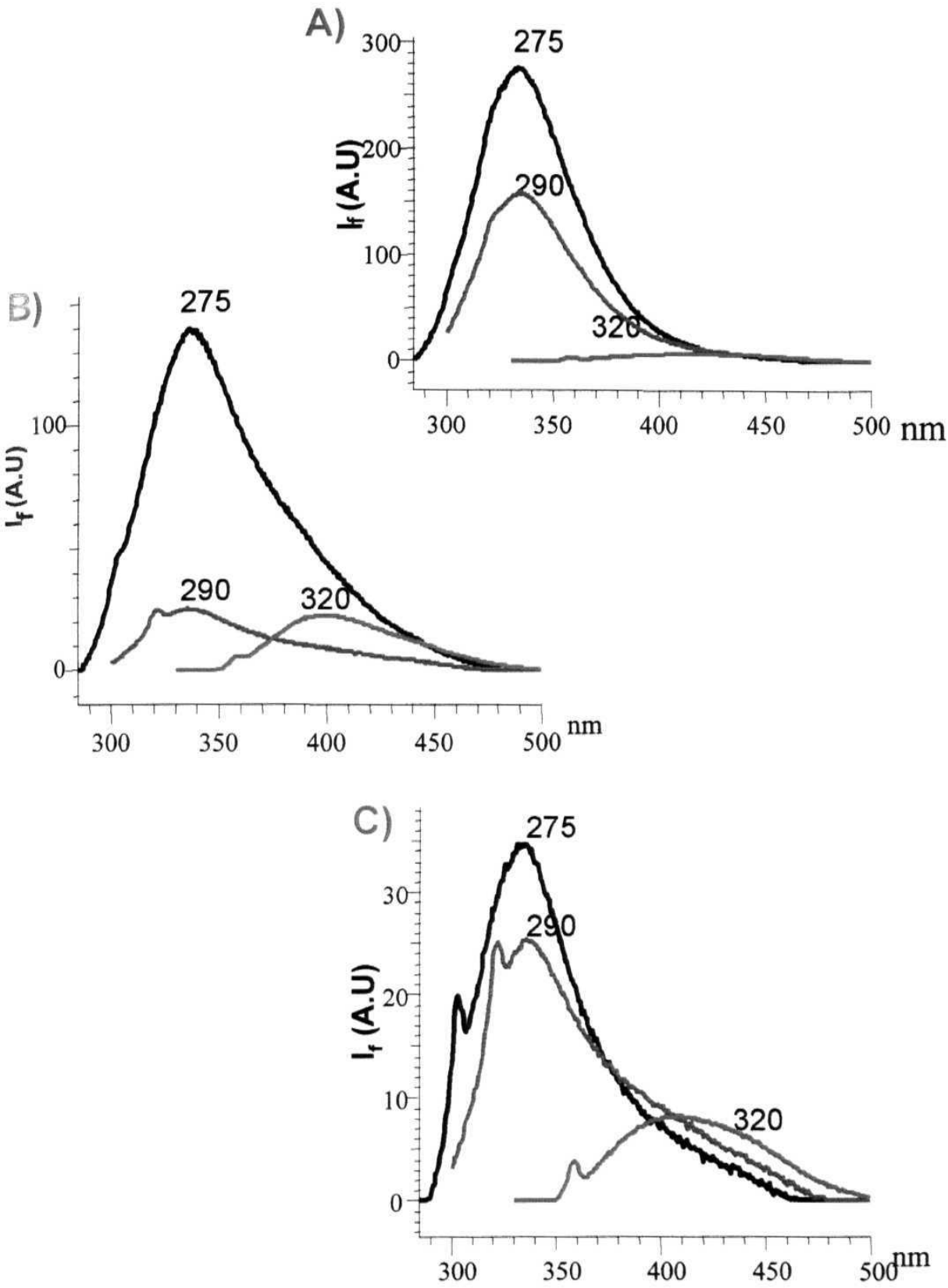


Figure 3.1.1. Fluorescence spectra of a-crystallin with λ_{ex}=275, 290 and 320nm

A = Unmodified a-crystallin
B = a-crystallin + 1 mM PON
C = a-crystallin + 1 mM PON + 10 mM HCO_3^-

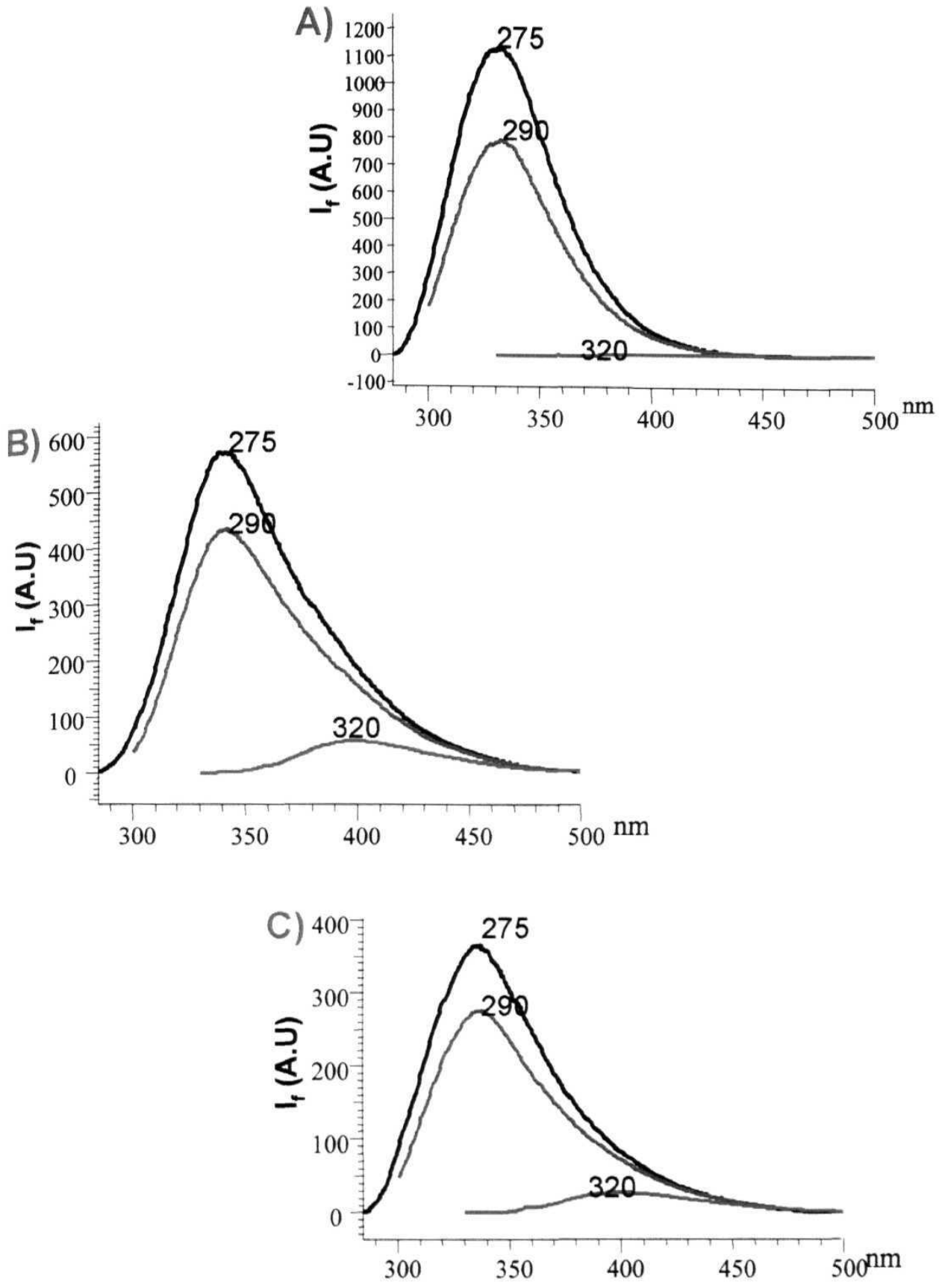


Figure 3.1.2. Fluorescence spectra of β-crystallin with λ_{ex}= 275, 290 and 320nm

A = Unmodified β-crystallin
B = β-crystallin + 1 mM PON
C = β-crystallin + 1 mM PON + 10 mM HCO_3^-

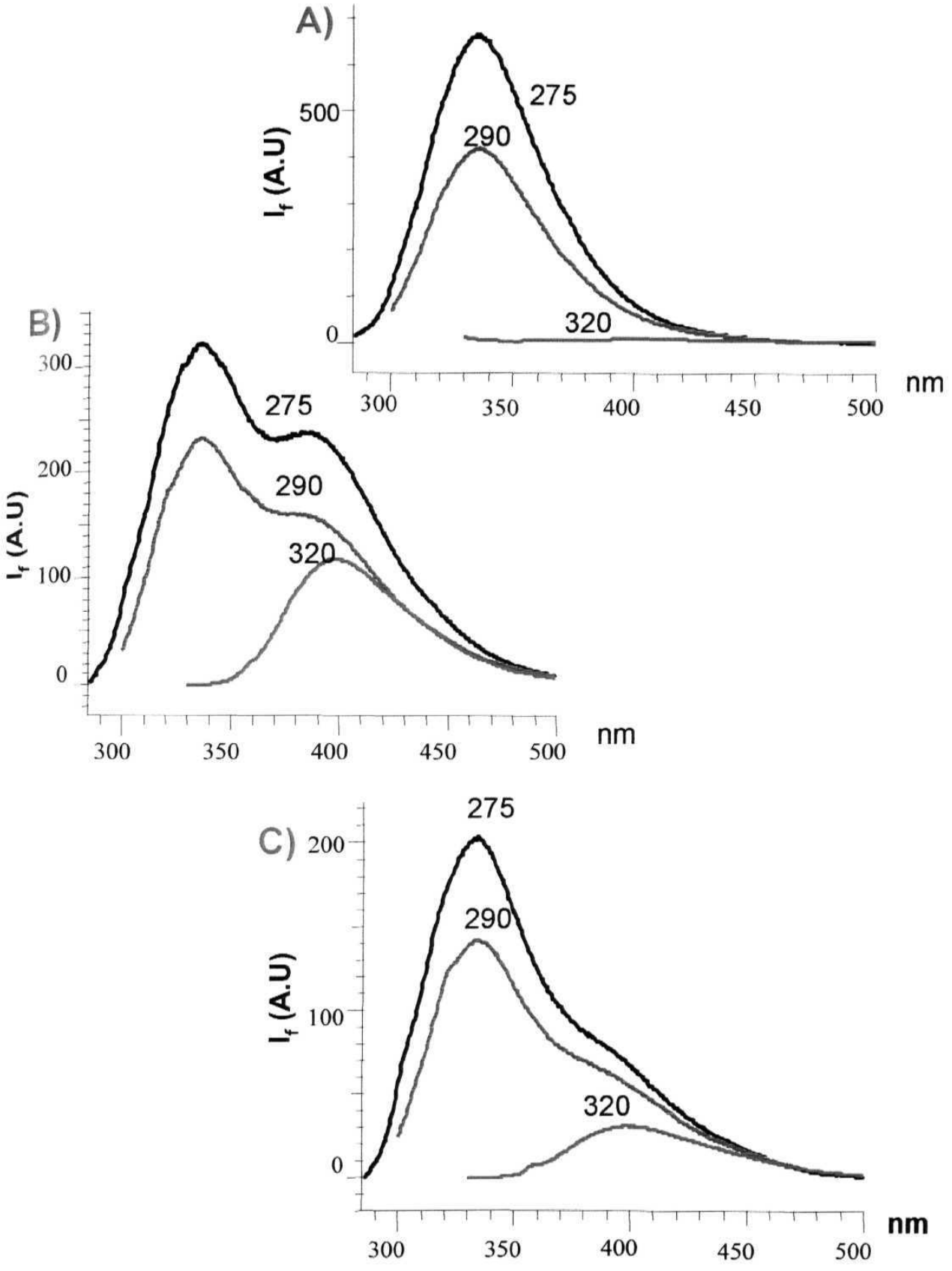


Figure 3.1.3. Fluorescence spectra of γ-crystallin with λ_{ex}= 275, 290 and 320nm

A = Unmodified γ-crystallin
B = γ-crystallin + 1 mM PON
C = γ-crystallin + 1 mM PON + 10 mM HCO_3^-

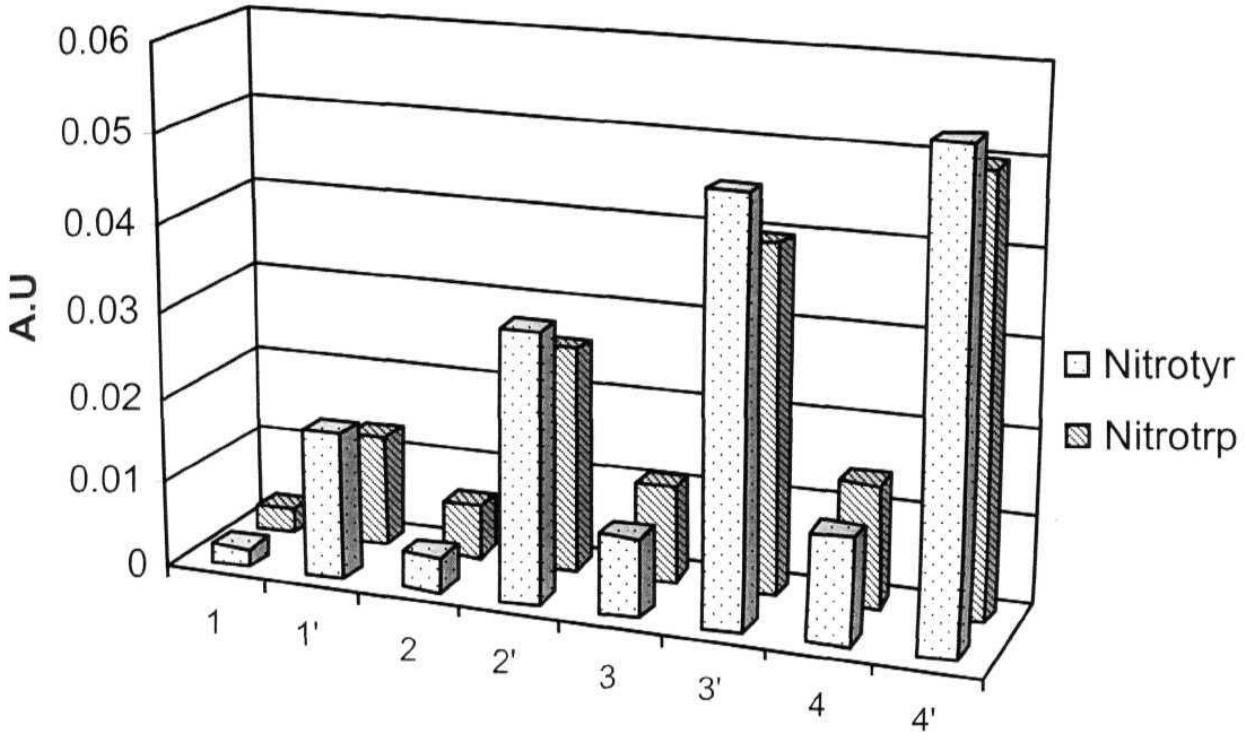


Figure 3.2.1. Nitration of tyr and trp in a-crystallin, upon reaction with:

1 = 0.25 mM PON
2 = 0.5 mM PON
3 = 1 mM PON
4 = 2 mM PON

V = 0.25 mM PON + 10 mM HCO_3^-
2' = 0.5 mM PON + 10 mM HCO_3^-
3' = 1 mM PON + 10 mM HCO_3^-
4' = 2 mM PON + 10 mM HCO_3^-

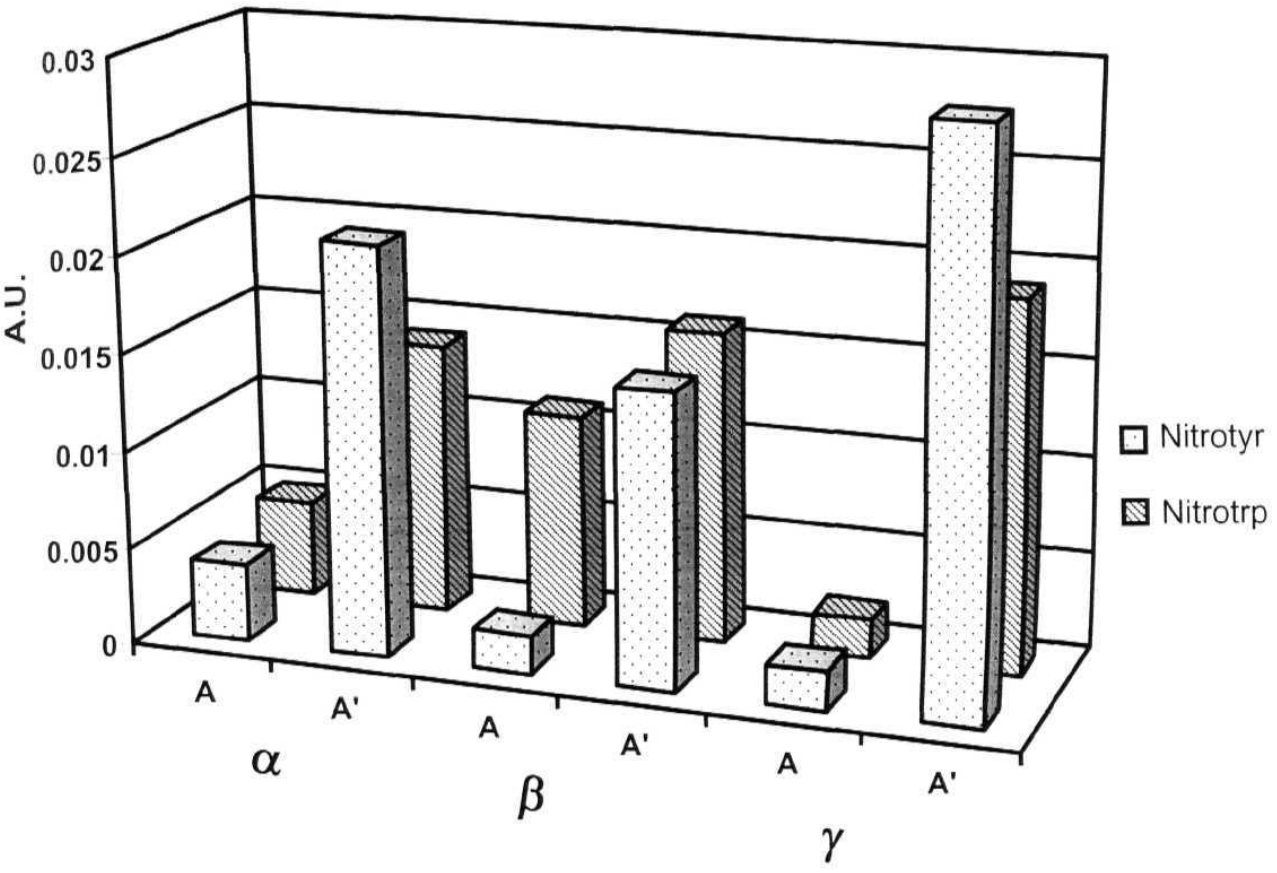


Figure 3.2.2. Nitration of lens crystallins, upon reaction with:

A = 1 mM PON A' = 1 mM PON + 10 mM HCO_3^-

quencher of this fluorophore (Fig. 3.3.1), (Malencik and Anderson, 1991) thus confirming the presence of dityrosine. Figure 3.3.2 shows the relative yields of dityrosine in a-, β- and γ-crystallins; γ-crystallins are seen to form most dityrosine linkages whereas α-crystallin forms the least, consistent with the constituent number of tyr and trp residues.

Amino acid analysis conducted on samples of a-crystallin revealed that the only residues affected by PON were Met and Tyr, though modifications to Cys and Trp could not be determined by this method.

3.2.3. SDS/PAGE WITH TEST PROTEINS

Figure 3.4 shows the SDS-PAGE profile of the reaction products of the crystallins with peroxynitrite. With all the proteins, reaction with peroxynitrite leads to both high molecular weight aggregates and degradation products. The proportion of the low molecular weight degradation products is reduced when the reaction is conducted in the presence of bicarbonate. Densitometric analysis of the protein bands in the gel revealed the maximum reduction in the degradation products intensity from 28% to 7% in the case of γ-crystallins, when the reaction was done in the presence of bicarbonate; with |3- it reduced from 41% to 34% and with a-crystallin the degradation itself was minor. Since peroxynitrite by itself disproportionates to produce $^{\bullet}OH$ but generates $CO_3^{\bullet-}$ when CO_2 is added ($^{\bullet}NO_2$ is produced in both instances), it would seem that $^{\bullet}OH$ causes more peptide chain degradation than $CO_3^{\bullet-}$. We found similar results when the reactions were conducted with RNase A. It

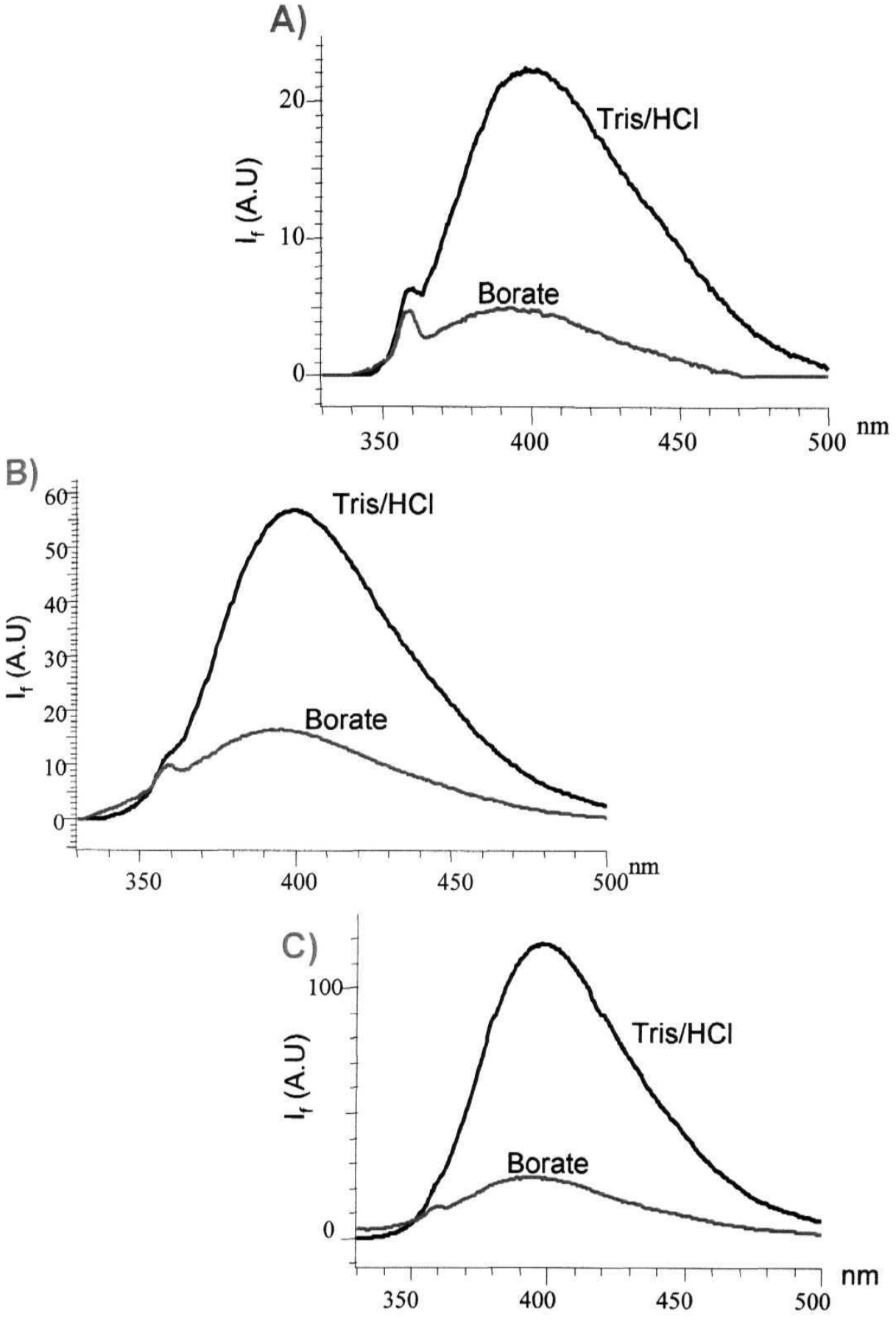


Figure 3.3.1. Borate quenching to confirm dityrosine formation with λ_{ex}= 320nm

A = α-crystallin B = β-crystallin C = γ-crystallin

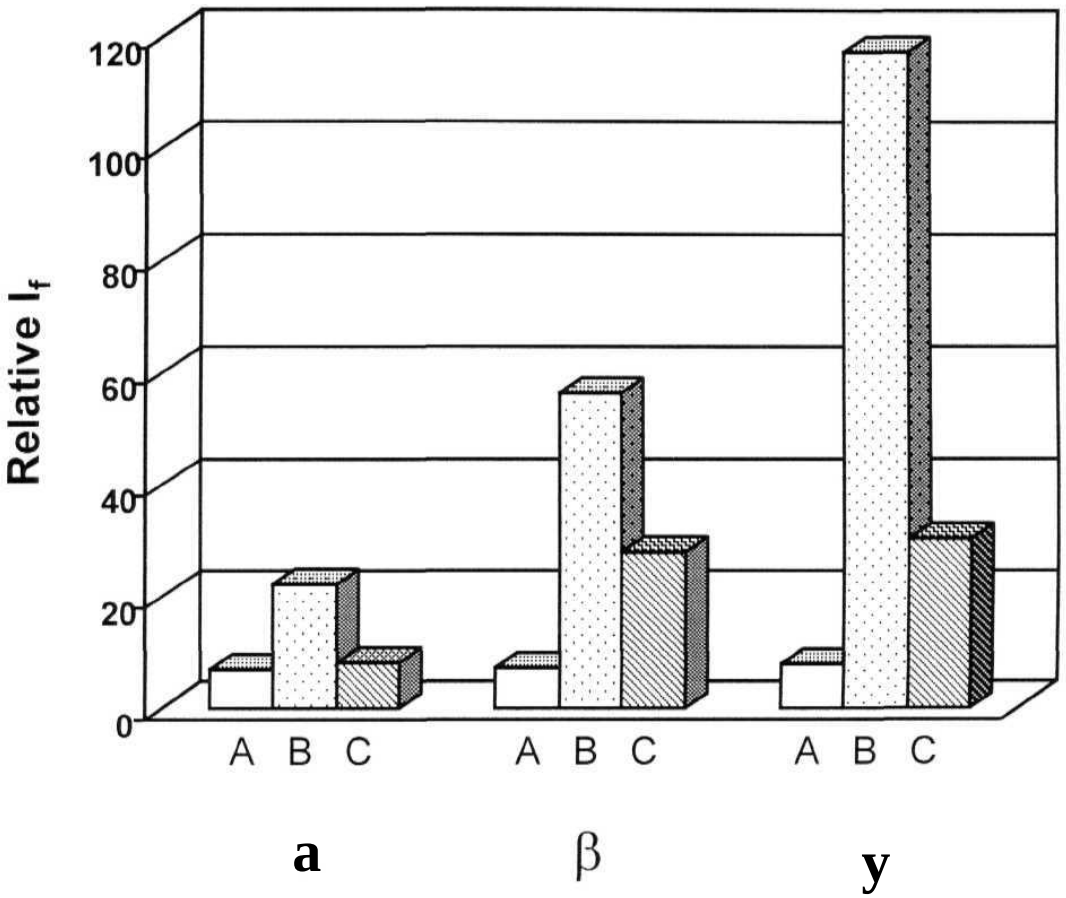


Figure 3.3.2. Dityrosine formation in lens crystallins

A = Control
B = upon reaction with 1 mM PON, and
C = upon reaction with 1 mM PON + 10 mM HCO_3^-

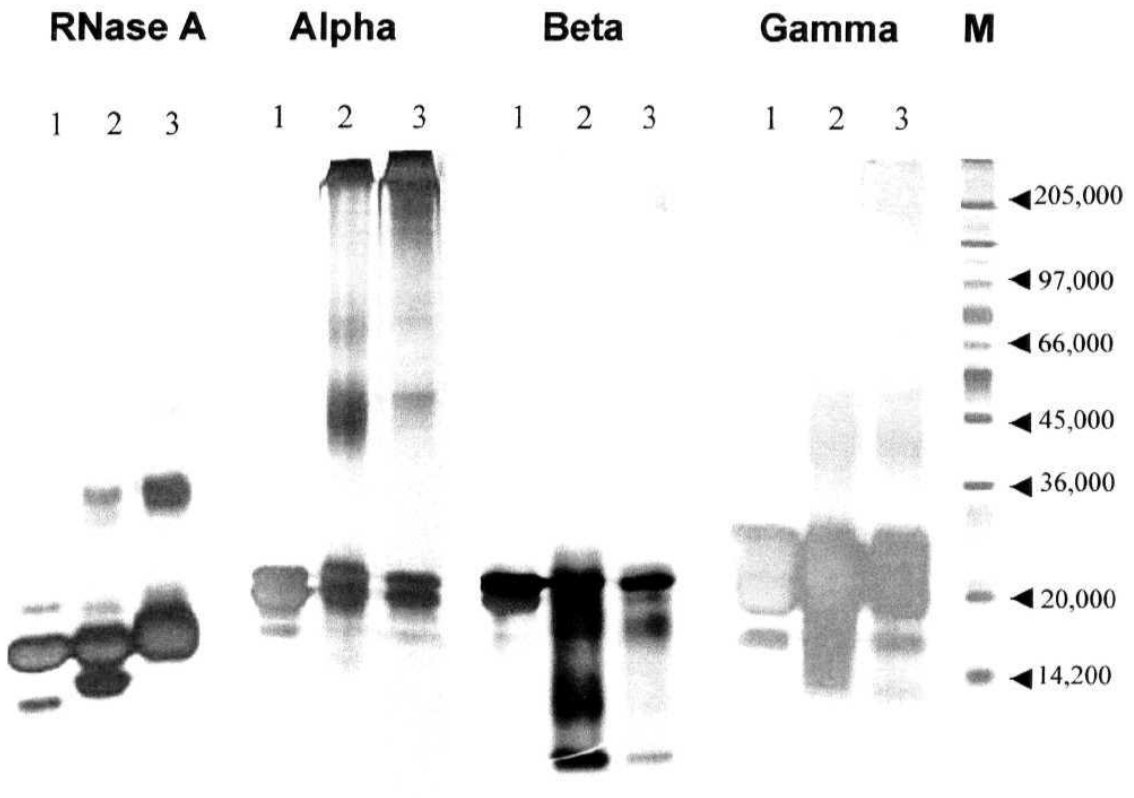


Figure 3.4. SDS/PAGE of RNase A, a-, p-, and y-crystallin

Lane 1 = Unmodified protein
Lane 2 = Protein + 1 mM PON
Lane 3 = Protein + 1 mM PON + 10 mM HCO_3^-

has been noted that 'OH is a stronger one- electron- oxidizing radical than $^{\bullet}NO_2$ or $CO_3^{\bullet-}$ (Hodges *et al.,* 2000).

3.2.4. STRUCTURAL ALTERATION IN α-CRYSTALLIN

α-Crystallin is thought to be involved in the maintenance of eye lens transparency via its chaperone-like function. Hence, we focussed our attention on the structure and function of α-crystallin after its reaction with PON. The effect of PON reaction on the conformation of a-crystallin was recorded by following the far-UV and near-UV CD spectra of the protein. As seen in Fig. 3.5, the largely (3-sheet conformation of the native protein was reduced (around 218 nm) and a greater degree of chain disorder (around 205 nm) was noticed in the far-UV CD spectrum upon PON modification. The tertiary structure was found to be altered too, where the band intensities around 250-260 nm (ascribable to Phe) were seen to increase, and the weak band around 290 nm (ascribable to Trp) also showed significant alteration. This could indicate some tertiary structural rearrangement around the aromatic residues upon reaction with PON.

3.2.5. CHAPERONE-LIKE ACTIVITY OF MODIFIED α-CRYSTALLIN

The conformation of a-crystallin having changed, we next studied the ability of PON-modified a-crystallin to act as a chaperone. Figure 3.6 shows the aggregation of test proteins, insulin and β_L-crystallin, and inhibition of this process upon the addition of a-crystallin. Despite the

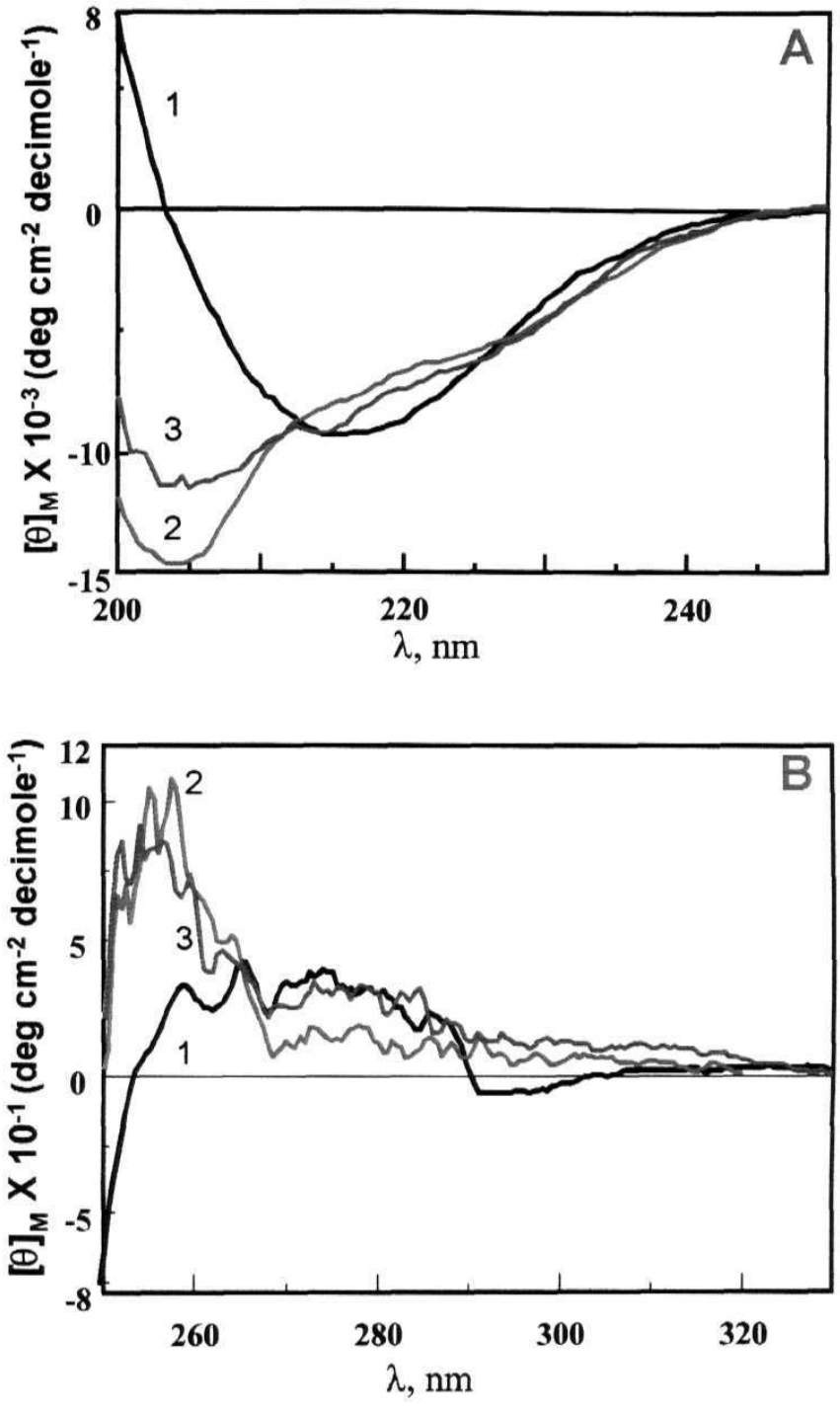


Figure 3.5. Far- UV (A) and Near-UV (B) CD spectra of α-crystallin

1 = Unmodified protein
2 = Protein + 1 mM PON
3 = Protein + 1 mM PON + 10 mM HCO_3^-

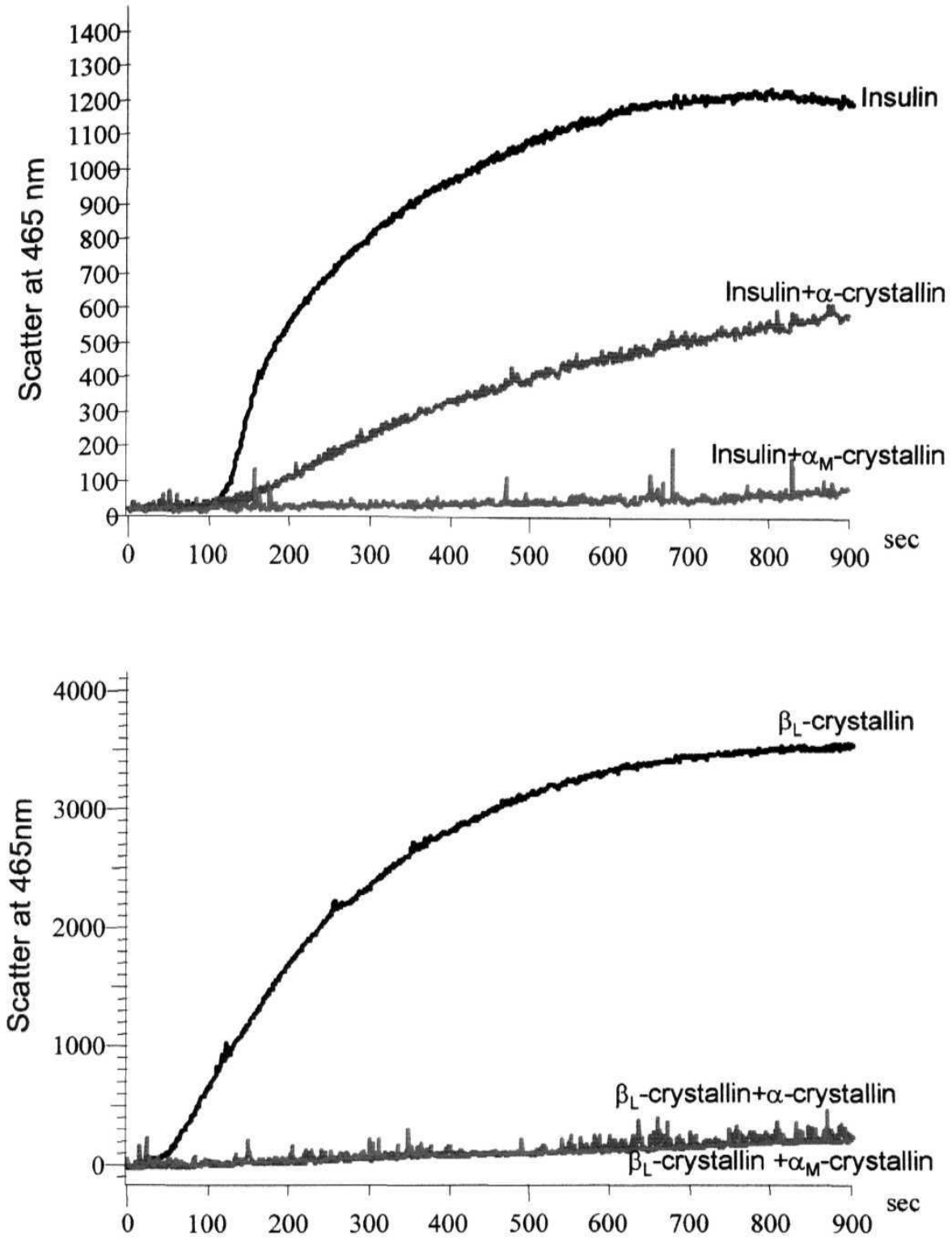


Figure 3.6. Chaperone-like activity of α-crystallin

Top: DTT-induced aggregation of insulin at 37°C

Bottom: Thermal aggregation of β_L-crystallin at 60 °C
α_M = a-crystallin modified with 1 mM PON

oxidative modification, and changes in the chain conformation, the peroxynitrite- treated protein is seen to be just as good as the parent molecule, if not somewhat better in protecting insulin from aggregation. Peroxynitrite-modified α-crystallin displays chaperone-like ability that is comparable to the unmodified parent molecule in preventing the thermal aggregation of β_L-crystallin as well.

α-Crystallin is thought to exert its chaperone-like action due to its ability to present a hydrophobic receptor surface to the aggregation-prone target protein (Raman and Rao, 1994; Raman *et al.,* 1995). This receptor surface can be blocked by the addition of the peptide melittin, thus leading to an inhibition of the chaperoning ability of a-crystallin (Sharma *et al.,* 2000). We found the chaperone-like activities of both parent a-crystallin and its peroxynitrite-modified product to be lost upon the addition of melittin to the solution, thus suggesting that both the proteins present a similar type of surface to the target protein.

To explain the retention of its chaperone function in spite of PON-modification, we considered the possibility of modified a-crystallin exposing a relatively greater hydrophobic surface. This possibility was checked by using the method of ANS-binding to the parent and modified a-crystallin, where the emission intensity of the probe increases in a hydrophobic environment (Stryer, 1968). However, we found ANS binding to be reduced in the case of the modified protein than with the parent a-crystallin (by as much as 45%, as estimated by the intensity of the fluorescence of protein-bound ANS). It thus appears that the modified

protein does not make available any more hydrophobic surfaces than the parent. To make sure that the activity was not due to the presence of any residual unmodified α-crystallin, we tried different ratios of target : α-crystallin, and we found that the modified protein could prevent aggregation even at ratios of 1:4. (Table 3.1).

It is being increasingly suggested that the presentation of a hydrophobic surface might not be the major determinant for the chaperone-like action of a-crystallin. Bhattacharyya *et al.* (2002) have recently shown that while αA-crystallin is more hydrophobic than aB-crystallin, their chaperone-like activities are not quantitatively related to subunit hydrophobicity. Farahbakhsh *et al.* (1995) have shown that the chaperone inhibitor melittin binds to the protein in non-hydrophobic regions as well. And van Rijk *et al.* (2000) have shown that the exon-duplicated product called "super a-crystallin" does not show chaperone ability, despite its increased exposure of hydrophobic patches, and a larger complex size. Indeed the results with insulin point to the possibility that the target protein-binding surface in the modified a-crystallin may comprise non-hydrophobic regions.

3.2.6. PEROXYNITRITE MODIFICATION OF MINI αA-CRYSTALLIN

Certain regions of a-crystallin have been suggested to be chaperone sites by crosslinking of melittin and bis-ANS binding studies. Such regions identified as functional chaperone elements are the segments KFVIFLDVKHFSPEDLTVK (70-88) in aA-crystallin and

Table 3.1. Light scattering at 465 nm, 450s after initiating the reaction

α-crystallin	a-crystallin : insulin				a-crystallin : β_L-crystallin				
	0:1	1:2	1:3	1:4	0:1	1:1	1:2	1:3	1:4
Unmodified	2400	900	1450	7280	1580	680	880	2080	2230
PON modified	2400	670	880	2430	1580	530	675	1100	1325

FSVNLDVK (75-82) in aB-crystallin. The 19 amino acid peptide sequence DFVIFLDVKHFSPEDLTVK from αA-crystallin was found to possess an anti-aggregating property similar to the aA-crystallin, and has therefore been called the mini-αA-crystallin (Sharma, 2000).

We took into consideration two possibilities to explain the chaperone-like activity of oxidized a-crystallin. Either the functionally active element of the chain (the 19 aa peptide mentioned above) is inert to the reaction of PON, or there are additional active sites in the protein. We therefore decided next to study the effect of peroxynitrite treatment on the structure and conformation of the functional element sequence 71-88 of aA-crystallin. Based on considerations of solubility, we chose to work with the sequence DFVIFLDVKHFSPEDLTVK, which has been shown to be just as active in its chaperoning ability as whole α-crystallin. Prof Krishna K. Sharma of the University of Missouri was kind enough to spare us a sample of this fragment. Circular dichroism spectra, shown in Fig. 3.7, revealed conformational changes in this peptide upon peroxynitrite treatment, which were quite similar to those observed in the parent protein. While we found this peptide to protect the target protein from aggregating and precipitating out of solution (Fig. 3.8, with lysozyme as the target protein, ratio of mini aA-crystallin:lysozyme being 1:1), it failed to do so after it was reacted with peroxynitrite. Its chaperoning ability was lost upon oxidation, a result that is in contrast to what was obesrved with a-crystallin. This raised the possibility that there might be other regions in the aA-

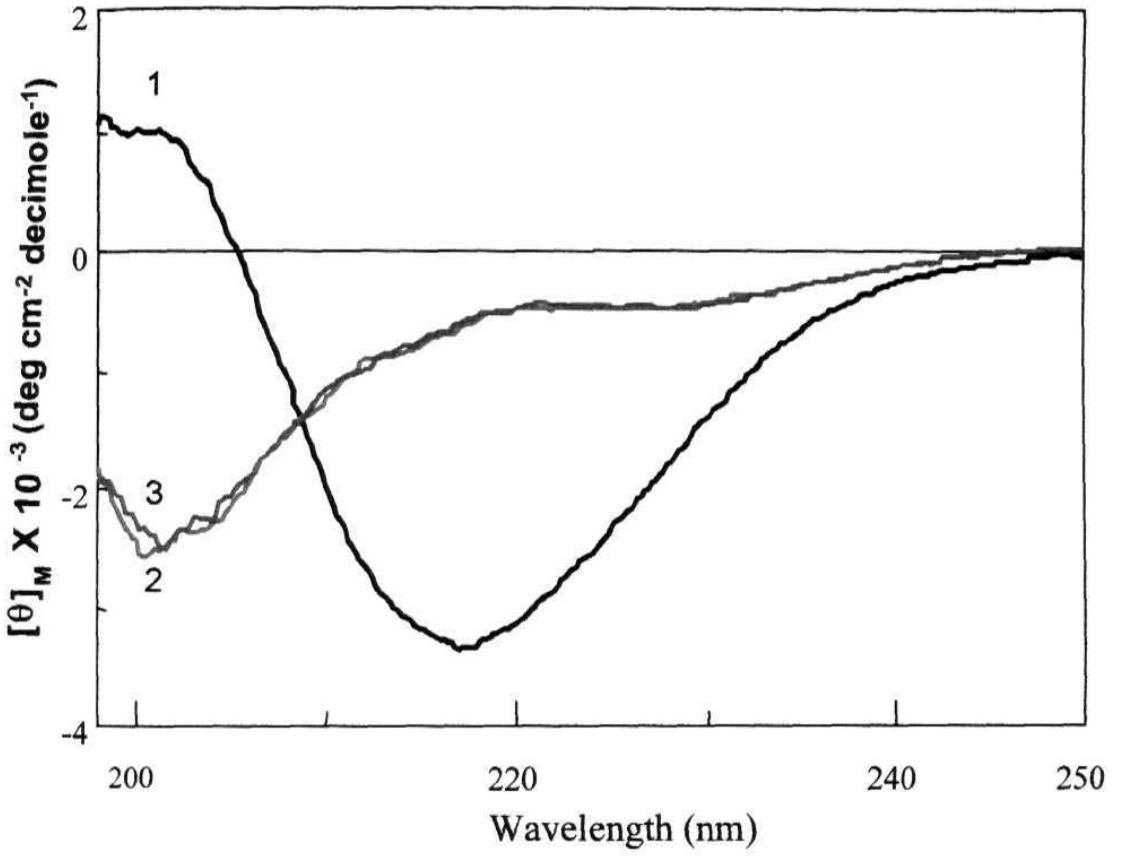


Figure 3.7. Far- UV CD spectra of chaperone-element peptide of αA-crystallin

1 = Unmodified protein
2 = Protein + 1 mM PON
3 = Protein + 1 mM PON + 10 mM HCO_3^-

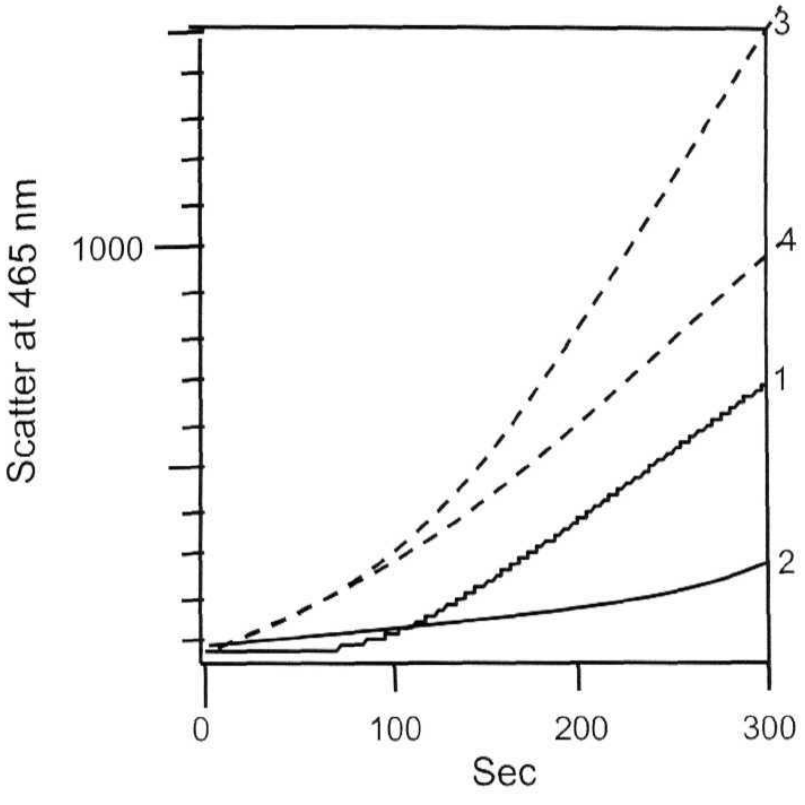


Figure 3.8. Chaperone-like activity of mini aA-crystallin

DTT-induced aggregation of lysozyme at 37°C.
1 = lysozyme alone
2 = lysozyme + unmodified mini aA-crystallin
3 = lysozyme + mini aA-crystallin modified with 1 mM PON
4 = lysozyme + mini aA-crystallin modified with 1 mM PON + 10 mM HCO_3^-

crystallin molecule, besides the sequence 71-88, which contribute to its chaperone-like ability.

3.2.7. TRYPSIN DIGESTION OF αA-CRYSTALLIN

Limit-digestion of human αA-crystallin using trypsin, isolation of individual peptide fragments and measuring the chaperone-like ability of each of these upon reaction with peroxynitrite, revealed one fraction displaying chaperone-like activity both against insulin and β_L-crystallin even when oxidized by peroxynitrite (Fig. 3.9). The peptide (called peptide X) performed better in preventing the DTT-induced aggregation of insulin (1:2 peptide:insulin) than against thermal aggregation of β_L-crystallin, indicating its sensitivity to high temperature. The structural consequence of the reaction of peroxynitrite with this peptide could not be assessed. When the formation of nitrated products of tyr and trp was measured in the peptide, 3-nitrotyrosine was detected which indicated the presence of at least one tyr within the peptide, while no 6-nitrotryptophan was detected. ANS binding ability of this peptide was similar before and after peroxynitrite modification. MALDI/MS of this fraction revealed multiple masses, suggesting that this fraction may comprise a mixture of peptides. Either one or all of these peptides may be responsible for the chaperone-like activity of oxidized α-crystallin. This fraction needs to be further purified and the active peptides should be identified.

Direct information regarding the structure of a-crystallin is lacking because neither the oligomer nor its composite subunits have been

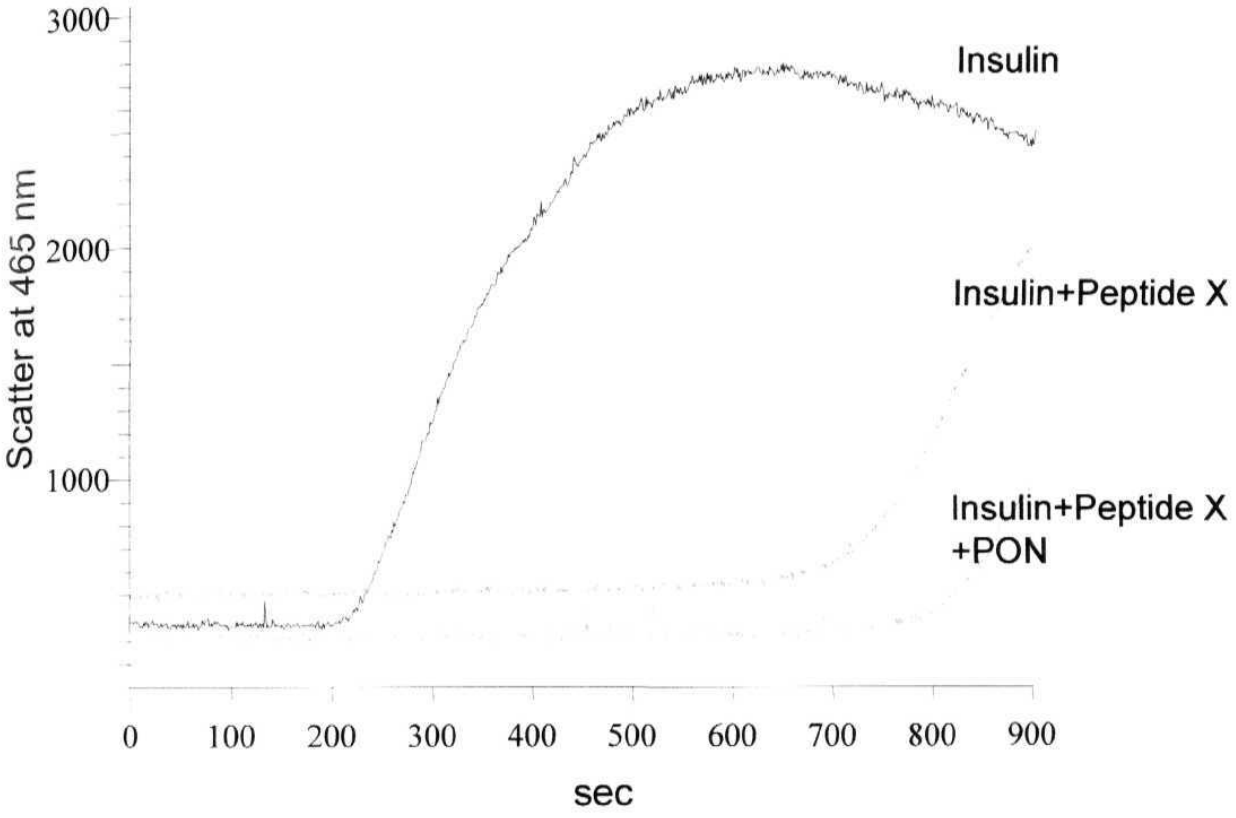


Figure 3.9. Chaperone-like activity of peptide X of a-crystallin

DTT-induced aggregation of insulin at 37°C.
Ratio of insulin: peptide X = 2:1

crystallized. Therefore, it is difficult to predict where the stretch of peptide(s) showing activity would lie in the protein. Perhaps, it lies in a region where it is inaccessible to reaction with peroxynitrite, or as shown by us it retains its ability to function as a chaperone despite oxidation. The ability of the peptide(s) to prevent protein aggregation needs to be confirmed by synthesizing the peptide(s) and looking for its chaperone-like activity.

But the overall results indicate that the lens protein α-crystallin can act as a chaperone under conditions of oxidative stress, decreasing the light scatter of other constituent proteins in the lens. Some earlier reports (Wang and Spector, 1995; Pal and Ghosh, 1998; Dhir *et al.,* 1999) had also indicated this possibility, and our present results bear this out. Also, as a comparison, we photo-oxidized a-crystallin in solution, using riboflavin as the sensitizer, and monitored the chaperoning ability of the oxidized protein. Here again, though spectral changes indicated modifications in the structure and conformation of the molecule, its chaperoning action was not affected in any significant manner, in comparison to the parent crystallin (the photo-oxidized molecule displayed about 90% chaperoning ability of the native unmodified a-crystallin, when tried against the target protein, βL-crystallin). These results are somewhat similar to those seen by Dhir *et al.* (1999), who found that a-crystallin photo-oxidized at 300 nm, and also the W9F mutant of the protein, retain chaperoning ability. Likewise, Weinreb *et al* (2000) found that the protein from young calf lenses does not lose its chaperoning ability after 24 h of UV-A light exposure, though the same from old lenses does show decreased activity after similar treatment.

Although it is assumed that small heat-shock proteins interact with denaturing proteins via their hydrophobic surfaces, Smulders *et al.* (1995) showed that charged residues in a-crystallin can also influence the efficiency of substrate binding. To investigate the structure and chaperone-like activity, four mutants of bovine αA-crystallin were generated by site-directed mutagenesis. In comparison with wild-type aA-crystallin, the D69S mutant, in which a highly conserved charged residue has been replaced, was seen to form larger multimers and display a three-fold reduced chaperone-like activity while the conformation and thermal stability was not noticeably affected. To study the hydrophobic inter-subunit interactions, three other mutations, replacing hydrophobic by uncharged hydrophilic residues, were studied. These mutations did not show major structural perturbations and only minor differences in chaperone activity were observed.

Site-directed mutagenesis has also been used earlier to identify regions of a-crystallin necessary for chaperone function. When chaperone function of the mutants was assessed most mutants were found to have full chaperone function showing the robust nature of a-crystallin. A mutant corresponding to a minor component of rodent aA-crystallin, αA^{ins}-crystallin, had decreased chaperone function. Decreased chaperone function was also found for human S139R, T144R, S59A mutants of aB-crystallin and double mutants S45A / S59A, K103L / H104I, and E110H / H111E (Derham *et al.*, 2001).

In order to investigate the differences between αA- and αB-crystallins, Kumar and Rao (2000) made chimeras by N-terminal domain swapping of aA and B which resulted in an engineered protein with significantly enhanced chaperone-like activity. These findings show the importance of the N- and C-terminal domains of aA- and αB-crystallins in subunit oligomerization and chaperone-like activity.

Likewise, a mutant of porcine αB-crystallin with C-terminal lysine truncated end was constructed by Liao ef *al.* (2002). This mutant possessed similar secondary and tertiary structures to recombinant aB-crystallins, but showed better chaperone-like function than wild-type aB-crystallin and was smaller than wild-type aB-crystallin in aggregation size and mass.

A point mutation of a highly conserved arginine residue in aA- and aB-crystallins was shown to cause autosomal dominant congenital cataract (Litt *et al.,* 1998) and desmin-related myopathy (Vicart *et al.,* 1998) respectively, in humans. The mutations R116C in αA-crystallin and R120G in aB-crystallin were seen to reduce the chaperone-like activity of these proteins significantly with an altered packing of the secondary structural elements and a change in tertiary structure of the mutants. These results suggest the importance of the conserved arginine of the a-crystallin domain of the small heat shock proteins in the structural integrity and subsequent *in vivo* function (Kumar ef *al.,* 1999).

The function of the eye lens is to receive and focus light, and it is thus under continual photic stress, which is largely oxidative in character. Because oxidative stress is known to be present under usual physiological conditions, it appears probable that the protein a-crystallin has evolved to be able to contribute to the mechanisms that maintain the lens in a transparent state. It has been shown earlier that the chaperone activity of a-crystallin is decreased by chemical agents like methylglyoxal and 1,2-cyclohexanedione, which cause extensive modification of the protein (Derham and Harding, 2002). This highlights the robust nature of α-crystallin. It is also worth noting here that while the other lens proteins, such as the y-, 8-, ε-, T-, ζ-, ρ-crystallins, show taxon specificity (Wistow and Kim, 1991; Tomarev and Piatigorsky, 1996), a-crystallin is found ubiquitously, and in high homology, across animal species. Also, it has high homology in its sequence with the small heat shock proteins or sHSPs (Ingolia and Craig, 1982) and, just like the latter, displays chaperone-like properties, which are of functional use in the lens. Its structural and functional robustness thus appears to have been selected through evolution, particularly for its role in the eye lens where molecular turnover is extremely sluggish.

Chapter 4

4.0.0. ROLE OF ANTIOXIDANTS IN DELAYING ONSET OF CATARACT

Age-related cataract is the leading cause of visual loss worldwide. The recently launched Vision 2020, a global initiative for the elimination of avoidable blindness introduced by the World Health Organization and other organizations singled out cataract as the priority disease (Thylefors, 1999). Effective surgical procedures are available for treatment, but besides the requirement of highly trained personnel, the problem of post-operative complications, cost of surgery and high number of people requiring surgery pose a substantial economic burden.

As seen earlier, oxidative stress, either as the primary event or secondary to risk factors like aging and smoking, is one of the predominant factors that lead to cataract. A major mode of damage to lens proteins, therefore, involves oxidative reactions (Zigler and Goosey, 1981; Zigler *et al.,* 1985; Young, 1991). For this reason, the possible role of antioxidants in delaying the onset or progression of age-related cataract has gained considerable interest. Endogenous defence mechanisms which protect the lens against oxidative damage include compounds like glutathione, ascorbate and antioxidant enzymes like catalase, SOD, GR, GPx etc. (Zigman *et al.,* 1979; Varma *et al.,* 1984; McCay, 1985). But with increasing age, the levels of these protective enzymes are known to decline in the human eye (Garland *et al.,* 1988; Berman, 1991). Supplementation with antioxidants thus appears to be an attractive possibility to delay the onset of age-related cataract. It has been estimated

that a delay in cataract formation of approximately 10 years would reduce the cataract surgical burden by perhaps 45% (Kupfer, 1984).

4.0.1. CAROTENES, VITAMIN A AND CATARACT: EPIDEMIOLOGICAL EVIDENCE

Most research on nutrition and cataract has been concerned with antioxidant vitamins (vitamins A, C and E). Consistently, several studies have found vitamin A intake to be associated with a reduced risk of cataract (Hankinson *et al.,* 1992; Knekt *et al.,* 1992; Mares-Perlman *et al.,* 1995; Cumming *et al.,* 2000). A prospective study on the effect of carotenes and vitamin A on the risk of cataract formation showed that women in the highest quintiie for consumption of lutein and zeaxanthin had a 22% decreased risk of cataract extraction (Chasan-Taber *et al.,* 1999) while men had a 19% decreased risk of cataract extraction when age, smoking and other risk factors were controlled for (Brown *et al.,* 1999). Another cohort of the Nurses' Health Study found women in the highest quintiie of vitamin A consumption had a 39% lower risk of developing cataracts compared to women in the lowest quintiie (Hankinson *et al*.. 1992).

4.0.2. VITAMIN E: ANIMAL, EPIDEMIOLOGICAL AND CLINICAL STUDIES

Several human studies found low levels of vitamin E intake to be associated with higher risk for cataract development (Tavani *et al.,* 1966;

Robertson *et al.,* 1989). The Vitamin E and Cataract prevention study (VECAT) found a statistically significant relationship between past vitamin E supplementation and prevention of cortical cataract but not nuclear cataract (Nadalin *et al.,* 1999). The Lens Opacities Case-Control Study, which was designed to determine the risk factors for cataract showed that the risk of developing cataract was reduced to less than half in subjects with higher levels of vitamin E (Leske *et al.,* 1995). Lens opacities were examined and the risk of development of cataract was 30% less in regular users of multivitamins and 57% less in regular users of supplemental vitamin E (Leske *et al.,* 1998). The 10-year Linxian Cataract Study found a significant reduction in the incidence of cataract in those taking a daily supplement of antioxidant vitamins. Supplementation also delayed the necessity of corrective surgery by as much as 10 years (Sperduto *et al.,* 1993).

A randomized trial with 50 subjects showing early cataracts, receiving either 100 mg vitamin E twice daily or placebo for 30 days, concluded that vitamin E decreases oxidative stress in cataractous lenses and that the protective effect of vitamin E is due to enhancement of GSH levels (Seth and Kharb, 1999). Though some evidence exists to support a relationship between nuclear cataract and low levels of dietary intake and plasma vitamin E, results are not consistent. Plasma α-tocopherol was inversely associated with nuclear opacities in the Baltimore Longitudinal Study on aging (Vitale *et al.,* 1993) and in the LOCS with both prevalent (Leske *et al.,* 1995) and incident (Leske *et al.,* 1998) nuclear opacities, but was not associated with nuclear opacities in the Italian American Cataract

Study (The Italian-American Cataract Study Group, 1991) or the India-US case-control study (Mohan *et al.,* 1989).

4.0.3. VITAMIN C AND RISK OF CATARACTS

Ascorbic acid is normally found in high concentrations in the aqueous humor (1.1 mM) and lens (3.6 mM) of humans (Taylor, 2000). Vitamin C is shown to prevent hydrocortisone-induced cataract in chick embryos (Nishigori *et al.,* 1985). In the Nurses' Health Study, vitamin C supplementation for a period of 10 years or greater was associated with a 77% lower incidence of early lens opacities and 83% lower incidence of moderate lens opacities (Jacques *et al.,* 1997). The most comprehensive epidemiological study of diet and cataract is the nutritional component of the Beaver Dam Eye Study (Mares-Perlman *et al.,* 1995) which found numerous nutrients to be associated with reduced risk of nuclear cataract, like vitamins A, C, E, riboflavin, thiamin, niacin, and folate.

To determine if a mixture of oral antioxidant micronutrients (beta-carotene (18 mg/day), vitamin C (750 mg/day), and vitamin E (600 mg/day)) could modify progression of age-related cataract, a 3-year multi-centered, prospective, double-masked, randomized, placebo-controlled trial was carried out with 445 patients, called the Roche European American Cataract Trial (REACT). After two years of treatment, there was a small positive treatment effect (P = 0.0001) and after three years a positive effect was apparent (p = 0.048) in the patients (Chylack *et al.,* 2002).

The Age-related Eye Disease Study (AREDS) Group conducted a randomized, placebo-controlled, clinical trial of high-dose supplementation for age-related cataract. After an average treatment time of 6.3 years, the antioxidant formulation containing high doses of vitamins C, E, beta-carotene and/or zinc had no statistically significant effect on the development or progression of age-related lens opacities or cataract surgery (Age-related Eye Disease Study Research Group, 2001). However, the AREDS design included relatively well-nourished participants compared with the general population. Also, interpreting the AREDS results requires a consideration of the time and duration of the antioxidant intervention. All AREDS participants were 55 years or older at enrollment, and it is therefore likely that cataracts had probably already started to develop.

4.0.4. ANTIOXIDANTS FROM PLANT SOURCES

While the connection between antioxidant intake and lowering the risk of cataract is clear from the above, supplementation of antioxidants on a regular basis is beyond the economic reach of people in the developing world who form the major fraction of cataract-afflicted across the globe- However, they include a range of plant material, rich in antioxidants and micronutrients, in their diet. Hence we have studied the role of certain plant extracts that are traditionally more acceptable and which form a part of the daily diet of many people, e.g. tea and greens. Earlier work from our laboratory (Thiagarajan *et al.,* 2001) had shown that both green tea and black tea display excellent antioxidant activities, and also exhibit

cataractostatic ability in animals in which oxidative cataract was induced through selenite. We chose to investigate the antioxidant properties of extracts of the Chinese tree *Ginkgo biloba,* and *Withania somnifera* (Ashwagandha) of the Indian pharmacopoeia.

4.0.4.1. Ginkgo biloba

Extracts of the leaves of *Ginkgo biloba* contain mixtures of bioactive ingredients used in medical practice as polyvalent therapeutics. A recent monograph has analyzed the available scientific information and has summarized four major 'concepts of action' of a standardized extract of *Ginkgo biloba,* called EGb761, namely vasoregulatory action, cognition enhancing action, stress alleviating action and gene regulatory action (De Feudis, 1998). The main chemical constituents of *Ginkgo biloba* extracts (GBE) are biflavonoids (such as procyanidin and prodelphinidin), flavonoids (such as quercetin, kaempferol, isorhamnetin and myricetin) and their glycosides, and terpenoids (such as ginkgolides A, B, C, J and M, and bilobalide), and some small phenolic compounds. The polyphenolic flavonoids are expected to confer antioxidant properties to the extract (Marcocci *et al.,* 1994; Kuse and Dogan, 1995; Seif- El- Nasr and El-Fattah, 1995) while the terpenoids act as antagonists of platelet activating factor (Braquet, 1986; De Feudis, 1998). Some apolar compounds present in *Ginkgo biloba* leaves have been reported to have antimicrobial properties (Mazzanti *et al.,* 2000). A major mode of action of GBE is through its antioxidant ability which is attributed to the flavonoids, since ginkgolide and bilobalide were found to be incapable of quenching

oxyradicals. Chen *et al.* (1999) also found that these terpenoids in GBE did not scavenge hydroxyl radicals.

4.0.4.2. Reported beneficial uses of Ginkgo biloba

Ginkgo biloba extract has been tried out in ocular pharmacology. It is reported to accelerate corneal wound healing in rabbits after excimer laser keratectomy (Juarez *et al.,* 1999), protect against retinal ischemia-reperfusion injury (Szabo *et al.,* 1991; Szabo *et al.,* 1992) and retinal damage in rats (Doly *et al.,* 1986; Besse *et al.,* 1994; De Feudis, 1998; Ranchon *et al.,* 1999). Chung *et al.* (1999) have shown that it increases ocular blood flow velocity in the ophthalmic artery of human glaucoma patients. Based on these promising results, Ritch (2000) has suggested it to be a potential drug for the treatment of glaucoma. Juarez *et al.* (2000) have found it to be an effective angiostatic inhibitor in experimental retinopathy of prematurity. Költringer *et al.* (1992) have suggested that the combination therapy of folic acid plus *Ginkgo biloba* extract may be useful in the treatment of diabetic retinopathy. GBE has been suggested to lead to improvement in long distance visual acuity in human patients suffering from senile macular degeneration (Lebuisson, 1986), however Evans (2000) has suggested that the number of volunteers being small, the beneficial effect of GBE on retarding the prevention of this disease is yet to be confirmed.

4.0.4.3. Withania somnifera (Ashwagandha)

Ever since ancient times, drugs derived from plant sources have been used to alleviate or cure human diseases. One such traditional ancient plant that finds immense importance in Indian *Ayurveda* is Ashwagandha *(Withania somnifera,* also called winter cherry). Ashwagandha is a dicotyledonous plant belonging to the family Solanaceae. The roots of Ashwagandha have been compared with ginseng roots, and the plant is thus also known as the "Indian Ginseng" (Singh and Kumar, 1998). Ayurvedic medicine includes *Withania somnifera* in the class of herbs called 'adaptogens' or 'vitalizers'. These relatively innocuous adaptogens cause adaptive reactions to disease and appear to produce a state of nonspecific increased resistance to adverse effects of biological agents (Mishra *et al.,* 2000). The medicinal properties of Ashwagandha have been attributed to its chemical constituents, mainly alkaloids and steroidal lactones (primarily of the withanolide class). A monograph on this plant describing the extensive characterization of its chemical and medicinal aspects has recently been published (Singh and Kumar, 1998). Found and used in Africa, the Arab world and in South Asia, the extract of *Withania somnifera* (WSE), is used for a variety of illnesses such as asthma, rheumatic pain, inflammation of joints, nervous disorders, epilepsy, as a uterine sedative, antispasmodic, sedative and hypnotic and against eye diseases.

4.0.4.4. Reported beneficial uses of Ashwagandha

Withania somnifera has been reported to have a wide range of biological activities. It shows antibacterial activity against gram positive and gram negative bacteria. However, most studies show Ashwagandha to have greater antibacterial activity against Gram positive than Gram negative bacteria (Kurup, 1958; Sethi *et al.,* 1974). The ethanolic extract of the plant shows antiviral activity against Ranikhet disease virus, Vaccinia virus and Tobacco Mosaic virus (Dhar *et al.,* 1968; Sastry and Singh, 1982). Other known activities of Ashwagandha are its anti-tumour and radiosensitizing effects, anti-inflammatory, immunomodulatory, anti-stress, anti-convulsive, hemopoetic, and rejuvenating properties. The antioxidant activity of the glycowithanilides of Ashwagandha have been reported earlier, where the effect of intraperitoneal injections of glycowithanolides on concentrations of antioxidant enzymes in brain frontal cortex and striatum of adult male Wistar rats was studied (Bhattacharya *et al.,* 1997). Recent literature suggests its efficacy as a cardioprotective agent (Dhuley, 2000), inhibitor of drug-induced urotoxicity (Davis and Kuttan, 2000), enhancer of white blood cell and platelet counts (Agarwal *et al.,* 1999), as an agent that enhances immunoprotection, cytokine production and stem cell proliferation (Davis and Kuttan, 1999) and as an antioxidant (Bhattacharya *et al.,* 1997).

In light of the interesting possibilities and applications of GBE and Ashwagandha, we have monitored their antioxidant and cytoprotective abilities.

4.1.0. MATERIALS AND METHODS

4.1.1. PREPARATION OF EXTRACTS

We have worked with the standardized extract preparation called EGb761, made by the Beaufour-Ipsen Company of France, which contains 24% flavonol glycosides and 6% terpene lactones (De Feudis, 1998). The sample of EGb761 was a kind gift from Dr. Katy Drieu of Beaufour-Ipsen. A sample of the root powder of Ashwagandha was obtained from Kotakkal Arya Vaidya Sala, Kerala, India. Stock solutions of GBE or Ashwagandha (100 mg/ml) were prepared in water and the soluble part was used to determine the antioxidant activity as well as for protein crosslinking studies. For experiments with cell lines, solutions were made in sterile phosphate buffered saline (PBS) and filtered using a 0.2 μm filter.

4.1.2. ABTS ANTIOXIDANT ASSAY

The ABTS assay was performed based on the procedure of Miller *et al.* (1993). Briefly, when azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) or ABTS is incubated with a peroxidase (such as metmyoglobin) and H_2O_2, the relatively long-lived radical cation $ABTS^{\bullet+}$ is formed. In the presence of an antioxidant, the absorption of this radical cation (at 734 nm) is quenched. In a typical experiment, ABTS (30 μl, 5 mM), 50 μl of metmyoglobin (50 μM), and 820 μl of phosphate buffer (50 mM, pH 7.4) (of which 10 μl is replaced with GBE or Ashwagandha (100 mg/ml stock) when the sample is being investigated) are mixed, and the reaction is

initiated by the addition of 100 μl of H_2O_2 (1 mM). The absorbance at 734 nm is measured as a function of time at 5-min. intervals for 30 min.

4.1.3. PROTEIN OXIDATION

(i) *Assay for inhibition of photo-oxidation*

The protein bovine pancreatic ribonuclease A, or RNase A, (1 mg/ml) was irradiated in the presence of the photosensitizer molecule bis(hydroperoxy)naphthaldiimide (also called the Photo-Fenton reagent), which generates hydroxyl radicals upon irradiation at 366 nm (Guptasarma *et al.,* 1992). The reagent was adsorbed on controlled pore glass beads and suspended in the protein solution. The mixture was taken in a fluorescence cuvette, placed in the sample compartment of a Hitachi F2500 spectrofluorimeter, and irradiated at 366 nm for 90 min., by tuning the excitation monochromator to this wavelength, with the excitation slit wide open. The light intensity falling on the sample has been earlier estimated to be about 0.5 mW/cm^2 (10^{14} photons incident/sec) (Murali Krishna *et al.,* 1991). The $^{\bullet}OH$ produced generate oxidative crosslinks in the protein, monitored by polyacrylamide gel electrophoresis (PAGE), using 12% acrylamide for setting the gel, and in the presence of sodium dodecylsulfate (SDS) and β-mercaptoethanol. Inhibition of the formation of high molecular weight products by the extract (GBE/Ashwagandha) was assayed by adding known amounts of the substance to the reaction mixture before irradiation. An identical procedure was adopted for the photosensitized oxidation of RNase A, using riboflavin which, upon

irradiation at 445 nm, yields singlet oxygen in high quantum yield (Murali Krishna *et al.,* 1991). The damage to the protein, and protection by the extract, was monitored by SDS/PAGE.

(ii) Assay for inhibition of peroxynitrite-mediated damage

The test protein bovine α-crystallin (1 mg/ml), was incubated with 1 mM peroxynitrite, with or without 10 mM bicarbonate, and series of increasing concentrations of the plant extract. Peroxynitrite was generated, as mentioned earlier. Protein crosslinking was studied by SDS/PAGE.

4.1.4. SINGLE CELL GEL ELECTROPHORESIS

DNA damage was evaluated by single cell gel electrophoresis, also called the comet assay (Singh *et al.,* 1988). Cultured HLE cells were trypsinized to obtain a single cell suspension. Cells were irradiated for 1 h with broad- band visible light in the presence of 25 μM riboflavin, with or without GBE/Ashwagandha. The cell suspension was then mixed with low melting agarose (2%, in Ca- and Mg-free PBS) and layered onto frosted glass slides and allowed to gel. Prepared slides were placed in lysis solution (154 mM NaCl, 10 mM EDTA and 0.5% N-laurylsarcosine, pH 7.0) for three minutes. Slides were then washed in distilled water for five minutes. Electrophoresis was carried out in buffer containing 25 mM NaCl and 1 mM EDTA at 7 V/cm^2, for three minutes. Finally, the slides were washed and stained with ethidium bromide (50 μg/ml). DNA damage was visualized using a fluorescence microscope equipped with an excitation

filter of 515-560 nm and a barrier filter of 590 nm. DNA migration, nuclear diameter and tail intensity were analyzed.

4.1.5. ASSAY OF CYTOPROTECTIVE ABILITY

The cytoprotective ability of *Ginkgo biloba* and Ashwagandha was studied using the MTT assay (Hansen *et al.,* 1989). This assay is based on the ability of living cells to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to form formazon products, which can be quantified at 540 nm, by the intensity of blue color. The rabbit corneal keratocyte cell line, SIRC, which was procured from the National Center for Cell Sciences, Pune, India or HLE cells (1 x 10^5 cells per well in 0.5 ml medium) were seeded in a 24-well tissue culture plate and incubated at 37°C, 5% CO_2. After 22 h of incubation, when cells were about 75 % confluent, oxidative stress was induced using riboflavin (20 μM) as the photosensitizer, in the presence or absence of GBE/ Ashwagandha. After exposing the cells to stress, they were incubated in fresh medium for 18 h at 37°C and 5% CO_2, after which 50 μl of 5 mg/ml MTT in PBS was added to each well. After 4 hours of incubation at 37°C, the medium was removed and 500 μl of 40 mM HCl/ isopropanol was added to stop MTT reduction. The blue color developed was read at 540 nm.

4.1.6. PHASE SEPARATION MODEL Of- CATARACT (COLD CATARACT)

The effect of GBE on the phase separation temperature of lens cytoplasm was studied using fish lens homogenate as per Hiraoka *et al.* (1996). Whole lenses of fish were dissected out, cut into small pieces, homogenized at 37°C and centrifuged at 20,000 rpm for 1h to remove membranes. The resulting homogenate was used to determine the phase separation temperature. A stock solution of GBE was prepared in 0.1 M phosphate buffer, pH 7.0 and one part of this stock solution was mixed with five parts of fish lens homogenate to yield a final extract concentration of 15 mg/ml. In the control, the extract was replaced with buffer. The sample tube was placed in a fluorescence spectrophotometer (Hitachi F2500) with a temperature-controlled circulating water bath and the scatter at 600/600 nm was recorded over the range of +20°C to –10°C. The transition from transparency to opacity was observed as a sharp increase in scatter, which occurred over a narrow temperature range. In case of Ashwagandha, the phase separation temperature was determined in a similar fashion using bovine lens homogenate, in the presence of sucrose (0.9 M) since sucrose is known to bring up the lower critical solution temperature (LCST) of liquid mixtures (Balasubramanian and Mitra, 1979).

Similarly, the effect of GBE and Ashwagandha on thermally induced self-aggregation of lens proteins was also studied. Scatter was recorded over the range of 25°C to 75°C.

4.2.0. RESULTS AND DISCUSSION

4.2.1. INHERENT ANTIOXIDANT ABILITY OF GBE/ASHWAGANDHA

While there are reports on the possible antioxidant nature of GBE in cell suspensions, tissues, organs and whole animals, the actual molecular effects have not been reported in detail, barring the early pioneering study of Marcocci *et al.* (1994) on the antioxidant ability of the standardized extract preparation EGb761. With respect to Ashwagandha, the free radical scavenging activity of the root powder has been reported earlier (Panda and Kar, 1997). We extend the antioxidant analysis of both GBE and Ashwagandha here, using the ABTS assay (Miller *et al.,* 1993). As shown in Fig. 4.2.1 addition of GBE/Ashwagandha (1 mg/ml) led to a rapid drop in the ABTS absorption at 734 nm within minutes, showing them to be efficient antioxidants.

4.2.2. PROTECTION FROM OXIDATIVE DAMAGE TO PROTEINS AND DNA

Figure 4.2.2 shows the electrophoresis pattern of test proteins subjected to oxidative stress by either riboflavin, or the Photo-Fenton reagent, or peroxynitrite. Both GBE and Ashwagandha are able to effectively inhibit oxidative damage induced by the Photo-Fenton reagent, which generates hydroxyl radicals. When riboflavin was used as the sensitizer, GBE as well as Ashwagandha were able to quench $^1O_2$ and protect the protein from damage. GBE and Ashwagandha were also able

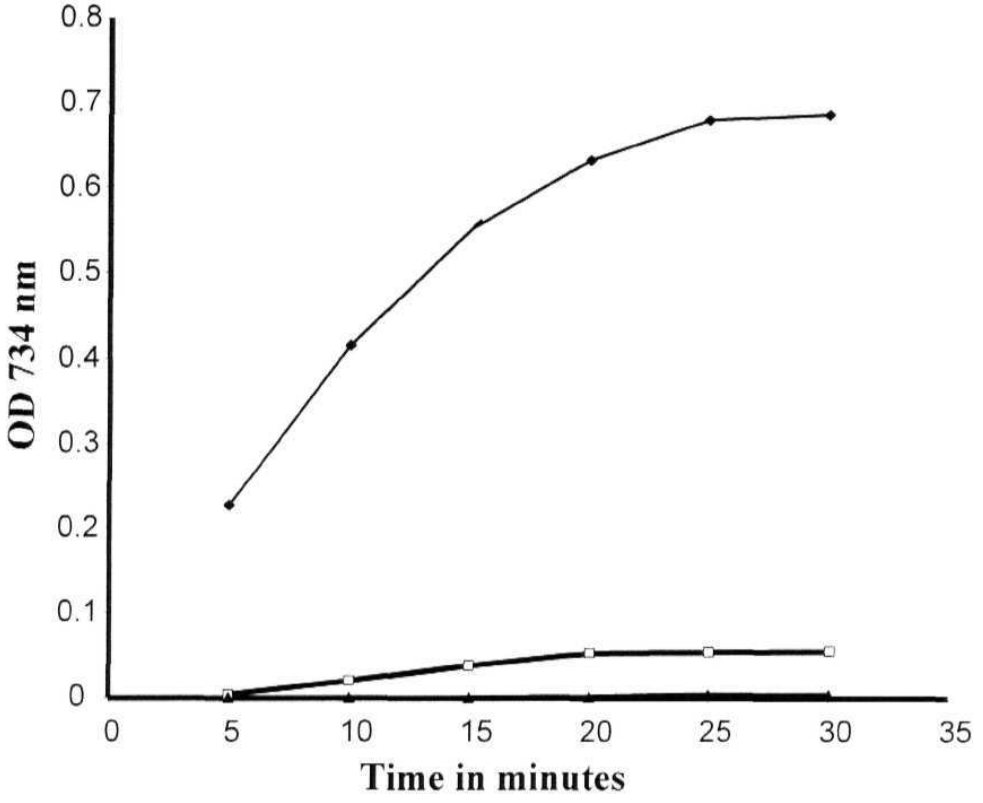


Figure 4.2.1. ABTS Assay of GBE and Ashwagandha (1 mg/ml)
Control (◆), GBE (▲), Ashwagandha (□)

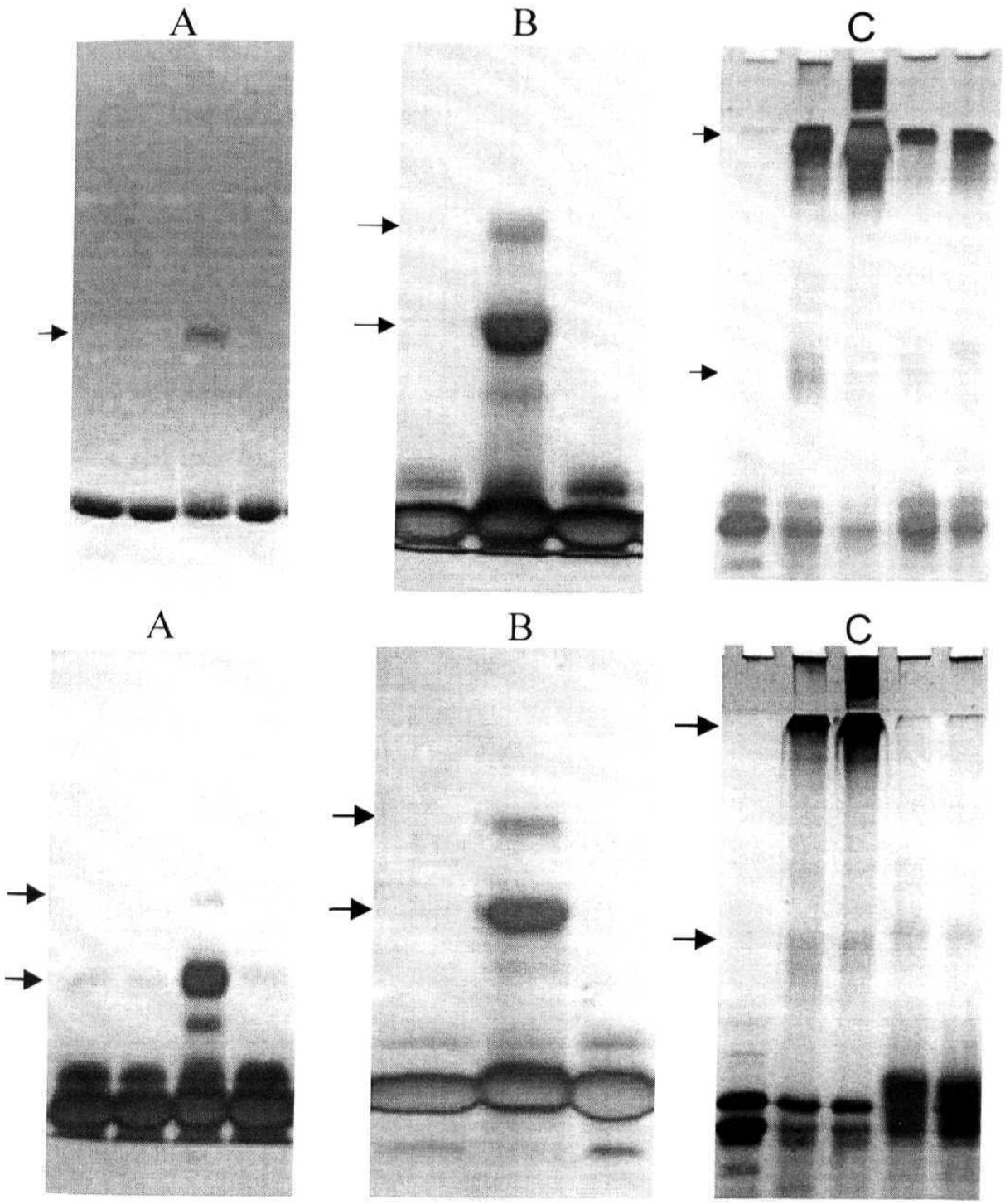


Figure 4.2.2. SDS/PAGE of oxidized proteins (1 mg/ml).

TOP: Protection by GBE **BOTTOM:** Protection by Ashwagandha

Riboflavin-induced photo-oxidation (A) - Lane 1 = Control RNase A, lane 2 = RNase A - irradiated, Lane 3 = RNase A +20 μM riboflavin, lane 4 = RNase A +20 μM riboflavin + 0.1 mg/ml GBE/Ashwagandha - irradiated

Photo-Fenton (B) - Lane 1 = Control RNase A, lane 2 = RNase A + beads - irradiated at 366 nm, lane 3 = RNase A + beads + 2mg/ml GBE/Ashwagandha - irradiated at 366 nm

Peroxynitrite-mediated damage (C) - Lane 1 = Control α-crystallin, lane 2 = α-crystallin+ 1mM PON, lane 3 = α-crystallin+1mM PON+10mM HCO_3^-, lane 4 = α-crystallin+1mM PON+ GBE (0.2mg/ml)/Ashwagandha (2mg/ml), lane 5 = α-crystallin+1mM PON+10mM HCO_3^-+ GBE (0.2mg/ml)/Ashwagandha (2mg/ml)

to effectively inhibit peroxynitrite-mediated covalent modifications in the test protein bovine α-crystallin.

Figure 4.2.3 shows the ability of the test compounds to protect oxidative strand-breaks in the nuclear DNA in intact human lens epithelial cells. When single cells are electrophoresed, the intact DNA in the nuclei is seen as a compact disk or a "moon"; when strand break and thus chain scission occurs by oxidative damage, it is visualized as a trailing "comet" in the electrophoregram. Addition of GBE/Ashwagandha is seen to inhibit the damage to DNA, reduce the trail and restore the "moon".

4.2.3. CYTOPROTECTIVE ABILITY

The ability of GBE/Ashwagandha to enter cells and protect the nuclear DNA led us to study the cytoprotective properties of the extract. We subjected HLE or SIRC to riboflavin-induced stress in the absence and presence of GBE/Ashwagandha, and assayed the viability of cells using the MTT metabolism method. Figure 4.2.4 shows that both the test compounds protect HLE cells from permanent damage and keeps them viable, and Fig. 4.2.5 shows that this protective ability holds well even for cells of corneal origin, SIRC.

Both the extracts are able to permeate cell membranes and exert their action on the nuclear DNA and cytoplasmic components, as the comet and MTT assays reveal. In case of GBE, the role of the non-flavonoid components appears to be important in these instances. The cytoprotective nature of the extracts and their ability to rescue cells from

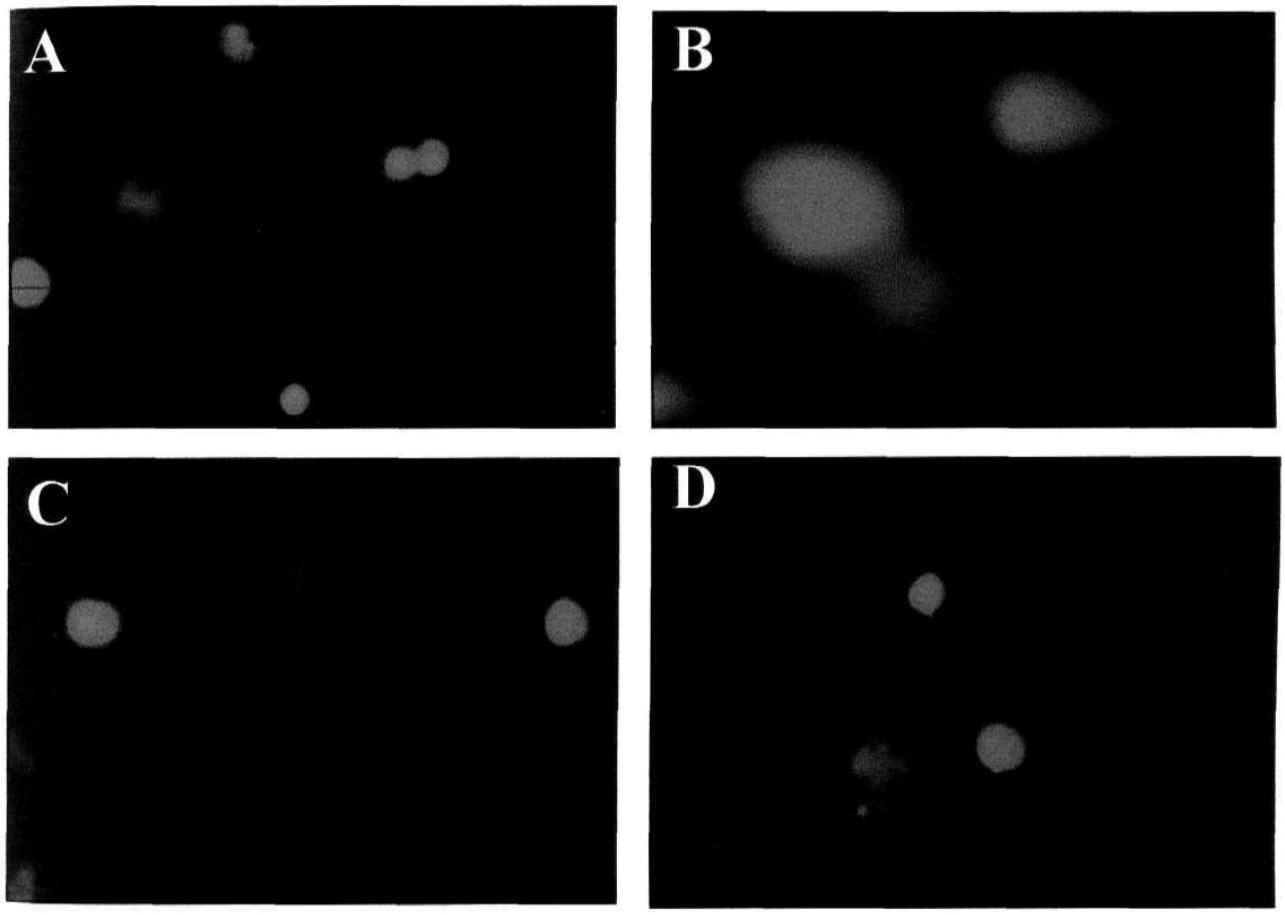
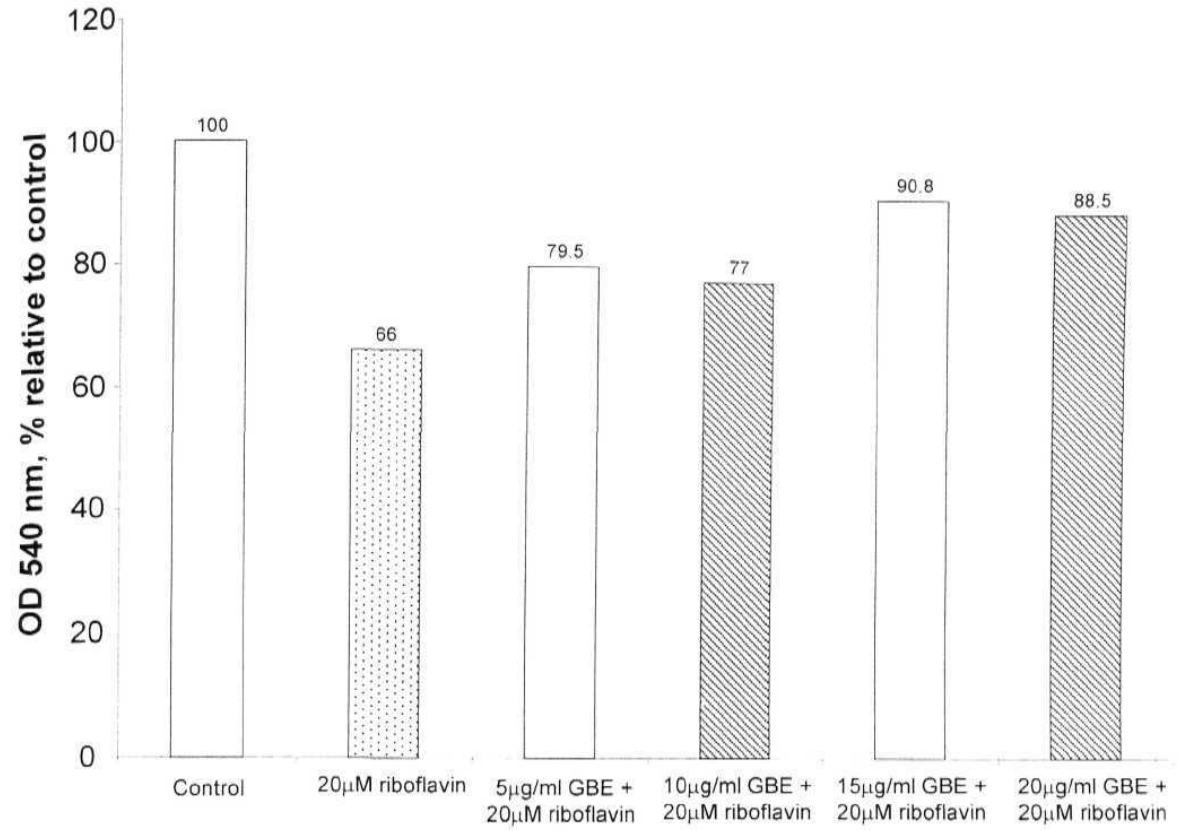


Figure 4.2.3. Comet assay of human lens epithelial cells

A) Control cells
B) Cells with 25 μM riboflavin
C) Cells with 25 μM riboflavin and 0.1 mg/ml GBE
D) Cells with 25 μM riboflavin and 0.2 mg/ml Ashwagandha



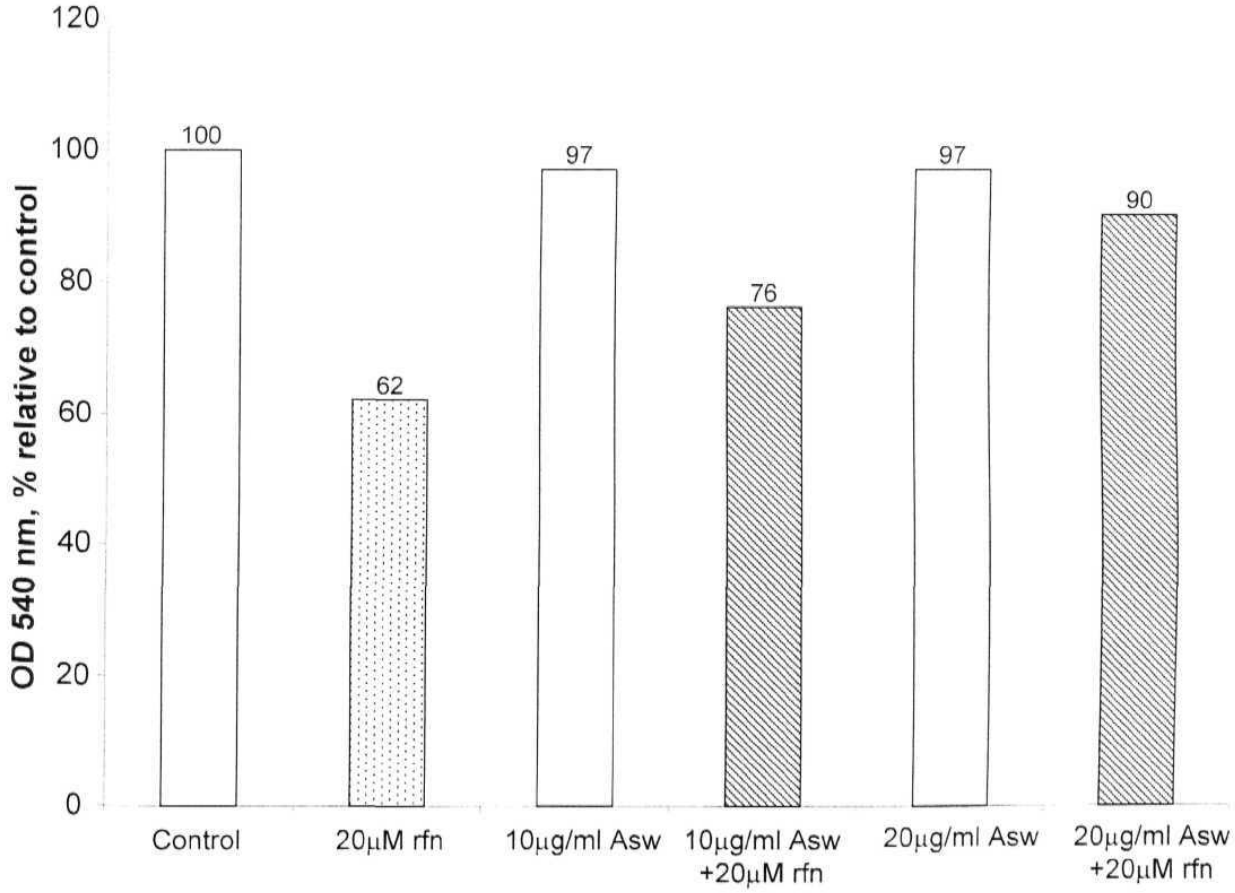


Figure 4.2.4. MTT cell viability assay with human lens epithelial cells (SRA01/04), oxidized with riboflavin

TOP: with GBE BOTTOM: with Ashwagandha

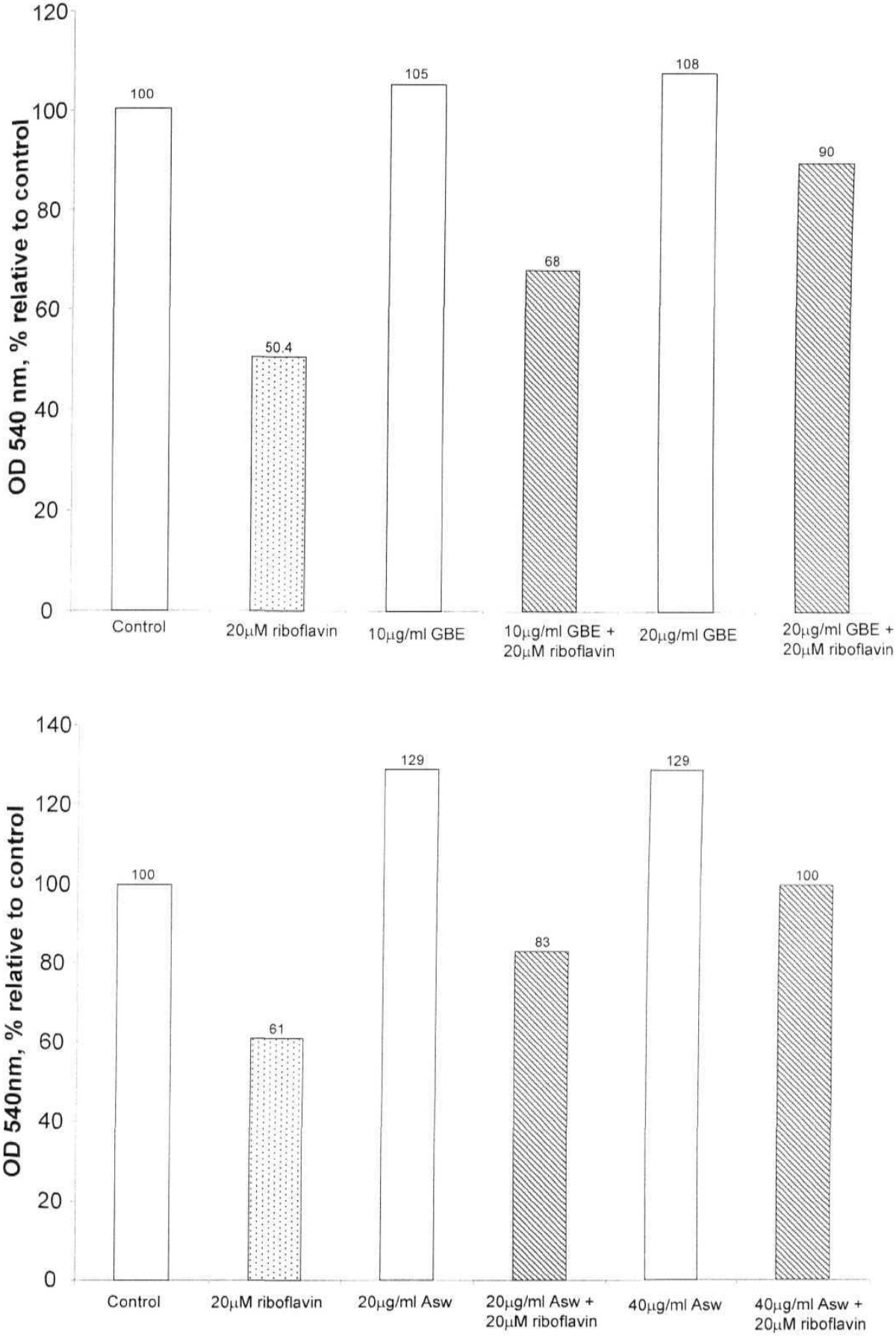


Figure 4.2.5. MTT cell viability assay with rabbit corneal keratocyte cell line (SIRC), oxidized with riboflavin

TOP: with GBE BOTTOM: with Ashwagandha

light-induced assault may largely be due to the antioxidant character. GBE has also been reported to possess anti-apoptotic properties. Ahlemeyer *et al.* (1999) have shown that bilobalide is the most potent anti-apoptotic factor in GBE. Ginkgolide B also shows this activity, while ginkgolide A is inactive in this regard. Yao *et al.* (2001) have recently shown that a terpene- and flavonoid- free extract of GBE, called HE208, failed to protect neuronal cells from beta-amyloid-induced cytotoxicity and cell death, and that the flavonoids and terpenoids present in GBE act in combination. Liebgott *et al.* (2000) have further shown that the terpenes and the flavonoids act complementarily in offering protection to the heart during ischemia and reperfusion. From these it appears that the beneficial action of GBE is derived from both its flavonoid and terpenoid components. With respect to Ashwagandha, the steroidal lactones appear to have useful biological properties. Though glycowithanolides are believed to be responsible for the antioxidant property, the chemistry needs to be studied in detail.

4.2.4. EFFECT ON THE THERMAL PHASE SEPARATION OF LENS PROTEINS

We next investigated the effect of the addition of GBE/Ashwagandha on the "cold cataract" phenomenon (Fig. 4.2.6). Solubility studies have shown that the early proteins (like γ-crystallin) readily phase separate, causing the phenomenon of cold cataract (Zigman and Lerman, 1965; Broide *et al.,* 1991). y-crystallin behaves like a cryoprotein, tending to precipitate from its solution on cooling below 4°C

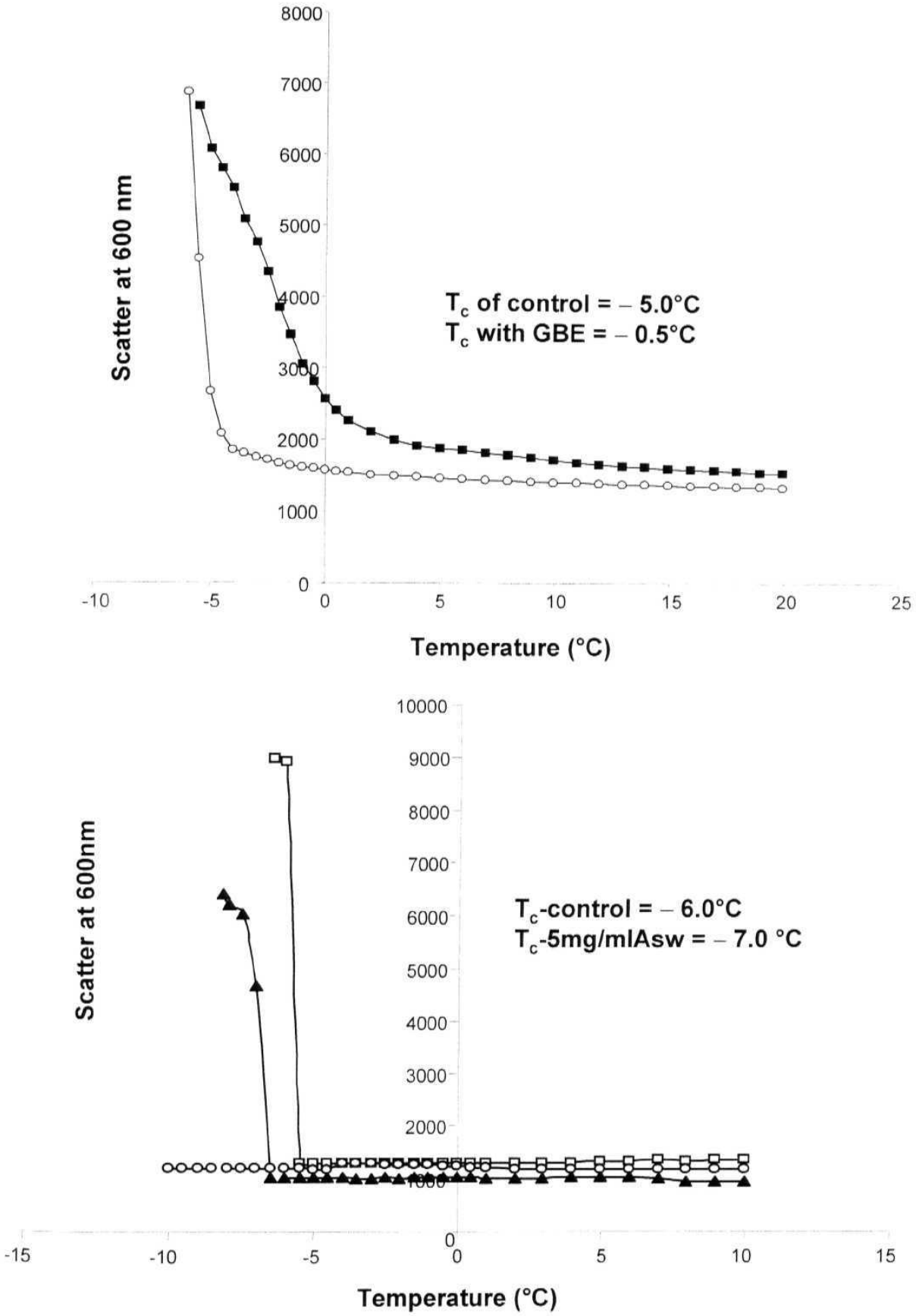


Figure 4.2.6. Phase-transition curves of lens proteins at low temperature

TOP: with 0 mg/ml (O) and 15 mg/ml (■) GBE
BOTTOM: with 0 mg/ml (□), 5 mg/ml (▲) and 20 mg/ml (O) Ashwagandha

but readily redissolving upon warming. Fish lens homogenate was found to dramatically increase light scattering when it was cooled to -5°C. When GBE was added to the homogenate at a final concentration of 15 mg/ml, this precipitation temperature was raised to –2°C, indicating that the extract was not able to inhibit the cold precipitation of the homogenate. On the other hand, Ashwagandha (5 mg/ml) was able to lower the phase transition temperature to –7°C *vs* –6°C for control, and 20 mg/ml did not cause its precipitation even up to -10°C. We then looked at the effect of the extracts on the high temperature precipitation of lens homogenate (Fig. 4.2.7). In the absence of any additive, the scattering of light sharply increased beyond 52°C for fish lens homogenate and around 70°C for bovine lens proteins. Addition of either GBE (15 mg/ml) or Ashwagandha (5 mg/ml, 20 mg/ml) did not inhibit this precipitation but in fact advanced it to lower temperatures. Addition of 5 mg/ml Ashwagandha lowered the Tc by 0.5°C while 20 mg/ml dropped it further to 60°C. Therefore, while GBE is not effective against the phase separation and precipitation that occurs on cooling of the lens crystallins, nor against the heat-induced aggregation of lens proteins; Ashwagandha could prevent only the "cold cataract" phenomenon. This can be explained since neither of these phenomena involve any oxidative or other covalent chemical modifications.

4.2.5. ASHWAGANDHA AND GBE ARE PHOTODYNAMICALLY INERT

Since some herbal extracts, e. g., hypericin from *St. John's wort,* used as drugs and health supplements have been demonstrated to have

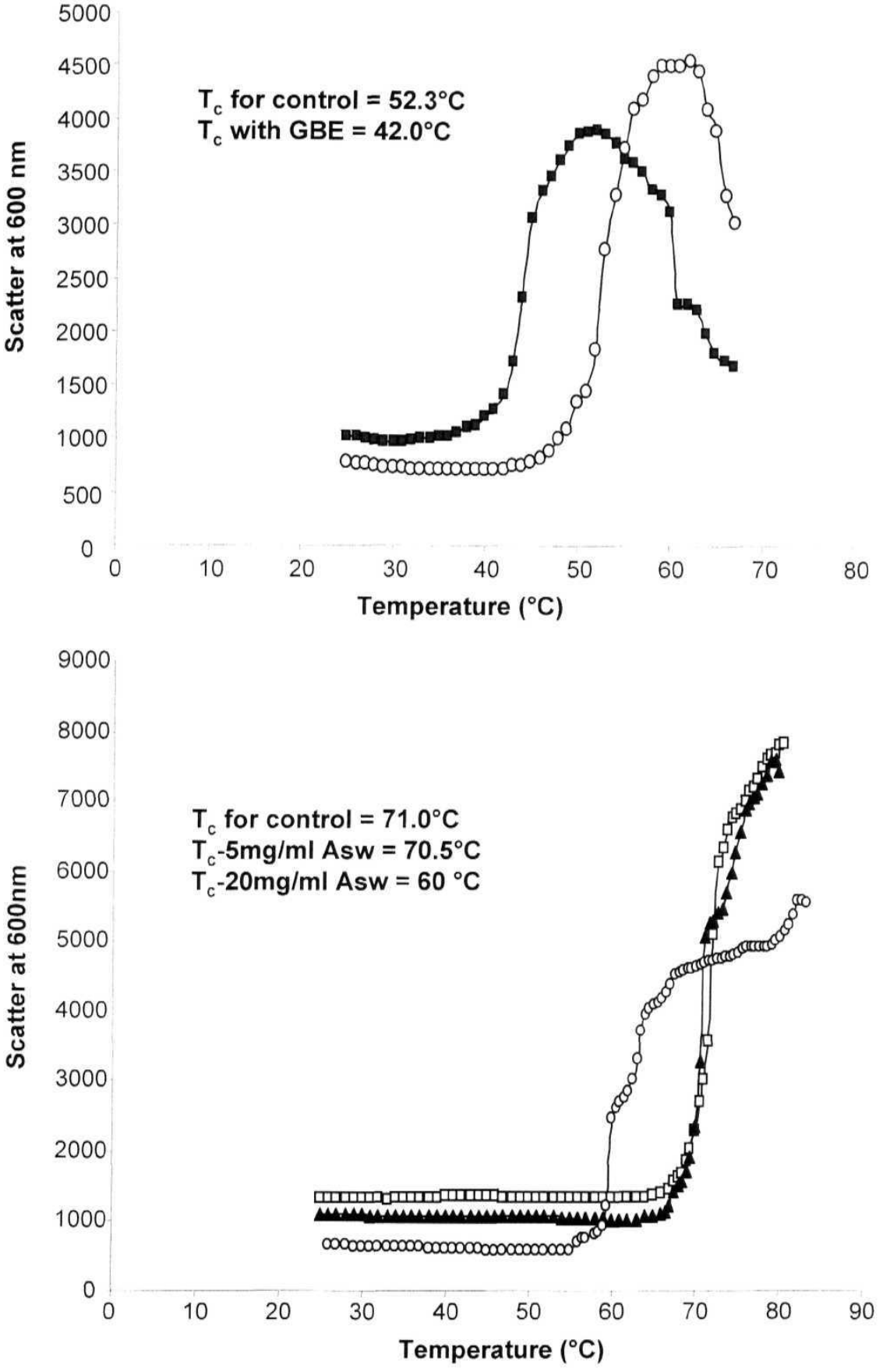


Figure 4.2.7. High temperature scatter of lens proteins

TOP: with 0 mg/ml (O) and 15 mg/ml (•) GBE
BOTTOM: with 0 mg/ml (□), 5 mg/ml (A) and 20 mg/ml (O) Ashwagandha

inherent photosensitizer properties (Schey *et al.,* 2000), we monitored the photodynamic properties of GBE and Ashwagandha. This was done by irradiating a 1 mg/ml solution of RNase A in the presence of the added extract (0.5 mg/ml) for 120 min (at 365 nm for GBE and 320 nm for Ashwagandha, wavelengths where the protein does not absorb but the extract does). The covalent damage to the protein was monitored by gel electrophoresis. No modifications were seen in the gel, indicating that neither GBE nor Ashwagandha are phototoxic, i. e., they do not act as sensitizers producing any reactive species upon excitation.

Though GBE could prevent selenite-induced cataract in rat pups, which is an oxidative model of cataractogenesis, the effect of Ashwagandha on an animal model of cataract is yet to be determined. The ability of GBE to retard other ocular conditions that are brought about by oxidative stress, such as senile macular degeneration (Lebuisson *et al.,* 1986; Evans, 2000), appears promising from our results on its antioxidant property. It has also been shown that GBE is able to protect cultured retinal pigment epithelial cells from oxidative stress. The antioxidant, antimicrobial and cytoprotective properties of Ashwagandha also make it an attractive drug of choice to be tried in ocular pharmacology.

Finally, the issue of toxicology and safety of GBE/Ashwagandha doses to humans needs to be addressed. A remarkably low rate of adverse drug events associated with the use of EGb761-containing products has been reported (De Feudis, 1998). Rhee *et al.* (2001), commenting about the use of complementary and alternative medicine for

glaucoma, have worried about possible toxicities of GBE and other herbal remedies. Le Bars and Kastelan (2000) have summarized the efficacy and safety of *Ginkgo biloba* extracts, and suggest that a dose of 120 mg EGb761 per day is acceptable. While this is reassuring, the material available over the counter in drug stores and nature food shops is not the standardized extract EGb761, but leaf powder, capsules and caplets. These contain alkylphenols (e.g., ginkgolic acids, ginkgol, bilobol), which EGb761 does not, and might have potential allergenic and toxic properties. Baron-Ruppert and Luepke (2001) have evaluated the toxicity of these alkylphenols, using the chicken egg test, and find that ginkgolic acids are lethal to chick embryos at very low levels (LD_{50}: 3.5 mg/egg, or 64 ppm). On the other hand, the biflavone fraction (that contains 1% or less of ginkgolic acids) has a LD_{50} value of 250 mg/egg, or 4540 ppm. Westendorf and Regan (2000) report that ginkgolic acids might cause DNA strand breaks in rat hepatocytes. There has also been the suggestion that GBE may contain constituents with immunotoxic properties, as monitored by the popliteal lymph node assay (Koch *et al.,* 2000), though it is not clear yet what these constituents are. Thus, while the flavonoids themselves are safe, these alkylphenols are best avoided. A preliminary toxicity study of *Withania somnifera* using total alkaloids from the roots showed an LD_{50} of 465 mg/kg body weight in rats and 432 mg/kg body weight in mice (Malhotra *et al.,* 1965).

Russo *et al.* (2001) investigated the free radical scavenging capacity of methanolic extracts of *Withania somnifera* and the effect on DNA cleavage induced by H_2O_2 UV-photolysis and whether this extract

was capable of reducing the hydrogen peroxide-induced cytotoxicity and DNA damage in human non-immortalized fibroblasts. The extract showed a dose-dependent free radical scavenging capacity and a protective effect on DNA cleavage. These results were confirmed by a significant protective effect on H_2O_2-induced cytotoxicity and DNA damage in human non-immortalized fibroblasts. These antioxidant effects of *Withania somnifera* may explain, at least in part, the reported anti-stress, immunomodulatory, cognition-facilitating, anti-inflammatory and anti-aging effects produced by them in experimental animals and in clinical situations and may justify further investigation of their other beneficial biological properties.

Chapter 5

5.0.0. CLONING AND EXPRESSION OF MUTANT βA3/A1-CRYSTALLIN IN *E.COLI*

Cataract is the commonest treatable cause of visual disability in childhood with an incidence of 1-6 per 10,000 live births (Lambert and Drack, 1996). Congenital cataracts cause 10-30 % of all blindness in children, with one-third of cases estimated to have a genetic cause (Lund *et al.,* 1992). Cataract may be inherited either as an isolated ocular abnormality or as part of a syndrome, majority of inherited non-syndromic cataract shows autosomal dominant inheritance, but X-linked and autosomal recessive forms are also seen (Francois, 1982).

The Twin Eye Study has suggested that the contributions of genetics and environment to the development of age-related nuclear cataract are 48% and 14% respectively (Hammond *et al.,* 2000), while in the case of cortical cataract, genetics accounts for 37-58% and environment 11-37% (Hammond *et al.,* 2001).

5.0.1. CRYSTALLIN GENES AND PROTEINS

The *Cry* genes code for crystallins, *Crya* for a-, *Cryb* for (3- and *Cryg* for γ-crystallins, In human lenses, expressed genes include *Cryaa, Cryab, Cryba1, Cryba3, Cryba4, Crybb1, Crybb2, Crybb3, Crygs, Crygc* and *Crygd.* The *Cryg* gene in all mammals consists of three exons; the first exon codes for only three amino acids while the subsequent two exons are responsible for two Greek-key motifs each. Six β-crystallins are expressed

in the human lens, βA1, pA3, pA4, pB1, βB2 and pB3. |3B1 and pB2 are the predominant components; pB3 is detected only in infant lenses. βA3 and pA1 have the same sequence except that the N-terminus of pA1 begins with residue 19 of the pA3 sequence. Of all the γ-crystallins, yC- and γD-crystallins are expressed in appreciable amounts in humans, and only γD-crystallin continues to be expressed until late childhood. The human yE and yF genes harbour early stop codons (Meakin *et al.,* 1987) and are fortunately not expressed, as the corresponding severely truncated γ-crystallin polypeptides would not be able to fold.

Lampi *et al.* (1997) corrected the sequence of the *CRYBA1/BA3* gene, which they designated 'beta-A1/beta-A3.' The calculated molecular weights of the two proteins matched the values determined experimentally by mass Spectrometry and by analysis of tryptic peptides. They also found that beta-A1 and beta-A3 are among 11 major soluble proteins in the young human lens. In addition, they reported that there is a third truncated form, known as 'beta-A3 (23-215),' in the lens that lacks the first four amino acids of beta-A1.

Structurally, the p- and y-crystallins share a common chain conformational characteristic, the Greek-key motif, joined by a linker segment. Crystallographic studies have shown that each of the p- and *y*-crystallins is composed of two domains, each built up by two Greek-key motifs. Each Greek-key motif is made up of four antiparallel p-sheets. In both classes of crystallins, the repeated py motifs can be classified as types A and B so that each two-domain crystallin monomer has the motif

pattern ABAB. The B-type motifs are generally highly conserved and include a small hydrophobic surface patch of two residues in the center of the β-sheet that makes up one side of the wedge-shaped β-sandwich and the two B-type motifs interact through their symmetrically related patches (Wistow, 1983, Bax *et al.,* 1990, Lapatto *et al.,* 1991). From the point of view of the role of crystallins in maintaining eye lens transparency, it is quite apparent that any alteration in the structure and interaction of the crystallins would affect transparency.

Crystallin βB2 is the only member of the β-crystallin gene cluster at the locus 22q, which is highly transcribed in the human lens. Missense mutations in this gene are known to result in the development of cerulean (Litt *et al.,* 1997) and Coppock-like (Gill *et al.,* 2000) cataracts - (see Table 5.1).

5.0.2. MOUSE MODELS OF CATARACT

The mouse has served as an appropriate animal model to identify genes responsible for cataract formation and to analyze the mechanisms leading to opacification of the lens. From the genetic point of view, the mouse is one of the best-characterized model systems and many observable pathological alterations there are comparable to those in man. A systematic approach to collecting murine cataract mutations was initiated about 20 years ago (Kratochvilova and Ehling, 1979) and since then several mouse models have been developed. The series of *Cat2* mutations have been mapped close to the γ-crystallin genes (Loster *et al.,*

Table 5.1. Some mutations identified in human congenital cataracts

Locus	Gene	Protein	Mutation	Phenotype	Reference
1q21-25	*GJA8*	Connexin 50	Missense	Pulverulent	Shiels *et al.*, 1998
			Missense	Pulverulent	Berry *et al.*, 1999
13q11-13	*GJA3*	Connexin 46	Missense	Pulverulent	Rees *et al.*, 2000
			Missense	Pulverulent	Mackay *et al.*, 1999
2q33-35	*CRYGC*	γC-crystallin	Missense	Coppock-like	Heon *et al.*, 1999.
			Insertion	Zonular pulverulent	Ren *et al.*, 2000.
			Missense	lamellar	Santhiya *et al.*, 2002
2q33-35	*CRYGD*	γD-crystallin	Missense	Nuclear, Punctate progressive	Stephan *et al.*, 1999.
			Missense	C87-lamellar	Santhiya *et al.*, 2002.
			Missense	Prismatic crystals	Kmoch *et al.*, 2000.
			Missense	Aculeiform	Heon *et al.*, 1999.

2q33-35	*CRYGD*	yD-crystallin	Nonsense	Central nuclear	Santhiya *et al.*, 2002.
10q24-25	*PITX3*	Pitx3	Missense	Total	Semina *et al.*, 1998.
21q22.3 (Autosomal Recessive)	*CRYAA*	αA-crystallin	Nonsense	-	Pras *et al.*, 2000.
			Missense	Zonular central nuclear	Litt *et al.*, 1998.
11q22-22.3	*CRYAB*	αB-crystallin	Frameshift	Posterior polar	Berry *et al.*, 2001.
17q11-12	*CRYBA1*	βA3/A1-crystallin	Splice site	Zonular cataract with sutural opacities	Kannabiran *et al.*, 1998.
22q11.2	*CRYBB2*	PB2-crystallin	Nonsense	Cerulean	Litt *et al.*, 1997.
			Missense	Coppock-like	Gill *et al.*, 2000.
	CRYBB1	βB1-crystallin	Nonsense	Pulverulent	Mackay *et al.*, 2002.
3q21.2-22.3	*BFSP2*	Phakinin	Missense	Progressive	Conley *et al.*, 2000.

1994). The *Cat2*^{nop} model is characterized by a mutation in the third exon of *Crygb*, leading to a truncated γB-crystallin and the termination of lens fiber cell differentiation. The *Tcm* mutation which leads to a cataract with iris dysplasia and coloboma (Zhou *et al.,* 1997) and the *Ccw* mutation, cataract and curly whiskers, are localized on mouse chromosome 4 (Kerscher *et al.,* 1996). The nuclear-posterior polar opacity (A/pp) maps to chromosome 5 and the total opacity *(To2)* to chromosome 10. The mutation leading to an opacity due to poor secondary fiber cell junctions (*Opj*) was mapped to chromosome 16 and affects the *Crygs* gene (Sinha *et al.,* 1998). The total opacity *(To3)* is placed on chromosome 7; this mutation is characterized as a single G→T transversion within the first exon of the *Lim2* gene coding for a lens-specific integral membrane protein, MP19 (Steele *et al.,* 1997). The *rlc* (rupture of lens) model was mapped to chromosome 14 (Matsushima *et al.,* 1996) and a similar phenotype *Ir2* (lens rupture 2) was mapped nearby (Song *et al.,* 1997). The Philly cataract is associated with the pB2-crystallin where a deletion mutation is seen in the *Crybb2* gene (Chambers and Russell, 1991). The *Aey2* mutant is also associated with the βB2-crystallin where a T→A nucleotide change has been identified in the *Crybb2* gene (Graw *et al.,* 2001). The *Iop10* (lens opacity 10) mutation has been mapped to chromosome 3, where a missense mutation (G→C) has been identified in the *Gja8* gene (Connexin 50) (Chang *et al.,* 2002). These examples of various cataract mutants in mice demonstrate the genetic heterogeneity of this eye disorder.

Reddy *et al.* (2001) have demonstrated the critical role of the enzyme Glutathione peroxidase-1 or GPx-1 in the lens as deficiency of this enzyme in gene knock-out mice led to increased nuclear light scattering associated with fiber membrane damage. However, in αB-crystallin gene knock-out mice, the lenses developed normally and were similar to those of wild-type mouse (Brady *et al.,* 2001).

5.0.3. CRYSTALLIN MUTATIONS IN HUMANS WITH INHERITED CATARACTS

Mutations in genes coding for all the three classes of crystallins, membrane instrinsic proteins, gap junction proteins and cytoskeletal proteins have all been shown to lead to cataract. Mutations at more than one locus may also give rise to similar forms of cataract. Recently, Bu *et al.* (2002) carried out a genome-wide linkage analysis on Chinese individuals with lamellar cataract and found the disorder to be associated with inheritance of a 5.11-cM locus on chromosome 16. Individuals of three Chinese families were screened for mutations in the heat-shock transcription factor HSF4 and in each family it was found that a distinct missense mutation segregated with the disorder (Leu115Pro; Ala20Asp; Ile87Val). They also discovered an association between a missense mutation (C362T in exon 3 - Arg120Cys) and Marner cataract in a Danish family. These mutations in HSF4 are predicted to affect the DNA-binding domain of the protein and this finding suggests the critical role of HSF4 in lens development. Table 5.1 shows some of the mutations identified in humans with hereditary cataract.

Basti *et al.* (1996) have described a family with autosomal dominant zonular cataract with significant sutural opacities. Linkage analysis localized the gene causing these opacities to a region of chromosome 17q11-q12 including the pA3/A1-crystallin gene with a lod score of 3.91 (Padma *et al.,* 1995). All six exons of the βA3/A1-crystallin gene, including the coding region and the splice sites, were amplified and their sequence determined. Affected individuals showed a G to A transition at the 5' donor splice site of intron 3 (Kannabiran *et al.,* 1998). The G at this position is part of the conserved splice donor dinucleotide GT and mutation of the splice site would be expected to lead to exon-skipping. The possibilities for the final mRNA product as delineated by Kannabiran *et al.,* (1998) are (a) skipping of exon 3 (b) skipping of exon 3 and one or more of the other exons and (c) usage of cryptic splice sites within intron 3.

In order to know the effect of this splice site mutation on the pA3/A1-crystallin mRNA, the mutant gene was amplified from blood leukocyte DNA of an affected individual in the family to produce a ≈ 9 kb product which was cloned by TOPO-TA cloning (Invitrogen, Inc) using the PCR2.1-TOPO vector. The insert was released by *EcoR*1 digestion and cloned into a transgenic expression vector pACP2 containing the mouse α-crystallin promoter. The pACP2/mutant βA3/A1 was used to generate transgenic mouse lines (Courtesy: E. F. Wawrousek, National Eye Institute, Bethesda, MD, USA). F1 and F2 progeny were taken and lenses removed for mRNA isolation. Reverse transcription of transgenic lens

mRNA using primers specific for human βA3/A1 cDNA was done to obtain the transgenic product. Upon sequencing of this cDNA, it was found that exon 2 was joined to exon 5. The cDNA was subcloned into pBB3 (bluebac vector) (Kannabiran and Hejtmancik, unpublished data).

In βA3/A1-crystallin, the first two exons encode sequence of the amino terminal region, while exons 3-6 code for Greek-key motifs 1-4 respectively. Due to the splice site mutation, the aberrant pA3/A1-crystallin thus lacks two of the four Greek-key motifs. Another mutation has been identified in a Brazilian family at the same position (intron 3 at the 5' donor splice junction), where the nucleotide change was G to C. This mutation cosegregated with the disease and led to autosomal dominant cataract (Bateman *et al.,* 2000). In light of these findings, we decided to subclone the mutant [3A3/A1-crystallin cDNA into an *E.coli* expression system and overexpress this mutant protein in *E.coli,* in order to study its structural features since this may provide an insight into its role in cataractogenesis.

5.1.0. MATERIALS AND METHODS

5.1.1. CLONING OF MUTANT BETA A3/A1-CRYSTALLIN cDNA

The human pA3/A1-crystallin cDNA (387 bp) was released from pBB3 by EcoR1 digestion and ligated into pET-21a(+) (Novagen) which had been linearized by digestion with *Eco*R1. Recombinant clones were selected by restriction digestion with *Eco*R1. The orientation of the insert was identified by *Bgl* II digestion and confirmed by direct sequencing of the plasmids using T7 forward primer on an automated DNA sequencer (ABI Prism 377 sequencer). Competent *E.coli* BL21 DE3 pLys(S) were transformed with the recombinant pET-21a(+) by heat shock at 42°C for 90 sec. Figure 5.1 shows the map of the plasmid showing the position of the insert and key restriction enzyme sites.

5.1.2. OVEREXPRESSION OF MUTANT BETA A3/A1-CRYSTALLIN

A single colony of *E. coli* BL 21 DE3 pLys (S) was inoculated in 5 ml LB with 50 μg/ml ampicillin, incubated at 37°C, 150 rpm, overnight. A 1% inoculum of the overnight culture was used to inoculate fresh 100 ml LB with 100μg/ml ampicillin. Culture was incubated at 37°C, 250 rpm, till the OD_{600} reached 0.2. Overexpression was induced using 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) and the culture was grown for further 3h at 37°C, 250 rpm. Both pre-induction and post-induction samples (1 ml each) were removed, centrifuged and cells resuspended in SDS sample buffer (100 μl). Expression of the mutant βA3/A1-crystallin was monitored

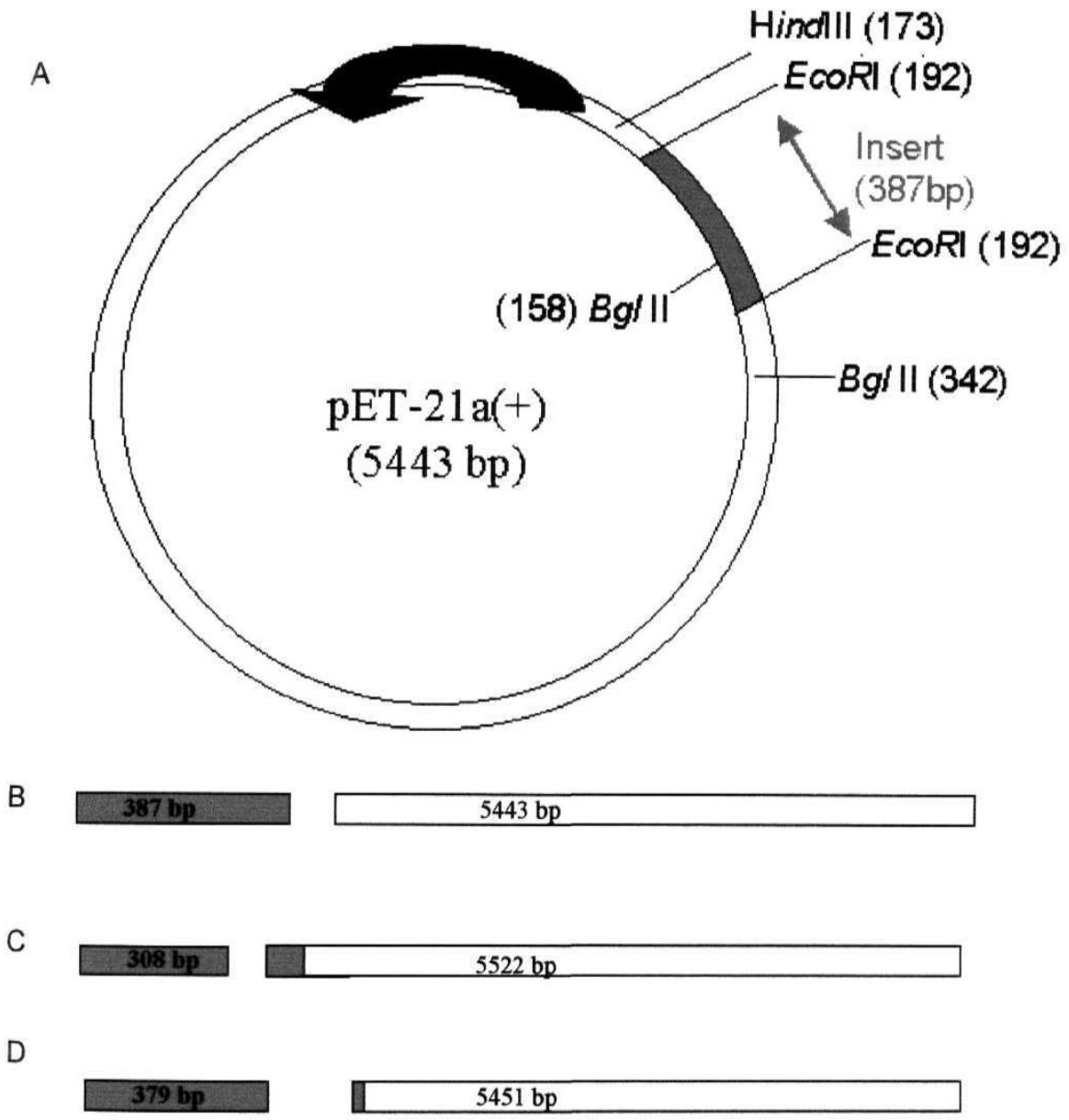


Figure 5.1. Map of pET-21a(+) vector showing mutant (3A3/A1 insert (A)

B) Release of insert after digestion with *Eco*RI

C) Release of insert after digestion with *BglW* (Right orientation)

D) Release of insert after digestion with *BglW* (Reverse orientation)

by SDS/PAGE on a 12% gel, when the volume loaded was normalized with respect to cell density.

5.1.3. EXTRACTION OF MUTANT βA3/A1-CRYSTALLIN

The procedure followed for extraction of mutant protein from *E.coli* was adapted from Marston (1987).

- Cells were harvested at 5000 rpm for 5 min at 4°C. The pellet was resuspended in 3 ml lysis buffer [50 mM Tris.Cl, pH 8.0, 1 mM EDTA, 100 mM NaCl] per gram of E. *coli*.
- For each gram of *E. coli*, 8 µl of 50 mM PMSF and 80 µl of lysozyme (10 mg/ml) was added and left for 20 min with occasional stirring.
- Deoxycholic acid (4 mg per gram of E. *coli)* was added while stirring continuously and left at 37°C. When the lysate became viscous, 20 µl of DNase I (1 mg/ml) per gram of E. *coli* was added, and lysate left at room temperature for 30 min till the solution was no longer viscous.
- The lysate was then centrifuged at 13,000 rpm for 15 min at 4°C. Pellet was dissolved in 100 µl water. Both supernatant and pellet were loaded on SDS/PAGE to know if the mutant βA3/A1-crystallin was soluble or formed inclusion bodies.

5.1.4. SGLUBILIZATION OF MUTANT βA3/A1-CRYSTALLIN FROM INCLUSION BODIES

- After inducing culture (1L), cells were harvested and resuspended in 40 ml ice cold Buffer A [20 mM Tris.Cl, pH 7.5, 20% (w/v) sucrose, 1 mM EDTA] and left on ice for 10 min.
- The suspension was spun at 11,000 rpm for 5 min at 4°C (Sorvall RC5 plus) and pellet resuspended in 40 ml ice cold water, left on ice for 10 min.
- The suspension was spun at 11,000 rpm for 5 min at 4°C. Pellet was resuspended in 10 ml ice cold Buffer P [PBS, 5 mM EDTA, 20 mg/ml aprotinin, 1 mM PMSF] and sonicated 12 x 15 s (with 30 s intervals) [Vibra Cell Sonicator, Danbury CT, USA].
- DNase I (250μg), RNase A (50μg) and 0.5 M $MgCl_2$ (200μl) was added and left at room temperature for 10 min.
- Inclusion bodies were harvested at 11,000 rpm for 30 min at 4°C. The pellet was resuspended in 1 ml buffer P and 40 ml Buffer W [PBS, 25% sucrose (w/v), 5 mM EDTA, 1% Triton X-100, 1 mM PMSF].
- Centrifuged at 20, 000 rpm for 10 min at 4°C. Washing step was repeated twice.
- Final pellet was resuspended in 5 ml Buffer D [50 mM Tris.Cl, pH 8.0, 5 mM EDTA, 8 M urea, 5 mM DTT] at room temperature and either stored at -20 °C or continued with folding immediately.

5.1.5. FOLDING OF MUTANT BETA A3/A1-CRYSTALLIN

All steps of folding were carried out at 4°C. The solubilized protein was diluted 1:10 in ice cold folding buffer [50 mM HEPES, pH 7.5, 0.2 M NaCl, 1 mM DTT, 400 mM L-Arginine, 1 mM PMSF]. The protein solution was dispensed into the folding buffer using a syringe, under vigorous stirring (using a magnetic stirrer). Stirring was continued for 2 min after addition and left at 4°C for 1 h. The protein mix was then dialysed against buffer containing 50 mM Tris.Cl, pH 8.0 at 4°C, with four changes of buffer.

5.1.6. PURIFICATION OF MUTANT BETA A3/A1-CRYSTALLIN

The folded protein was bound on DEAE-Cellulose using 50 mM Tris.Cl buffer, pH 8.0. The proteins were eluted using a salt gradient of 0-1M NaCl. OD 280 nm of all fractions was noted, protein fractions were pooled, concentrated and checked by SDS/PAGE on a 12% gel. The fraction with mutant βA3/A1-crystallin was dialyzed against buffer containing Tris (0.1 M), NaCl (0.5M), EDTA (1 mM) and NaN_3 (0.1 %), pH 7.4. It was then purified to homogeneity by gel filtration chromatography on Sephadex G-200. Purity of the protein was assessed by appearance of a single band on SDS/PAGE corresponding to ≈ 14 kDa.

5.2.0. RESULTS AND DISCUSSION

The β- and γ-crystallins share common structural elements and are therefore regarded as members of the βγ-superfamily of proteins. However, β- and y-crystallins differ in three primary ways:

(i) β-crystallins have N- and sometimes C-terminal extensions, while γ-crystallins do not (Wistow *et al*., 1981; Slingsby *et al.,* 1988). These arms have been implicated in molecular interactions between β-crystallins and with membrane or structural components of the cell.

(ii) Distribution of surface charges on the β- and γ-crystallin core domains differ (Lapatto *et al.,* 1991).

(iii) The connecting peptide linking the two domains is distinctly different in p- and y-crystallins. The connecting peptide in y-crystallins folds back on itself allowing the domains to interact, while in p-crystallins the connecting peptide is extended. This led to the suggestion that the connecting peptide of p-crystallins assumes a rigid structure, which prevents intramolecular association of the two domains and results in dimer formation (Bax *et al.,* 1990). However, Hope *et al.* (1994) have examined the role of the connecting peptide of β-crystallins in aggregation by constructing recombinant mouse βA3-crystallin containing the connecting peptide of mouse γB-crystallin. They conclude that the sequence of the connecting peptide is not critical for the association of pA3-crystallin into dimers and higher order aggregates.

Hence, the unique structural features of β-crystallins that distinguish them from γ-crystallins, which do not aggregate, include N- and C-terminal extensions from the globular domains and a distinctive linker sequence. There is evidence supporting the importance of both these features in the formation of aggregates. However, contrary to Hope *et al.* (1994), Kroone *et al.* (1994) reported that replacement of the linker sequence of rat pB2 with the γ-crystallin linker prevented dimer formation.

5.2.1. OVEREXPRESSION OF MUTANT pA3/A1-CRYSTALLIN

The cDNA of mutant βA3/A1-crystallin was cloned into the expression vector pET-21a(+). To pick clones having the insert in the right orientation, all recombinant clones were digested with *Bgl* II. The insert has a *Bgl* II site at position 158 bp and the vector (pET-21a(+)) has the site at 342 bp. Therefore, upon *Bgl* II digestion, clones with the insert in the right orientation would give a fragment size of 308 bp while reverse orientation would produce a 379 bp fragment. Positive clones were further confirmed by direct sequencing using the T7 forward primer. Figure 5.2.1 shows the orientation of the insert in the vector when sequenced with the T7 forward primer. Selected recombinant plasmids with the insert in the right orientation were transformed into *E.coli* BL21(DE3). Induction of the plasmids with IPTG resulted in overexpression of the recombinant protein, which migrated on SDS/PAGE at about 14 kDa. (Fig. 5.2.2). The expression levels of the mutant protein increased with time, reaching maximum at 3h post induction.

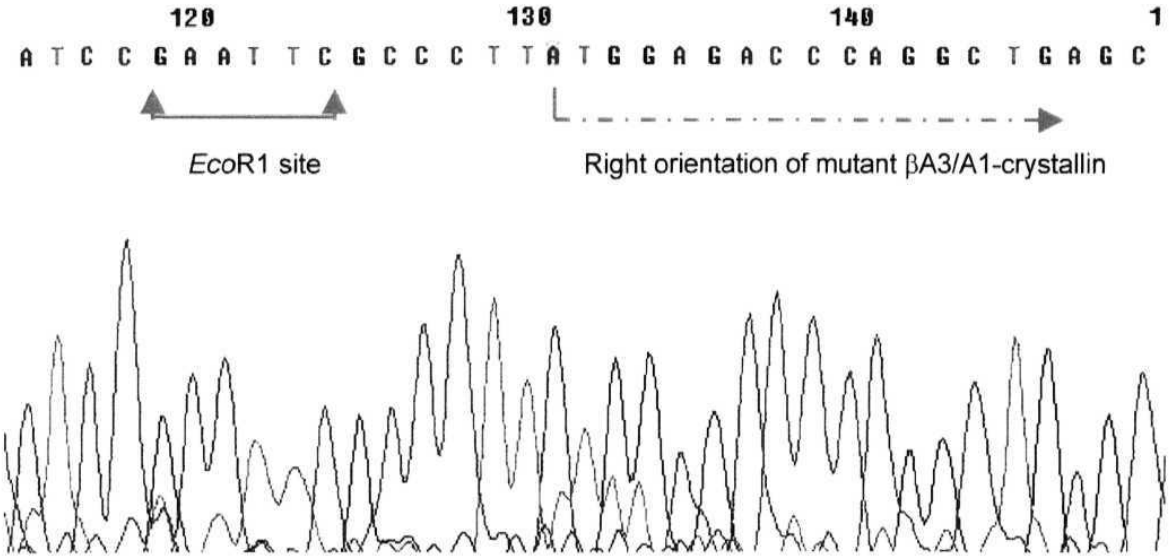


Figure 5.2.1. Electropherogram of mutant βA3/A1-crystallin gene in pET-21a (+)

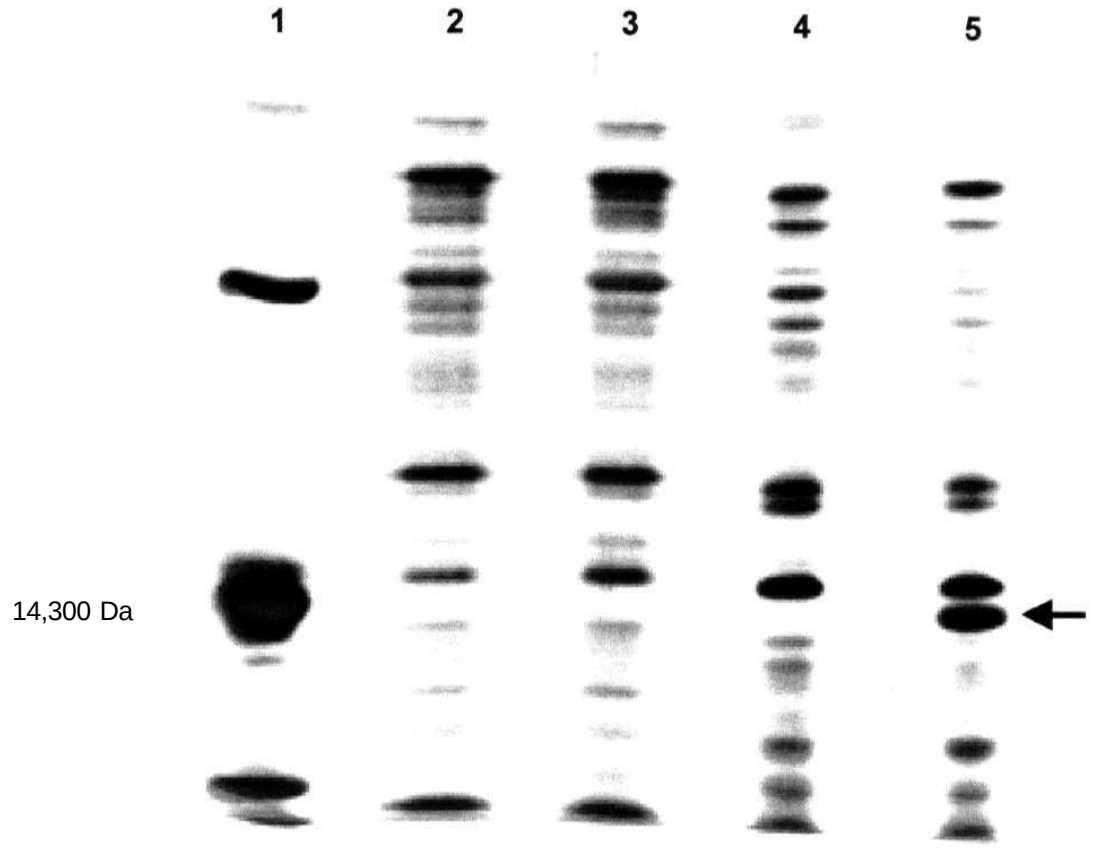


Figure 5.2.2. Overexpression of mutant βA3/A1-crystallin

Lane 1: Molecular weight marker
Lane 2: pET-21a (+) - pre-induction
Lane 3: pET-21a (+) with mutant βA3/A1 -pre-induction
Lane 4: pET-21a (+) - 3h post induction
Lane 5: pET-21a (+) with mutant βA3/A1 - 3h post induction

5.2.2. ACCUMULATION OF MUTANT βA3/A1-CRYSTALLIN IN THE AGGREGATED FORM

To determine if the protein was soluble or formed inclusion bodies, the cells were collected 3h after induction, lysed completely and the soluble and insoluble fractions were analyzed by SDS/PAGE. All of the expressed mutant protein partitioned into the insoluble fraction even at 30 min. post induction (Fig. 5.2.3). The accumulation of mutant protein as inclusion bodies increased as protein expression increased, with the maximum amount reaching at 3h. It appears that the deletion of about 88 residues in the mutant protein may alter its ability to fold appropriately, resulting in its deposition as inclusion bodies, even at low concentrations.

5.2.3. REFOLDING AND PURIFICATION OF MUTANT βA3/A1-CRYSTALLIN

The mutant protein was extracted from inclusion bodies by sonication and finally solubilizing it in 8M urea and 5 mM DTT (Fig. 5.2.3). Denatured protein was folded at 4°C in folding buffer and subsequently dialysed to remove residual urea. This refolded protein was then purified in two steps; first by ion exchange chromatography followed by gel filtration. Figure 5.2.4 shows the electropherogram (SDS/PAGE) of the fractions after column purification. A single band of mutant βA3/A1-crystallin, corresponding to a molecular weight of 14 kDa was seen after gel filtration on Sephadex G-200.

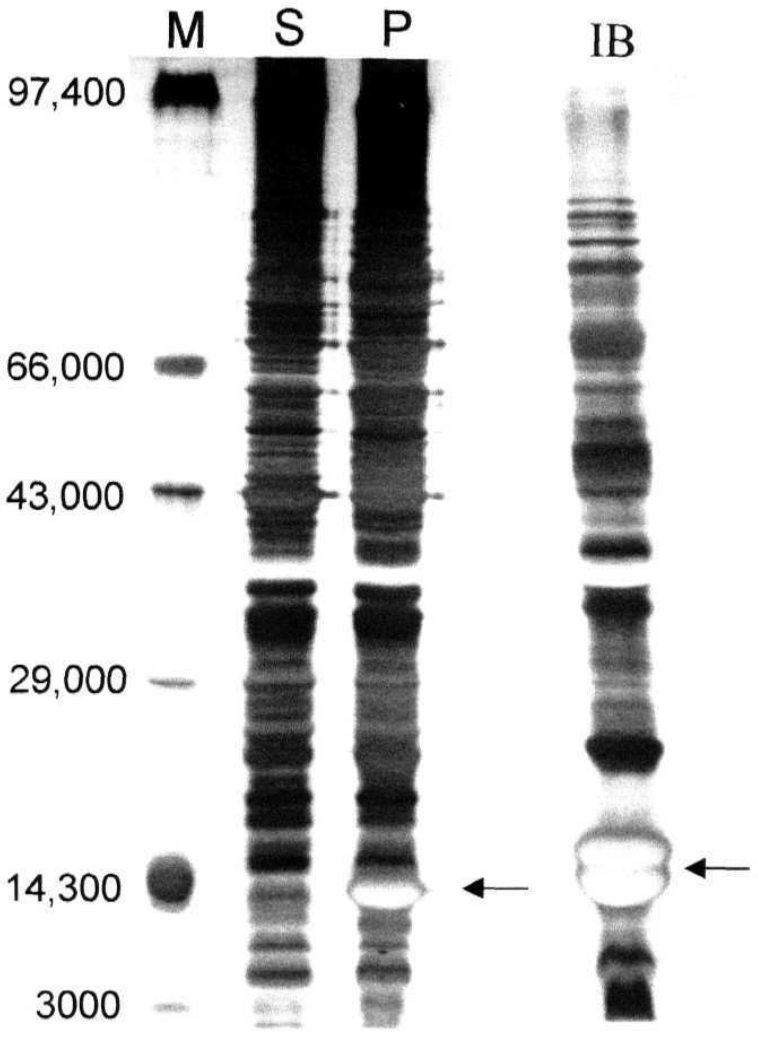


Figure 5.2.3. Solubility of mutant βA3/A1-crystallin

M = Molecular weight marker
S = Soluble proteins from cell lysate
P = Insoluble proteins from cell lysate
IB = Mutant βA3/A1-crystallin extracted from inclusion bodies

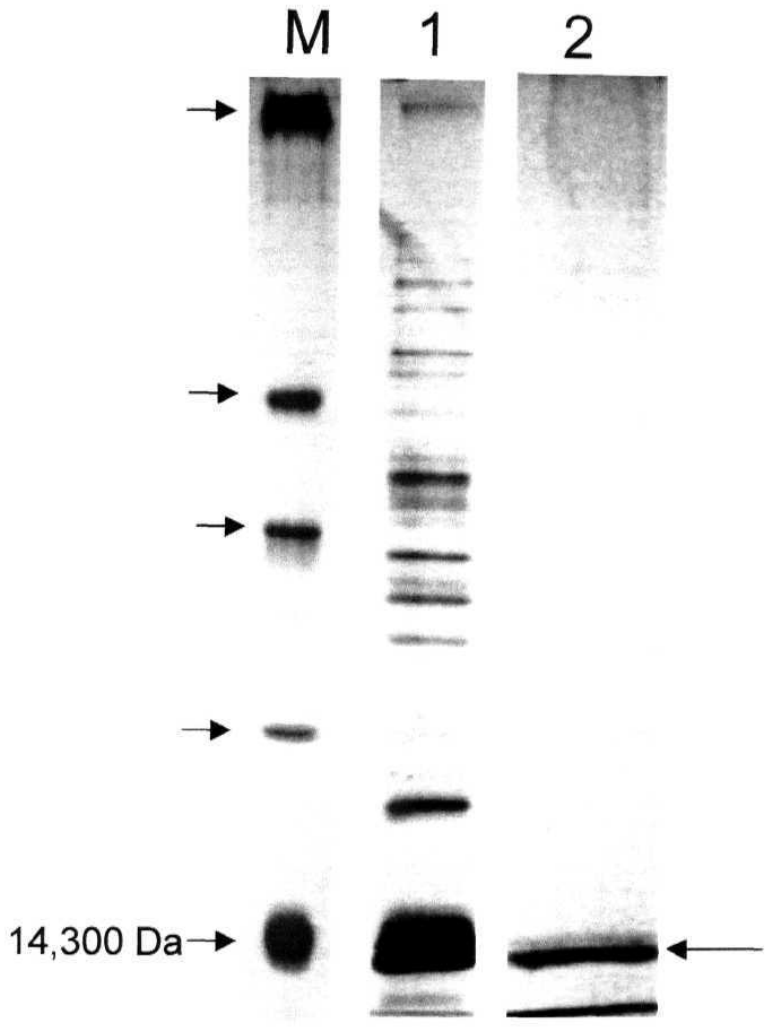


Figure 5.2.4. Purification of mutant βA3/A1-crystallin

M = Marker
1 = Post-DEAE-Cellulose
2 = Post-Sephadex G-200

5.2.4. AGGREGATION OF MUTANT pA3/A1-CRYSTALLIN

The protein obtained after purification showed increased turbidity compared to other β-crystallins at the same concentration. Comparison of the elution profiles on Sephadex G-200 of mutant βA3/A1-crystallin with that of human lens proteins showed the mutant protein to elute much earlier than expected, almost along with human β_H-crystallin (Fig. 5.2.5). This indicates that the protein has not folded appropriately and is thus not soluble. Another method to facilitate appropriate folding was tried out by adding dextrin-10 (50 mM) to the folding buffer, since it has been shown earlier that linear dextrins interact with denatured proteins and prevent self-aggregation (Sundari *et al.,* 1999); dextrin enhanced the solubility of the mutant, but only at low concentrations of the protein. Considering the large deletion in the protein, its inherent instability may be responsible, at least in part to cause its aggregation, since the protein was found to form inclusion bodies even at low concentrations. Structural details of the mutant could not be studied due to increased turbidity of solutions.

All [3- and γ-crystallins have the characteristic supersecondary structures called the 'Greek key motif. Due to the splice-site mutation (G to A transition) at the 5' donor splice site of intron 3, two exons (exon 3 and exon 4) are deleted resulting in loss of two Greek key motifs in the protein. To model the mutant protein, the amino acid sequence was submitted to Swiss-Model protein modelling server (www.swissmodel.expasy.org) where the template was selected

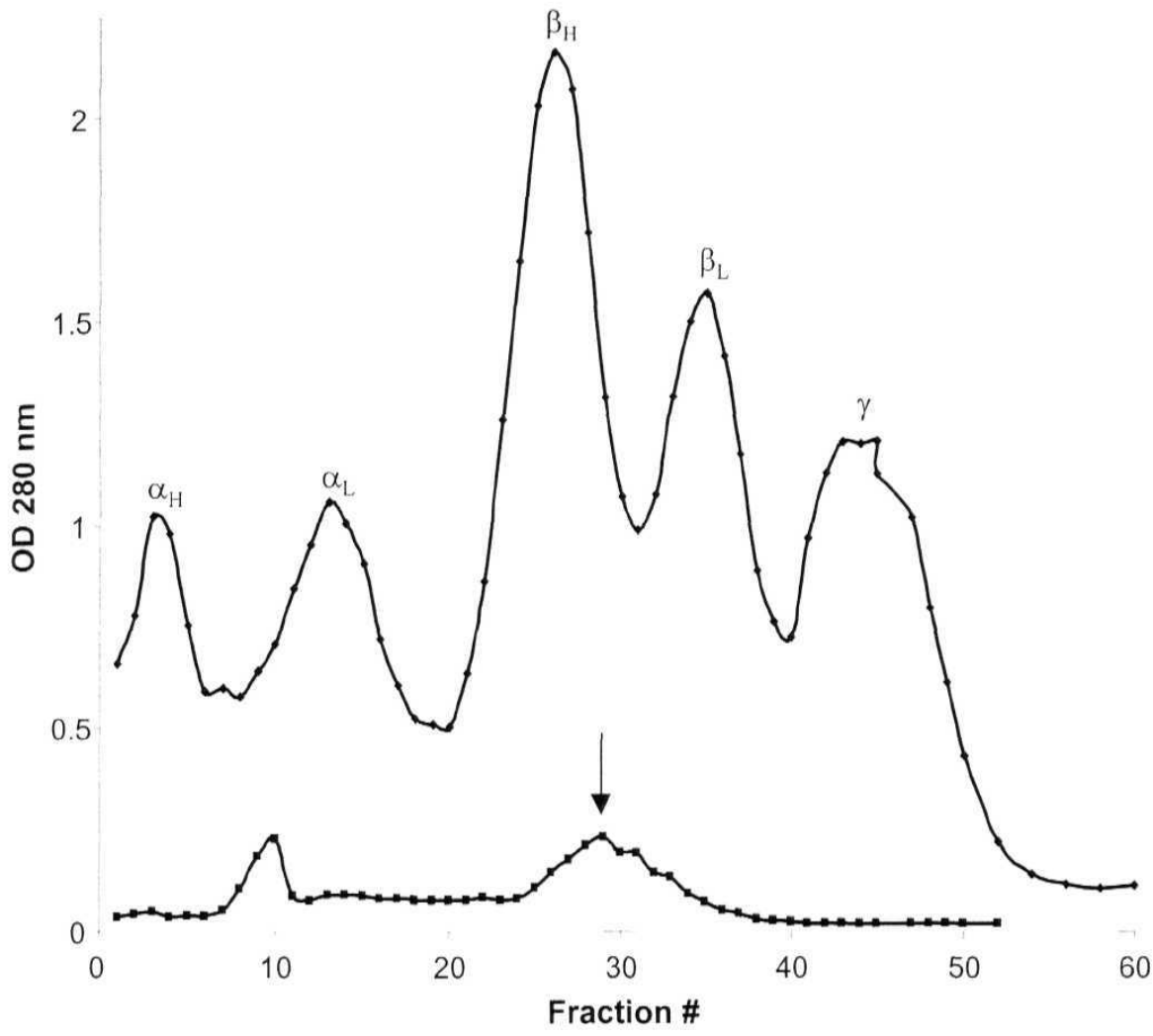


Figure 5.2.5. Elution profile of mutant βA3/A1-crystallin from Sephadex G-200

Human lens proteins (♦—-♦)
Mutant βA3/A1-crystallin (■—■)

automatically by the program (Peitsch, 1995; Peitsch, 1996; Guex and Peitsch, 1997). Refinement of the structure was carried out on a Silicon Graphics workstation using Insight II. Energy minimization was performed using charge valence force field with 500 steps of steepest gradient and 1000 steps of conjugate. As predicted, the mutant protein was found to have lost one complete domain and had one domain, composed of two Greek-Key motifs, intact along with an overhanging N-terminal arm (Fig. 5.2.6).

Random mutations in proteins are known to dramatically affect their solubility. Pande *et al.* (2001) have shown that the R58H (linked to aculeiform cataract) and R36S (linked to another form of congenital cataract) mutants of human γD-crystallin spontaneously crystallize and lower the solubility of the protein though no difference was observed between mutants and wild-type protein in their phase behavior, thermal stability and protein conformation.

If misfolding and aggregation of the mutant βA3/A1-crystallin are responsible for the cataract phenotype, it is interesting to note that despite the presence of large amounts of α-crystallin, which acts like a chaperone and helps to keep aggregation-prone proteins in solution, the mutant βA3/A1-crystallin forms insoluble aggregates.

The effect of the mutation on protein folding can be explained by comparing the structures of the mutant βA3/A1-crystallin with the wild type protein, which has earlier been expressed and purified in the soluble form

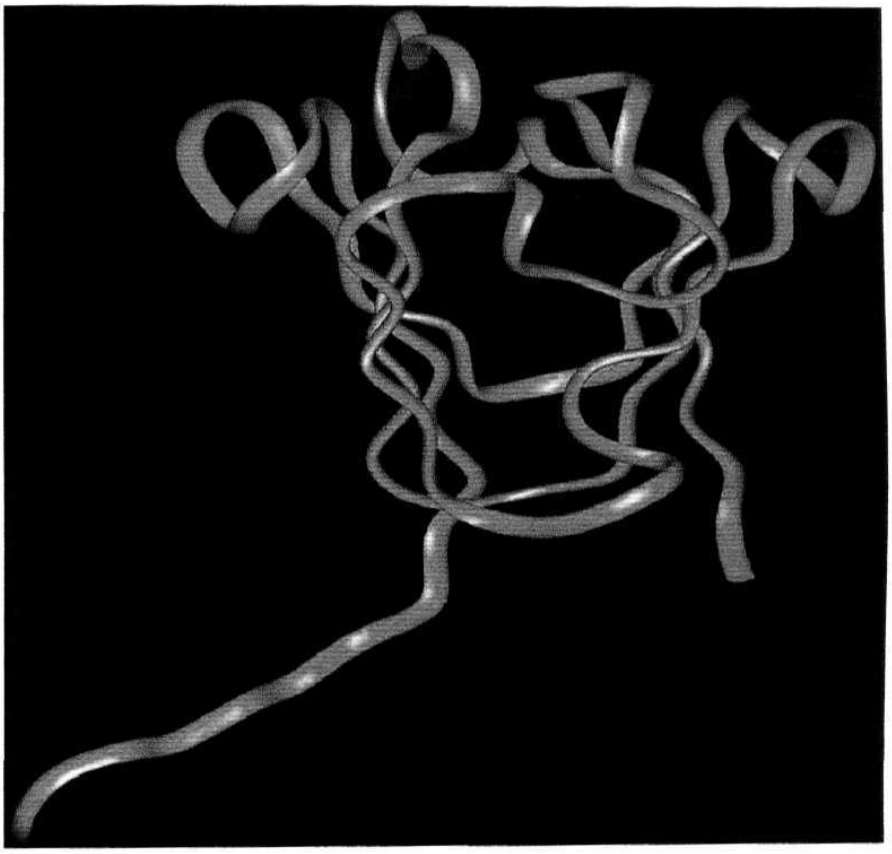


Figure 5.2.6. Model of the mutant βA3/A1-crystallin

using the baculovirus expression system (Hope *et al.,* 1994). However, the model structure of the mutant protein showed an overhanging NH_2-terminus, which could also aid aggregation. We found that the heterologous expression of the mutant βA3/A1-crystallin in *E.coli* caused the protein to aggregate, which could be responsible for this splice-site mutation to manifest as an autosomal dominant zonular cataract.

Summary

I) **Chapter 1** is a general introduction on the eye lens and its proteins, namely the crystallins. It also describes the different types of cataracts and the risk factors involved in cataractogenesis.

II) **Chapter 2** addresses the role of fluorophores/chromophores that accumulate in the lens with age.

- We confirm that 3-HK and 3-HKG are photochemically inert.
- XA is an efficient photosensitizer, capable of generating both singlet oxygen and superoxide radicals.
- XA8OG is also a UV-A region sensitizer, capable of efficiently generating singlet oxygen species but not much of superoxide.
- XA8OG has a high fluorescence quantum yield (φ) of 0.22 and a major emission lifetime of 12 ns, both favouring its photodynamic action.
- We also demonstrate that both XA and XA8OG oxidize proteins when irradiated with UV-A light, causing photodynamic covalent chemical damage to proteins and induce apoptosis in cultured HLE cells.

The accumulation of XA8OG in the aging human lens (and the attendant decrease in its precursor 3-HKG) can add to the oxidative burden on the system. XA8OG thus appears to be an endogenous accumulant in the lens that can act as a cataractogenic agent.

III) **Chapter 3** deals with the reaction of peroxynitrite with α-crystallin and the structure-function correlation of oxidized a-crystallin.

- Upon reaction with peroxynitrite, γ-crystallins produce the most nitrated products and dityrosine while a-crystallin produces the least, as expected from their tyr and trp contents.

- Spectral analysis showed a partial unfolding of α-crystallin, with some loss of secondary and tertiary structure. However, its chaperone-like ability, monitored using β-crystallin, insulin and lysozyme as target proteins, was not affected.
- Fluorescence probe analysis did not reveal any enhancement in surface hydrophobicity upon peroxynitrite treatment, while the hydrophobic peptide melittin bound to both parent and modified α-crystallin equally well, and abolished their chaperone-like activity.
- We then investigated the putative chaperone-functional-element (i. e., the sequence 71-88) of αA-crystallin. This peptide was seen to partially unfold upon peroxynitrite modification and lose its chaperone-like activity. To address the possibility of other chaperone-like-functional-elements in a-crystallin we digested human αA-crystallin with trypsin, isolated the individual peptides by reverse-phase HPLC and checked the chaperone-like activity of each fraction before and after peroxynitrite modification. We identified one fraction, containing a mixture of peptides, which retained chaperone-like activity despite oxidation.

These results reinforce earlier suggestions that oxidation or chemical modification of some residues of a-crystallin does not affect its chaperone-like ability.

IV) **Chapter 4** describes the use of dietary antioxidants in delaying **the** progression of age-related cataract.

- *Ginkgo biloba* and *Withania somnifera* are good antioxidants, capable of preventing crosslinking in proteins and strand breaks in DNA.
- Apart from being antioxidant, the extracts are also cytoprotective.
- Neither *Ginkgo biloba* nor *Withania somnifera* extracts are phototoxic.
- Though *Withania somnifera* could prevent the phase separation at low temperature, neither of them prevented high-temperature precipitation of proteins.

As oxidative reactions are involved in almost all tissues of the body, long term use of antioxidants could help not only in controlling the progression of cataract, but also in other age-related pathologies.

V) **Chapter 5** includes the cloning and expression of mutant βA3/A1-crystallin in *E.coli* to study the effect of a splice site mutation on its structure.

- Maximum expression of mutant βA3/A1-crystallin was obtained 3h post induction. The protein formed inclusion bodies in *E.coli* and was extracted using sonication and denaturing conditions.
- Upon refolding, the protein was found to aggregate.
- The mutant (3A3/A1-crystallin showed a higher tendency to aggregate as compared to normal β A3/A1 -crystallin.

Mutations in lens proteins resulting in aggregation may thus lead to cataract.

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LIST OF PUBLICATIONS

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Antioxidant Properties of Green and Black Tea, and their Potential Ability to Retard the Progression of Eye Lens Cataract

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Aqueous extracts of green and black lea are shown to quench reactive oxygen species such as singlet oxygen, superoxide and hydroxyl radicals, prevent the oxidative cross-linking of test proteins and inhibit single strand breakage of DNA in whole cells. They are also seen to be able to counteract the oxidative insult mounted by cigarette smoke. In rats in which cataract was induced by subcutaneous injection ol' selenite, administration of green or black lea extracts led to a retardation of the progression of lens opacity, suggesting the potential cataracto-static ability of tea. © 2001 Academic Press

Key words: tea extracts; antioxidants; comet assay: eye lens proteins: selenite cataract.

1, Introduction

Tea is a widely popular beverage around the world for centuries. It is estimated that on an average, about 6 g of tea is consumed per day by a 60 kg human being. Tea leaves contain about 55% polyphenols by dry weight. Green lea, popular in the Orient, is a rich source ol' llavonoids such as catechin (C), epicatechin (EC), epigallocatechin (EGC) and their gallate esters CG, ECG, EGCG, respectively. In black tea, which is more popular in India, Africa, Europe and the U.S., the catechins are converted to complex condensation products, namely theaflavins (TFs) and thearubigins (TGs), which impart the brew its characteristic rich color.

Green lea and black tea, as well as their extracts (GTE and BTE) are known to be beneficial to health. Hertog, Hollman and van de Patte (1993). Katiyar and Mukhtar (1996), Keli et al. (1996). Tijburg el al. (1997), and Zeyuan et al. (199S) have recently summarized the reported role of tea llavonoids in reducing the risk of hyperglycemia, hyperlipidemia, stroke, cardiovascular diseases, coronary heart diseases and cancer. Vinson et al. (1995) have shown that tea tlavonoids are powerful antioxidants (ten- to 2(-fold more powerful than vitamin C), using an in vitro oxidation model for heart disease. A major m chartism that is implicated here is the inhibition ol' low-density lipoprotein or LDL oxidation by lea flavanoids, which has been demonstrated in vitro (Serafini, Ghiselli and Ferro-Luzzi, 1996). The antioxidant properties of C, CG, ECG, EGCG, TFs and TGs have been reviewed recently by Wiseman, Balentine and Frei (1997). These compounds quench, to varying degrees of efficiency, reactive oxygen species and appear to be 2·9–6·2 times more efficient than Trolox in the Rice-Evans assay of antioxidant ability (Miller et al., 1995).

The anlioxidant properties ol' these tea extracts and llavonoids have been monitored by and large through lipid peroxidation assays. Their effects on the oxidative damage to DNA and protein molecules have been less monitored—barring some studies on cellular changes such as sister chromatid exchanges, micronuclei formation, microfiltration of strand broken DNA, or 8-hydroxydeoxyguanidine adducts or excretion (Xu et al., 1992: Yokozawa et al., 1992; Hasegawa et al., 1995: Leanderson, Faresjo and Tagesson, 1997: Wei et al., 1999). Direct molecular studies evaluating the protective action of tea components against protein and DNA damage would be of value in understanding the underlying mechanisms, particularly in relation to protein associated pathologies such as cataract or prion diseases (Balasubramanian, Chandani and Sivakama Sundari, 1998).

Cataract of the human eye lens accounts for over 42 % of blindness around the world. There are about 1 7 million people today who are cataract blind, and it is estimated that there are 28 000 new cataract cases every day. People over 50 years of age are most susceptible to this disease, and this population is expected to increase significantly in the near future. Also, even after cataract surgery, some post-operative complications, e.g. posterior capsular opacity, retinal

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This paper is dedicated to the memory of our dead friend and colleague Dr Mrs Darshini Ranganathan.

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detachment, conical edema, or endolphthalmitis, are not to be easily dismissed. Thus, cataract requires not just a surgical solution, but a chemical and pharmacological complement as well. Since oxidative stress is a common initiator of many age related conditions. including cataract, chemical approach to delaying the onset or retarding the progression of cataract is valuable. Robertson, Donner and Trevithick (1989) had compared the self reported consumption of supplementary vitamins by 175 cataract patients with th at of 1 75 individually matched cataract free subjects, and found that the latter group used significantly more supplementary vitamins C and E. In traditional societies, it is more acceptable, accessible and affordable to have antioxidant substances in indigenous, user friendly and user accessible forms than administer them as pills, drugs or capsules. Tea is one such food item that is consumed by a very large number of people across the world, and its potential benefits to the eye are worth studying.

It is with these in mind that we have looked at the in vitro inhibition of oxidative damage to proteins and DNA, by green tea and black tea extracts. In addition, we report on the effect of tea on the development and progress of experimentally induced cataracts on laboratory rats. Our studies show that *GTE* and BTE (i) inhibit the oxidative cross-linking of test proteins, (ii) inhibit the DNA strand breakage in the nuclei of whole cells and (iii) affect the progress of selenite-induced cataract in rats. They also show some differences in the behavior of GTE and HTM, presumably because of the extra components present in the latter and/or the differential levels of glycosidation of the polyphenols in the two.

2. Materials and Methods

Preparation oj Tea Extracts

Mxtracts were prepared following the method ol' Wang et al. (1989). Essentially the same extraction procedure has been followed by Leanderson et al. (1997), who found 47% of the compound mixture to be EGCG, and by Wei et al. (1999). Chinese green tea (brand Gunpowder, which is used as a standard) or black tea (Mipton) was suspended in water (7·5 ml g" ' tea, 75 C) for 5 min. and filtered. The tea residue was then suspended in 80% ethanol (7·5 ml g^{-1}, 50 C) and filtered. The procedure was repeated three times, with nitrogen being bubbled through the liquid continuously. The aqueous and ethanolic extracts were pooled and concentrated to 20% of their original volume using a rotary vacuum evaporator. This concentrate was thoroughly mixed with chloroform (1:1 v/v) in a separating funnel, and the aqueous phase collected and extracted three times with ethyl acetate (1 : 1 v/v). The collected ethyl acetate extract was vacuum dried and the green and black tea extract (GTE or BTE) thus obtained was dissolved in water. The concentration of the extra(was determined from the optical density of t aqueous solution at 270 nm (e.g. 100 U meani OD of 100).

Reactions using Singlet Oxygen

The SSS method (Midden and Wang, 1983) w; used to generate singlet oxygen species. Immobilize sensitizer was prepared by the physical adsorption < Rose Bengal onto diazotized glass beads (Pierc Chemical Co.. Rockford, IL, U.S.A.). Typically, 0·2 of the dye was added to 1 g of the beads in ml methanol and stirred. The solvent was removed on rotary evaporator at 40 C. The dry beads were now intense pink in colour. Transparent double-sided adhesive tape was applied to a glass plate, the beads spread over the sticking surface, and the excess beads shaken off. Samples (total volume 125 //I) were placed in the wells of a Boerner microscopic slide (well volume. 200 μl). The wells were covered with the sensitizer coated plates placed with the bead coated surface facing the wells. Samples were illuminated with a 200 W tungsten lamp, the light first passing through a shallow glass tray containing water (to absorb infrared radiation) and a 540 nm filter. Following irradiation, protein solutions, at a final concentration of 1 mg ml^{-1}, were aliquoted al various time intervals and subjected to SDS–PAGE analysis.

Reactions usiiuj Hydroxyl Radicals

The photo-Fenton reagent (Sailo. Takayama and Matsuura, 1990), namely bis(hydroperoxy)naphthaldiimide, which generates hydroxyl radicals upon irradiation, was coaled onto Kieselgel-60 beads, as described above. The coated beads were added to an aqueous solution of the protein (1 mg beads per ml ol solution) in a quartz cuvette, which was then irradiated al 366 nm (bandwidth 20 nm) for 90 min with constant stirring in the cell compartment of a Hitachi M-4000 spectrofluorimeter. Protein concentration was 1 mg ml" '. Following irradiation, aliquots of the protein were analysed on SDS–PAGE.

Electron Paramagnetic Resonance (EPR) Measurements

When riboflavin was used as the radical generator, an aliquot from a stock aqueous solution was added to the test solution. When cigarette smoke condensates were tried, these were prepared by bubbling smoke from one cigarette through 1 ml of H_2O under suction, until 70 mm of the cigarette had been burnt, and used immediately. EPR spectra were recorded on 1 ml mixtures containing a final concentration of 50 //M of riboflavin, or 0·7 ml of the smoke condensate, and 200 OD_{270} U of GTE in 100 mM 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), used as

spin trap. Radicals were generated by irradiating the solution for 2 min with broad band visible light from a xenon lamp source, with the UV range (and heat) cut out using a glass beaker of water as the filter. In experiments aimed at quenching specific oxyradicals, superoxide and hydroxyl radicals were produced using 0·1 mM xanthine, 50 mM DMPO, 0·1 mM DETAPAC, 0·1 mM Fe_3: EDTA, 100 U ml^{-1} catalase and 0·1 U ml^{-1} xanthine oxidase (superoxide under metal free conditions and hydroxyls in presence of metal ion). Spectra were recorded at 25° C at a microwave frequency of 9·385 GHz and power of 20 mW, time constant 0·128, modulation amplitude 1·25 G, and receiver gain $3·2 \times 10^4$, following Muralikrishna et al. (1991).

Single Cell Gel Electrophoresis

Cells of the CCRF cell line (human T cell lymphoma line maintained at the CCMB Hyderabad) were grown in culture and collected at sub-confluent stages, washed and suspended in phosphate buffered saline. Fresh cigarette smoke condensate was prepared as described above, and 65 μl of the condensate was mixed with 100 OD_{270} U of GTE or BTE. The osmolarity was adjusted to that of PBS, using a 10 × preparation of the buffer, to a final volume of 100 μl. To this, 5×10^4 CCRF cells in 5 μl of PBS were then added. Microtiter plates containing this mixture were irradiated for 45 min with visible light from a 200 W tungsten lamp. For sensitization with singlet oxygen, the mixture was placed in the well of a Boerner microscopic slide and irradiated for 45 min as described above. The cells were then centrifuged, washed once in PBS, transferred to 50 μl of low melting agarose at 37° C which was layered on ground glass microscopic slides previously coated with a thin layer of 1% agarose. After allowing to set, another layer of 1% agarose was applied over the low melting agarose layer containing the cells. Subsequent preparations and electrophoresis were performed using the method described by Singh et al. (1988). Ethidium bromide stained slides were examined under a fluorescence microscope, with an excitation filter of 515–560 nm and a barrier filter of 590 nm.

Evaluation of Antibacterial Activity of Tea Extracts

The antibacterial activity of aqueous extract of green tea was tested against ATCC strains of *Staphylococcus aureus* (ATCC29213), *Escherichia coli* (ATCC25922), and a laboratory isolate of *Pseudomonas aeruginosa*. The test organisms were subcultured in peptone broth for 1 hr at 37° C. They were then spread on MHA (Mueller Hinton Agar) plates and sterile filter paper discs containing varying concentrations (20, 10, 5, 2·5, 1, 0·5, 0·2 and 0·1 μg) of the extract were placed on it. The plates were incubated at 37°C for 24 hr and then evaluated for zones of inhibition.

Evaluation of Antiviral Activity

The antiviral activity of aqueous tea extract was tested against Herpes Simplex Virus 1 (laboratory isolate). Monolayers of SIRC cells (rabbit corneal keratocyte cell line, obtained from the National Centre for Cell Science, Pune) were infected with HSV1 in the presence of 10, 20, 30, 40 and 50 μg ml^{-1} of the extract. Reduction in cytopathic effect was evaluated after 72 hr by comparing with cells incubated with virus alone.

Induction of Selenite Cataracts

Selenite cataracts were induced using a modification of the method of Devamanoharan et al. (1991). Three litters of 8 day old Wistar rat pups (average weight 15 g) were administered intraperitoneal injections of 0·2 ml of physiological saline or 0·2 ml of freshly prepared aqueous green or black tea extracts (1 g of green or black tea suspended in 10 ml of physiological saline at 75° C for 5 min, filtered, centrifuged at 3000 *g*, and the volume adjusted to 100 OD_{270} U ml^{-1}). Each litter was subjected to one of these three treatments. The injections were repeated at 24 hr intervals until postnatal day 20. On postnatal day 10, 30 min after the i.p. injections, sodium selenite (2·2 mg kg^{-1} body weight) freshly dissolved in physiological saline was administered to all the pups subcutaneously. After their eyes had opened (postnatal day 15–17), the lenses were observed daily. Cataracts could be visualized with a penlight examination, or with a slit lamp. The extent of cataract was quantified using a scale of 1–6 (Hiraoka and Clark, 1995). When the animals were 6 weeks old, they were killed and the lenses excised. Animal experiments were performed following the NIH Guidelines on the Care and Use of Laboratory Animals in Research and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

3. Results

Antioxidant Ability

We have looked at the effect of GTE and BTE in inhibiting the oxidative stress imposed under a variety of conditions, such as (i) through singlet oxygen, (ii) by direct photolytic reactions in the UV region, (iii) through the photodynamic effect of sensitizer molecules such as riboflavin that can occur in the near UV or visible region of light, (iv) through the Fenton reaction that generates hydroxyl radicals, (v) by the ferryl (Fe-IV) system of metmyoglobin/ H_2O_2, (vi) via cigarette smoke exposure and (vii) by the administration of selenite to animals. In each situation, we

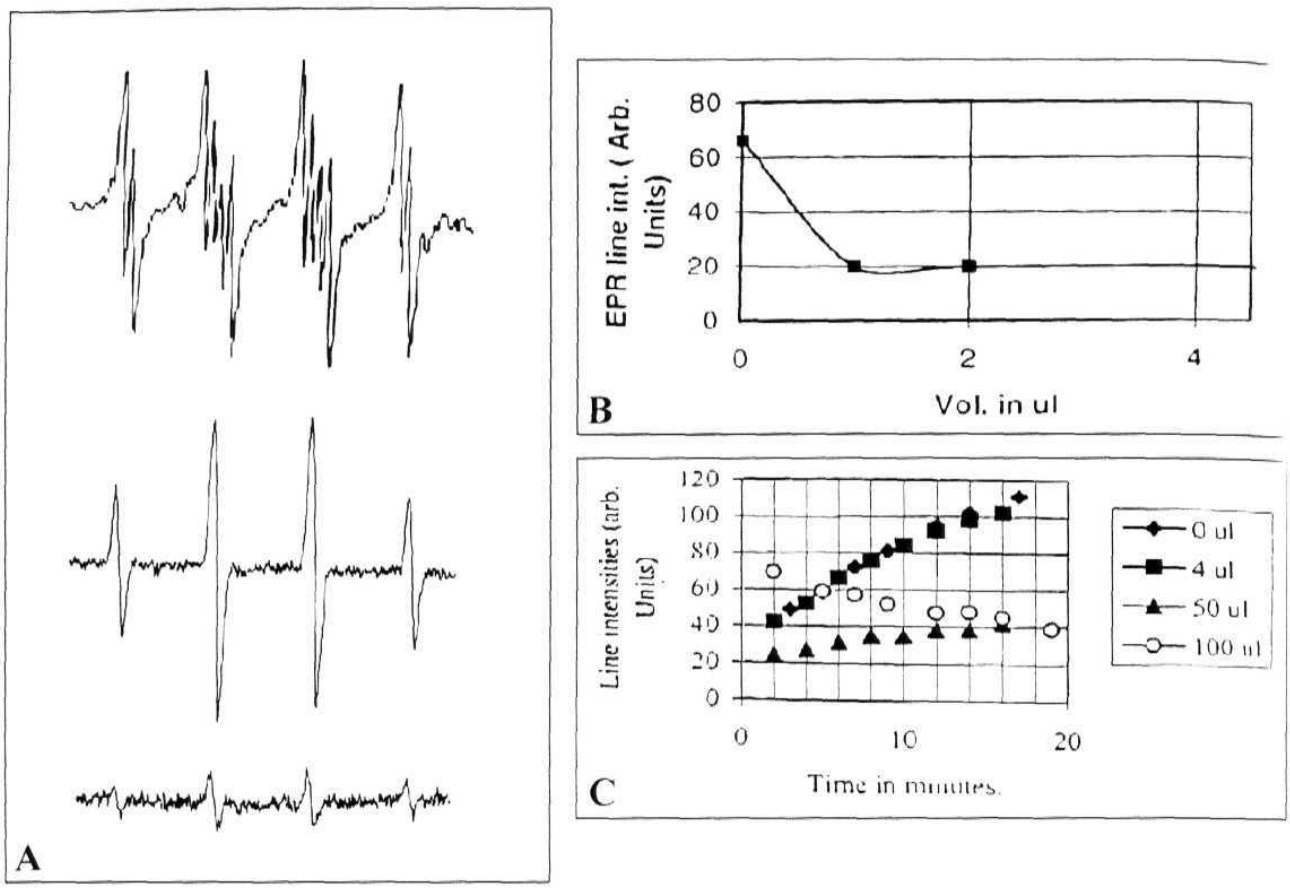

FIG. 1. (A) Free radicals produced upon irradiation of the photo-sensitizer riboflavin (25 μM in water) with broad band visible light, trapped as stable DMPO free radicals. Top: superoxide radicals. Middle: hydroxyl radicals. Bottom: addition of GTE (50 μl of 200 U concentration), prior to irradiation quenches the radicals. (B) Dose-dependent quenching of superoxide by GTE (concentration as above). (C) Dose-dependent quenching of hydroxyl radicals by GTE (as above).

find the extracts to be efficient in relieving oxidative stress.

Fig. 1(A) shows that the free radicals generated photochemically by the sensitizer molecule riboflavin are effectively quenched by GTE. The EPR signals produced by the sensitizer upon irradiation with visible light, and trapped by the spin trap molecule DMPO, are reduced almost completely when 50 μl of a 0·5 mg ml^{-1} solution of GTE are added to the riboflavin solution prior to irradiation. Fig. 1(B) and (C) show that GTE is able to destroy both superoxide and hydroxyl radicals effectively. Similar results were obtained with BTE. Both of them are also able to quench the other commonly encountered reactive oxygen species, namely singlet oxygen ($^1O_2$). Fig. 2(A) shows the effect of GTE and BTE on the singlet oxygen mediated oxidative cross-linking of bovine pancreatic ribonuclease A (RNase A) monitored by SDS–PAGE analysis. Singlet oxygen was generated by the SSS method (Midden and Wang, 1983); the longer the exposure the greater the amount of singlet oxygen generated and the consequent cross-linking of the protein (as shown by lanes 1, 2 and 5). GTE and BTE are both efficient in preventing the cross-linking as well as the chain degradation of the protein as lanes 3, 6 and 4 and 7 reveal. This inhibitory ability of these extracts is consistent with an earlier report (Tournaire et al., 1993) that flavonoids quench singlet oxygen, with catechin being the most efficient amongst the ones tried. Fig. 2(B) shows that the green and black tea extracts inhibit the hydroxyl radical mediated cross-linking in the test protein as well. This is consistent with their ability to quench hydroxyl radicals, as discussed above.

Inhibition of Protein Photo-aggregation

Protein damage can also occur by direct photolysis, a reaction that is important to tissues such as the skin and the eye. The eye lens protein γ-crystallin is known to aggregate and cross-link upon irradiation in the near UV region where its tryptophan residues absorb, leading to increased light scattering and the precipitation of the protein from solution (Chakrabarti, Bose and Mandal, 1986). As Fig. 3 shows, GTE is able to inhibit the photoprecipitation of γ-crystallin. This ability is of possible relevance to the risk of light mediated cataract or opacification of the eye lens, a tissue that contains crystallins as about 40% of its wet weight. Of the two, GTE appears better in this regard, since BTE tends to coagulate the protein even in the absence of irradiation; some component in GTE appears to complex with proteins. The crystallins, particularly α-crystallin, bind to a variety of cellular

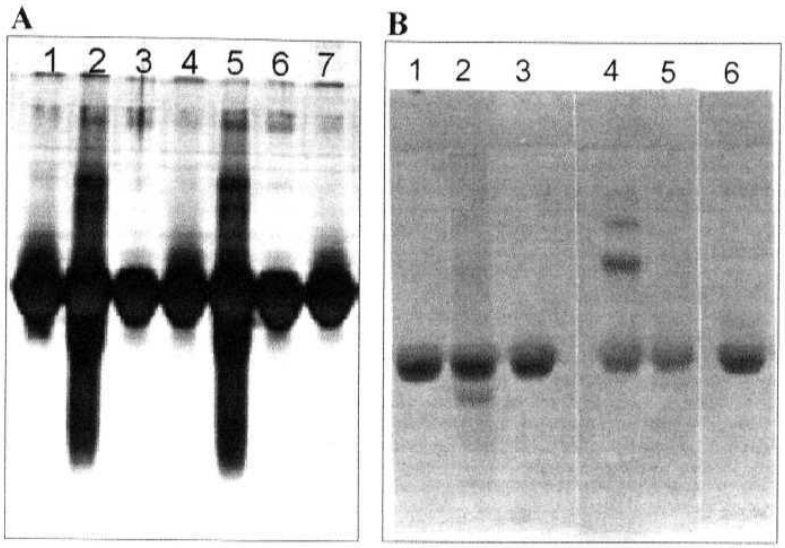

FIG. 2. (A) The effect of exposure to singlet oxygen on bovine pancreatic RNase A, analysed by SDS–PAGE (10% gel, stained with silver). Lanes 1, 2 and 5, RNase A exposed for 0, 15 and 30 min; lanes 3 and 6, RNase A + GTE (50 μl of 100 OD U) exposed for 15 and 30 min; lanes 4 and 7, RNase A + BTE (50 μl of 100 OD U) exposed for 15 and 30 min. (B) The effect of hydroxyl radicals on RNase A (1 mg ml^{-1}), analysed by SDS–PAGE (7·5% gel, stained with Coomassie blue R-250). Lanes 1 and 4, RNase A exposed for 0 and 90 min; lanes 2 and 5, RNase A + GTE (50 μl of 100 U) exposed for 0 and 90 min; lanes 3 and 6, RNase A + BTE (50 μl of 100 U) exposed for 0 and 90 min.

molecules, big and small, with facility (Raman and Rao, 1994). That polyphenols and tannins particularly those containing many -OH groups and hydrophobic moieties, bind to proteins has been known (Spencer et al., 1988; Haslam, 1996).

Counteracting the Toxic Effects of Smoke

We next studied protein damage mediated photodynamically, through the use of sensitizers such as riboflavin. Here too we found GTE and BTE to inhibit the oxidative cross-linking and chain degradation of test proteins such as RNase A. On a similar vein are our experiments monitoring the oxidative damage effected by cigarette smoke and its inhibition by the tea extracts. Cigarette smoke condensate (CSC) has been earlier shown to be an active source of reactive oxygen species both in the dark, and more so upon the exposure to light due to the photodynamic action of its polycyclic aromatic constituents (Shalini et al., 1994). Fig. 4(A) illustrates this point by showing the production of oxyradicals when CSC is irradiated with visible light: the EPR spin trap DMPO that was added to the reaction medium shows the presence of these oxyradicals (both O_2^- and OH^-, as analysis of the EPR spectral profile reveals). Addition of GTE to the mixture causes the quenching of these radicals. In a related experiment, when CSC was added to a solution of γ-crystallin and the solution irradiated with broad band white light for 30 min, high molecular weight aggregates were formed that could be separated by SDS–PAGE. These aggregates constituted approximately 30% of the band intensity as measured by densitometric analysis (70% being the monomer). GTE was able to inhibit the oxidative damage mediated by CSC under these conditions; densitometry showed

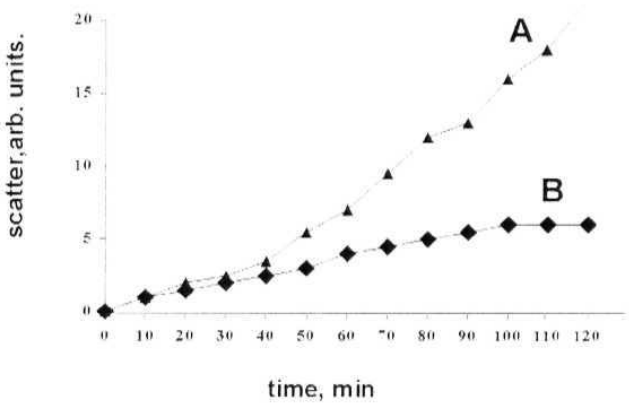

FIG. 3. The effect of GTE (50 μl of 100 U) on the light-induced aggregation of γ-crystallin (1 mg ml^{-1}). Protein samples were irradiated at 350 nm for varying time intervals, and right angle scatter measured at 600 nm. Top curve: γ-crystallin alone, bottom curve: γ-crystallin + GTE.

less than 10% of the higher molecular weight aggregates and about 87% monomeric protein [Fig. 4(B)].

Inhibition of DNA Strand Breaks in Intact Cells

Next we turn to the ability of tea extracts to protect DNA from damage. Fig. 5 shows the results of single cell electrophoresis studies, called the comet assay (Singh et al., 1988). When a suspension of cells is incubated in a medium under oxidative stress, single strand breaks occur to the DNA in the nucleus of the cells, causing the DNA to move electrophoretically not in a compact 'moonlike' manner, but in trails or comet like fashion indicating DNA strand breaks (A).

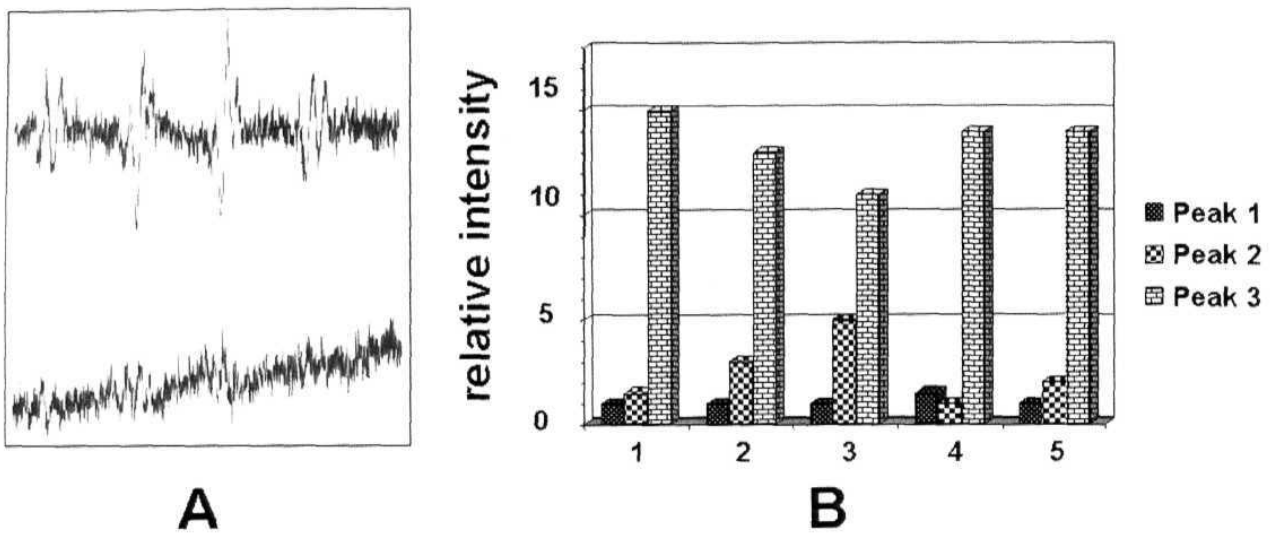

FIG. 4. (A) EPR spectra of cigarette smoke condensate (CSC) collected in water as described in Materials and Methods, recorded following irradiation for 5 min with broad band visible light. Top curve: CSC, diluted 2 : 1 with H_2O and containing 0·1 M DMPO; Bottom curve: 1 : 1 diluted CSC containing 0·1 M DMPO and 100 U of GTE. (B) A quantitation of the intensity of SDS gel electrophoresis bands obtained after the irradiation of γ-crystallin at 350 nm, in the presence of CSC and tea extracts. (1) γ-crystallin + CSC, no irradiation; (2) γ-crystallin + CSC, irradiated for 10 min; (3) γ-crystallin + CSC, irradiated for 30 min; (4) γ-crystallin + CSC + GTE (100 U), irradiated for 10 min; (5) γ-crystallin + CSC + GTE (100 U), irradiated for 30 min. For each lane, gel peak 3 corresponds to the monomer form of the protein, peaks 1 and 2 to higher multimeric forms.

When the experiment is done in the presence of GTE in the medium, the comet pattern vanishes and the nuclei are seen to be compact and moonlike (B). Interestingly, Leanderson et al. (1997) also found, using cultured lung cells and the microfiltration assay, that oxidative damage (through cigarette smoke, H_2O_2 or $FeCl_3$ solution) to DNA was inhibited by GTE. We found that black tea extract was also able to protect the DNA from single strand breaks and the comet like mobility pattern in a similar manner (C). Zigman et al. (1998, 2000) have reported that UV mediated cellular damage is inhibited by tea polyphenols. Wei et al. (1999) found that protection from oxidative damage (by H_2O_2 and UV-induced formation of 8-hydroxy 2′-deoxyguanosine) was offered by GTE and even better by BTE.

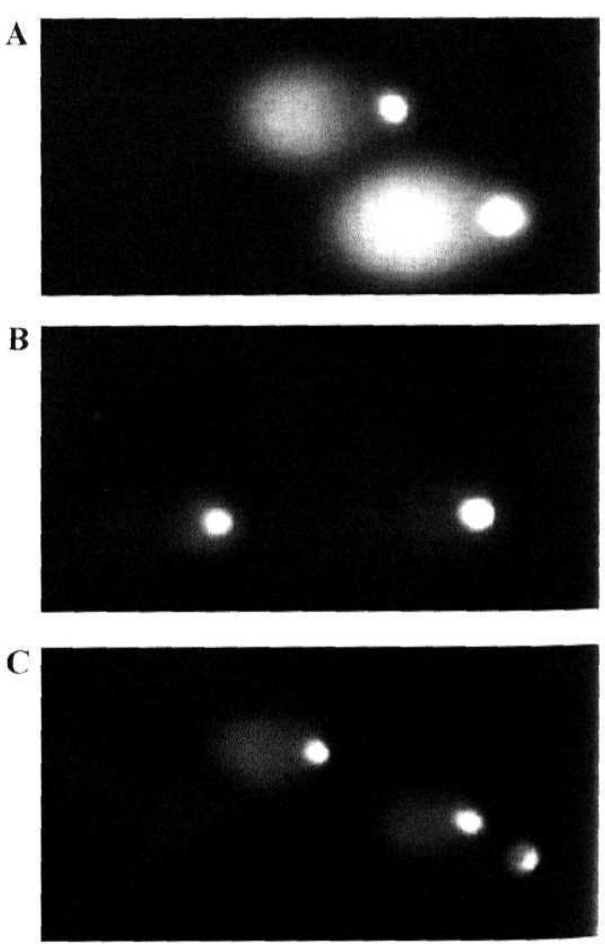

FIG. 5. Single cell gel electrophoresis of CCRF cells. (A) Cells irradiated with broad band visible light for 45 min in the presence of CSC; (B) cells irradiated as in (A), but additionally containing GTE (50 μl of 100 U); (C) cells irradiated as in (A), but additionally containing BTE (50 μl of 100 U).

4. Discussion

These results suggest that tea extracts, through their antioxidant action, might be able to counteract the oxidative insult that smoke constituents mount, and thus would be beneficial in this regard. It is interesting in this connection to note other reports highlighting the effect of tea on smokers (Xue et al., 1992; Shim et al., 1995).

Antimicrobial Activity

In the concentration range tested by us, green tea did not appear to inhibit growth of *S. aureus*, *P. aeruginosa* and *E. coli*. We could not evaluate the antiviral activity since concentrations above 20 μg ml^{-1} was found to be cytotoxic, associated

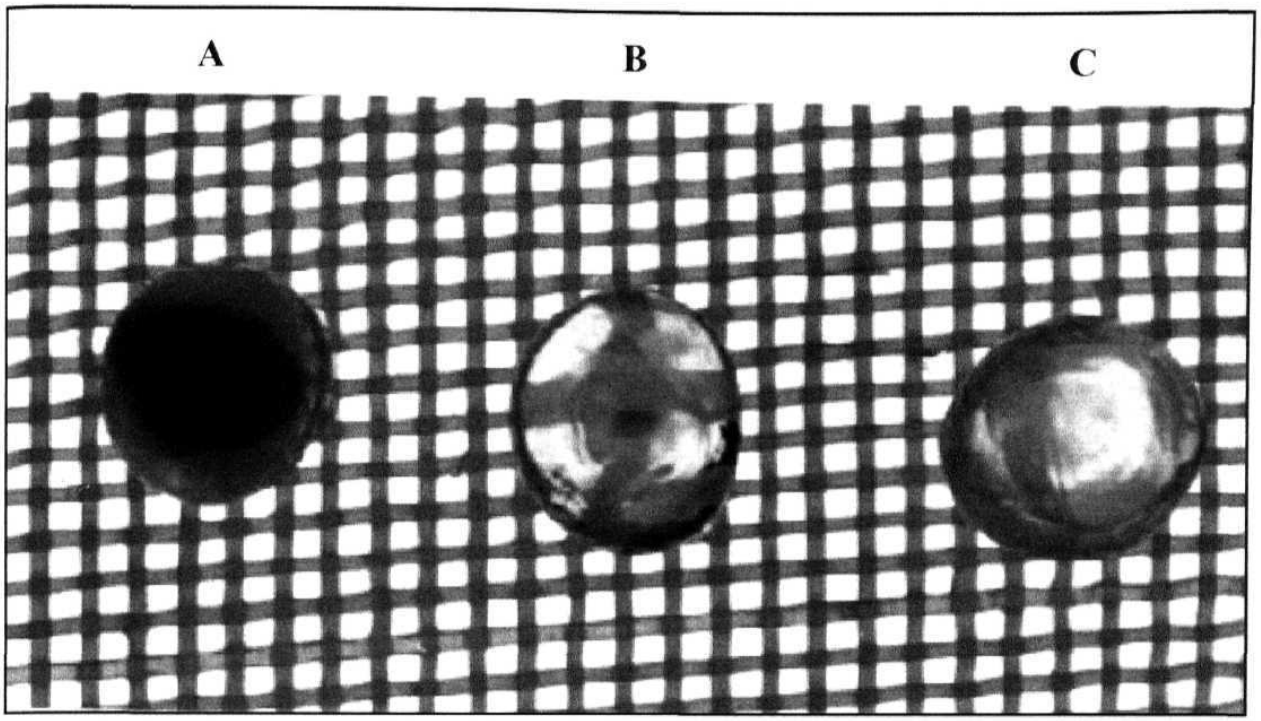

FIG. 6. Excised rat lenses. (A) Is typical of a control group animal lens, treated with selenite; (B) is that of an animal that was also given GTE; and (C) that of an animal that was also given BTE.

with granularity and peeling of the monolayer. Though no cytotoxicity was observed with 10 μg ml^{-1} of the extract, it did not show significant antiviral activity. Saeki et al. (1993) and Yao et al. (1998) had earlier given some indications that tea extracts have antimicrobial activity, but the concentrations used by them were as high as 1100 μg ml^{-1} for green tea and 510 μg ml^{-1} for black tea.

Cataracto-static Ability

Finally, we have examined the antioxidative effects of green and black tea components in vivo. Selenite has been used as a model cataractogenic agent in young animals, and appears to manifest its effect on the eye lens by inducing oxidative stress in the tissue (Bhuyan, Bhuyan and Podos, 1981a,b; Shearer et al., 1997). Ascorbic acid and pantethine have earlier been shown to inhibit the progression of selenite-induced cataract (Devamanoharan et al., 1991; Matsushima et al., 1997). Our results on rat pup lenses are presented in Fig. 6. In the control group (selenite only), 50% of the lenses show cataracts at stage 2 or higher, as measured on postnatal day 20. In the group to which aqueous green tea extract was administered along with selenite, 75% of the lenses had cataract at stage 1 or less. In the black tea treated group, nearly 90% of the lenses had cataract formation at stages 0·5–1·5. These results indicate that tea extracts can contribute to the inhibition of cataract in living systems.

Conclusions

In related studies carried out independently, Lin et al. (1998) and Ibaraki et al. (2000) have presented preliminary results that suggest that green tea polyphenols protect dog lens epithelial cells against H_2O_2, UVB and X-ray induced damage. Zigman et al. (1999, 2000) have shown using optical spectroscopy that systemically administered tea polyphenols do reach the animal lens, and offer it protection against photo-oxidative stress. Our results, taken together with these two, suggest that tea polyphenols may well be cataracto-static agents. It is interesting that two Japanese firms are now producing and selling green tea extract as a health aid. We further note that Grunberger and Frenkel (1997) have obtained a U.S. patent for a formulation for the inhibition of cataract formation, diseases resulting from oxidative stress, and HIV replication, by caffeic acid esters (components of tea and coffee).

In our studies, we have noted some differences in the behavior of GTE and BTE. For example, BTE appears to bind to proteins such as α-crystallin (see previously; and also to bovine serum albumin, results not shown). BTE also tends to coagulate solutions of γ-crystallin (see previously). These differences observed between GTE and BTE might arise for two reasons. One is because black tea is prepared by processing green tea leaves, the content and glycosylation levels of the polyphenols in the two forms of tea might differ. The fermentation step used in preparing black tea tends to remove the sugar residues from

glycosylated polyphenols. The resultant polyphenols, no longer sterically protected by the sugar, would be expected to have altered potency as antioxidants (e.g. Wei et al., 1999). The second is related to the presence of theaflavin, thearubigin and related compounds in black tea, which apparently tend to complex with proteins and other substrates. They are reported to chelate with metal ions such as Fe^{2+} and Cu^{2+} (Miller et al., 1996) more efficiently than the green tea constituents.

Finally, the analysis of Robertson et al. (1989) noted that people consuming five cups of tea per day for a period of 5 years had a significant univariate odds ratio (0·39, P[0·02) indicating a reduction of 61 % in risk of cataracts for long term tea consumption. Our results presented here on the antioxidant, cytoprotective and cataracto static abilities of tea seem to provide a molecular and cellular basis for this epidemiological connection.

Acknowledgements

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Role of Xanthurenic Acid 8-*O*-β-D-Glucoside, a Novel Fluorophore that Accumulates in the Brunescent Human Eye Lens¶

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ABSTRACT

We have been able to identify a blue fluorophore from the low-molecular weight soluble fraction of human adult nondiabetic brunescent cataract lenses as xanthurenic acid 8-*O*-β-D-glucoside (XA8OG) (excitation = 338 nm and emission = 440 nm). To determine the role of this fluorophore in the lens, we have examined its photophysical and photodynamic properties. We found XA8OG to have a fluorescence quantum yield (φ) of 0.22 and a major emission lifetime of 12 ns. We found it to be a UVA-region sensitizer, capable of efficiently generating singlet oxygen species but little of superoxide. We also demonstrated that XA8OG oxidizes proteins when irradiated with UVA light, causing photodynamic covalent chemical damage to proteins. Its accumulation in the aging human lens (and the attendant decrease of its precursor *O*-β-D-glucoside of 3-hydroxykynurenine) can, thus, add to the oxidative burden on the system. XA8OG, thus, appears to be an endogenous chromophore in the lens, which can act as a cataractogenic agent.

INTRODUCTION

The lens is an essentially transparent part of the eye, which aids in focusing incident light on the retina. It is, thus, necessarily scatter-free and colorless. With age or because of a variety of etiological factors (or both), clouding of the lens occurs leading to a progressive loss of vision (1). This condition, known as cataract, is generally characterized by (a) increased light scattering and opacification and (b) accumulation of pigments or molecules that absorb light in the visible (400–750 nm range) and ultraviolet A (315–400 nm) regions of the electromagnetic spectrum (2). Opacification of the lens is caused by the development of light-scattering centers of sizes 100 nm or larger, arising usually because of the aggregation of the constituent proteins into large particles, or by the formation of vacuoles with trapped material (*e.g.* water) within (3).

As the human lens ages, the chromophores present in it are modified, such that the pale yellow lens becomes brown, changing transmission spectra of the lens (1). These modified chromophores display a distinct fluorescence in the UVA–visible region (4). These are seen both in diabetic and nondiabetic cataract cases. In the former, the browning of the lens occurs largely because of the Maillard reaction between the accumulated sugar and lys and arg residues of lenticular proteins. As the reaction proceeds, advanced glycation end products are noted, which resulted not only in the brown color but also in the insolubility and precipitation of the constituent proteins (5,6).

The browning of the lens occurs even in nondiabetic conditions, particularly in age-related cataract lens nuclei, and it is this aspect that we address in this article. Many of the pigments in the human lens have been identified as oxidized and metabolized products of tryptophan. They include the kynurenine family of metabolites (kynurenine [Ky], *N*-formylkynurenine [NFK], 3-hydroxykynurenine [3HK] and its *O*-β-D-glucoside [3HKG], and 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-β-D-glucoside [AHBG]); quinolines (quinaldic acid, kynurenic acid [KA], xanthurenic acid [XA], oxoxanthurenic acid and 4-(hydroxy 3-glycine)-quinoline); and β-carbolines (2,4,7–11). Some of these occur in the free form in the lens, whereas the others are protein bound. The photophysical properties of these chromophores determine their role in the lens. For instance Ky, 3HK, 3HKG, AHBG and β-carboline are photochemically inert (11–13). These compounds absorb in the UVB (above 295 nm) and UVA, which are transmitted to the lens through the cornea (1), and dissipate this energy, thus, protecting the vitreous and retina from light-induced damage. Some of them may behave as antioxidants *in vivo* because they are able to quench oxyradicals and inhibit oxidative cross-linking of test proteins *in vitro*. On the other hand, NFK, KA (11,12) and XA (14,15) generate reactive oxygen species

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Abbreviations: AHBG, 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-β-D-glucoside; CCRG, cooperative cataract research group; EDTA, ethylenediaminetetraacetic acid; 3HK, 3-hydroxykynurenine; 3HKG, *O*-β-D-glucoside of 3HK; KA, kynurenic acid; Ky, kynurenine; NFK, *N*-formylkynurenine; PAGE, polyacrylamide gel electrophoresis; RF, riboflavin; RNase A, ribonuclease A; RNO, *N,N*-dimethyl-4-nitrosoaniline; SDS, sodium dodecylsulfate; XA, xanthurenic acid; XA8OG, xanthurenic acid-8-*O*-β-D-glucoside

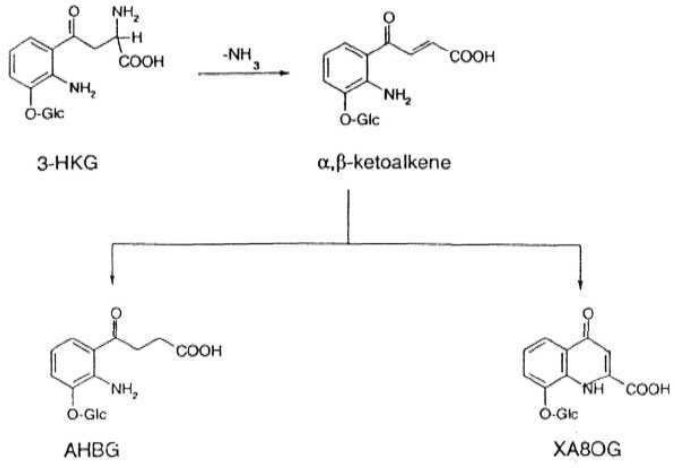

Figure 1. Suggested pathway of the formation of AHBG and XA8OG from 3HKG.

when irradiated with UVA light and can photodynamically inactivate proteins. These photochemically active compounds are likely to be chromophores involved in the induction of age-related cataracts.

In addition to these chromophores, Bessems and Hoenders (16) had found a new fluorescent chromophore that accumulates in the brunescent human lens. We have recently isolated this fluorophore from brunescent aged lenses (17) and have identified it as the glucoside of XA, xanthurenic acid-8-*O*-β-D-glucoside (XA8OG). We found that the endogenous levels of this fluorophore (which we had labeled as Fl-glc) markedly increased with time, and unlike other lenticular chromophores (2,18–22) it is not protein bound. We have shown (17) that it is formed by the initial deamination of 3HKG, followed by ring closure to give the 4-oxoquinoline product (see Fig. 1).

Because the amount of XA8OG present in the lens is in submicromolar amounts, we decided to chemically synthesize it, establish its identity with the naturally occurring substance and study its photophysical and photodynamic properties. Our results show XA8OG to be an effective sensitizer that generates reactive oxygen species photodynamically and, therefore, potential cataractogenic agent.

MATERIALS AND METHODS

Chemical synthesis of XA8OG and 3HKG. We had earlier discussed the isolation and structural characterization of XA8OG from nondiabetic brunescent human cataract lenses (Grade IV, as per the cooperative cataract research group [CCRG] grading system [23]) obtained from the L. V. Prasad Eye Institute and from the Kanazawa University Hospital. Because the amount of XA8OG obtained from even a pool of over 50 lenses was not sufficient for photophysical studies, we decided on chemical synthesis. XA8OG and 3HKG were synthesized from XA and 3HK as a starting material, respectively, and D-glucose, according to the procedure described by Real and Ferré (24,25), and their identities with the naturally occurring substances were confirmed by high performance liquid chromatography, nuclear magnetic resonance and mass spectrometry (17). 3HK, XA and glucose were obtained from Sigma Aldrich Chemical Co, St. Louis, MO.

Fluorescence spectroscopy. Steady-state fluorescence measurements were taken using a Hitachi F-2500 spectrofluorimeter. Quantum yields were estimated according to the procedure of Parker and Rees (26). Fluorescence lifetimes were measured using an IBH model 5000 U single photon counting spectrofluorimeter.

Singlet oxygen generation assay. The method of bleaching, or reduction in the absorption band at 440 nm, of *N,N*-dimethyl-4-nitrosoaniline (RNO) by singlet oxygen ($^1O_2$), devised by Kraljic and El Mohsni (27), was used to monitor $^1O_2$ production. The candidate sensitizer molecule (100 μ*M*) was exposed to light for increasing periods of time (usually 0–30 min) at its absorption wavelength in the presence of imidazole (10 m*M*) and RNO (50 μ*M*) in a 50 m*M* phosphate buffer, pH 7.4. The reaction mixture also contained superoxide dismutase (25 U/mL) and catalase (25 U/mL) to prevent the contributions of $O_2^{\cdot-}$ and H_2O_2. Air-saturated solution of the reaction mixture in a quartz cuvette (1 × 1 cm) was placed in the sample compartment of a Hitachi F2500 spectrofluorimeter and was irradiated at the chosen wavelength for 30 min by tuning the excitation monochromator to this wavelength, with the excitation slit set to 20 nm. The wavelengths used for excitation were 338 nm for XA8OG, 342 nm for XA, 371 nm for 3HK and 365 nm for 3HKG. The $^1O_2$ generated by the photoexcitation of the molecule reacts with imidazole to form a transannular peroxide that bleaches RNO. This is monitored spectrophotometrically by following the drop in the absorption at 440 nm as a function of time.

Superoxide generation assay. The ferricytochrome *c* reduction method of McCord and Fridovich (28), monitored by change in the absorption intensity at 550 nm, was used to assay the superoxide ($O_2^{\cdot-}$) generating abilities of the lens pigments. The rates of cytochrome *c* reduction were followed with time as air-saturated solutions of the test compounds (100 μ*M* each in 50 m*M* phosphate buffer [pH 7.4] containing 20 μ*M* ferricytochrome *c*) were illuminated with light at their absorption maxima (as mentioned above) for 0–30 min. To study the effect of electron donors on the production of superoxide by XA8OG, 1 m*M* ethylenediaminetetraacetic acid (EDTA) was added to the reaction mixture, and a drop in absorbance at 550 nm was monitored with time.

Electrophoresis. Each sensitizer (100 μ*M*) was added to the candidate protein, bovine pancreatic ribonuclease A (1 mg/mL) (RNase A; Sigma Aldrich), and was irradiated at the UVA absorption maximum wavelength of the additive (as mentioned above) for 120 min. The photodynamic reactions of the test compounds with the protein were monitored by following the products electrophoretically. Polyacrylamide gel electrophoresis (PAGE) (12% gel) was done in the presence of sodium dodecylsulfate (SDS) and β-mercaptoethanol, and the bands in the gel were observed through silver staining.

RESULTS

Figure 2a shows the photograph of the nuclear button of a brunescent lens excised from a representative nondiabetic patient, who came for cataract extraction at the clinic. It is classified according to the CCRG system (23) as Grade IV or brunescent. Figure 2b shows the absorption and fluorescence spectra of the aqueous homogenate of this sample. Broad emission, characteristic of XA8OG, is seen in the 400–500 nm region, as described earlier by Bessems and Hoenders (16).

We have determined the fluorescence parameters of 3HK, 3HKG, XA and XA8OG (Table 1). Note that 3HK and 3HKG both have very short emission lifetimes and quantum yields. The quantum yield of XA8OG is comparatively large ($\varphi = 0.22$), which is about three orders of magnitude greater than that of XA itself. The fluorescence of 3HKG, XA and XA8OG is complex, displaying a two-component emission mode. The lifetime of XA8OG is significantly longer (12 ns) than the longest lifetime of XA (6 ns).

Figure 3a compares the relative abilities of several lenticular pigments (100 μ*M* solutions in water) to generate $^1O_2$ with those of riboflavin (RF), a known photosensitizer and generator of $^1O_2$. This ability is seen to vary in the order XA8OG > RF > XA ≫ 3HK ~ 3HKG. We had earlier estimated the quantum yield of $^1O_2$ production of RF to be about 0.49 (12), whereas that of XA has been estimated to

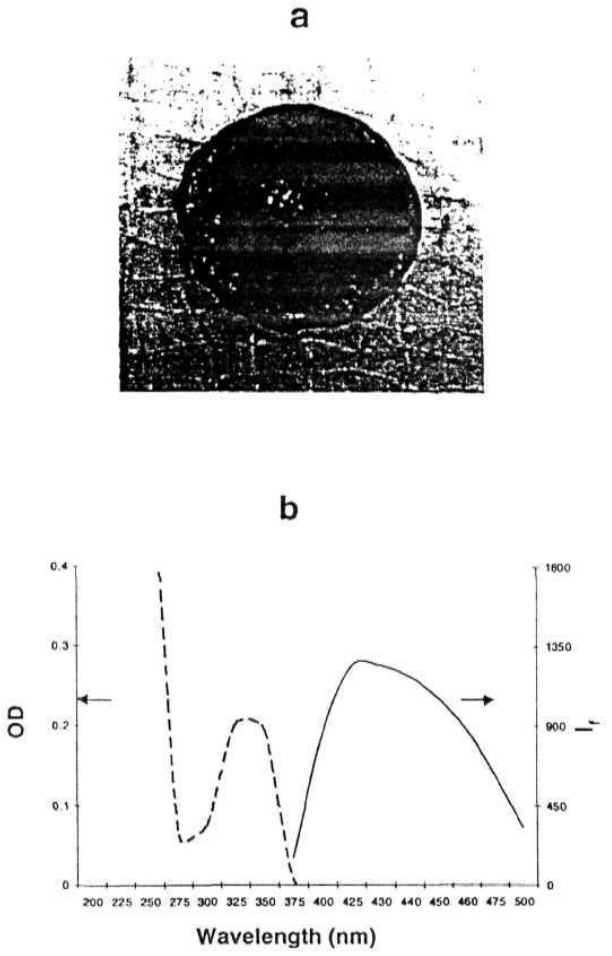

Figure 2. Panel a: Photograph of a brunescent nondiabetic human adult lens extracted at the clinic. Panel b: The absorption and fluorescence spectra of XA8OG isolated from such a lens.

be 0.17 by Roberts *et al.* (14). Figure 3b compares the photodynamic abilities of these compounds to generate the superoxide anion radical ($O_2^{\cdot-}$). XA is leading the rest in this property, whereas interestingly enough its glucoside, XA8OG, is not as efficient. Here again, 3HK and 3HKG are seen to be inactive.

We next tested the abilities of these compounds to impart oxidative damage to a test protein in solution. When RNase A was irradiated in the presence of 100 μM of the test compound for 2 h in the UVA (at wavelengths corresponding to the absorption bands of the additives, where the protein does not absorb), high-molecular weight aggregates of the protein were seen on SDS-PAGE. Protein irradiated in the absence of sensitizers did not show any additional bands on the gel. Figure 4 shows that both XA8OG and XA photooxidize the protein, whereas 3HK and 3HKG do not. Even higher concentrations (500 μM) of 3HK and 3HKG did not induce any photodamage.

DISCUSSION

Among the tryptophan metabolites present in the lens, Ky, 3HK and 3HKG (and some β-carbolines) have been shown to be photodynamically inert (11–13). They may, therefore, act as UVA filters to the posterior regions of the eye, and some of them may even act as endogenous antioxidants

Table 1. Fluorescence parameters of some lenticular pigments

Compound	Emission lifetimes τ_1	Emission lifetimes τ_2	Quantum yield (ϕ)
3HK	<0.5 ns	—	1.2×10^{-4}
3HKG	0.62 ns (94%)	7.7 ns (6%)	3.1×10^{-4}
XA	0.77 ns (79%)	6.0 ns (21%)	2.9×10^{-4}
XA8OG	12.3 ns (77%)	1.1 ns (23%)	2.2×10^{-1}

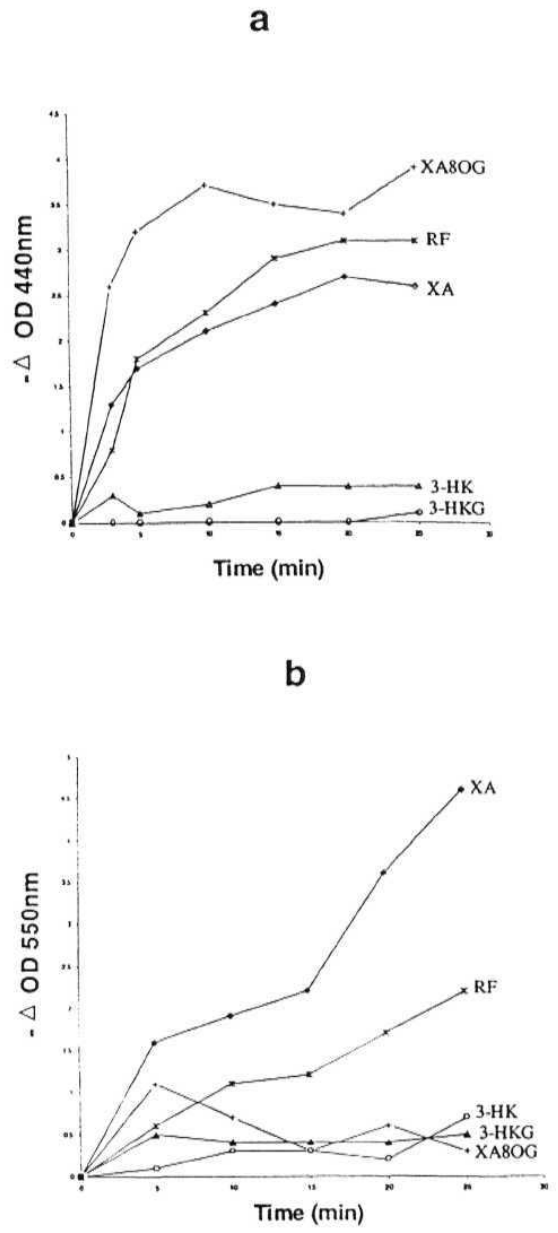

Figure 3. Panel a: RNO bleaching assay of the photodynamic abilities of various compounds (100 μM each in water) to generate singlet oxygen. Each compound was irradiated at its UVA absorption band maximum, and an aliquot was drawn every 5 min and assayed. Panel b: Fridovich assay of the photodynamic abilities of various compounds to generate superoxide in solution. Each compound was irradiated at its UVA absorption band maximum, and an aliquot was drawn every 5 min and assayed.

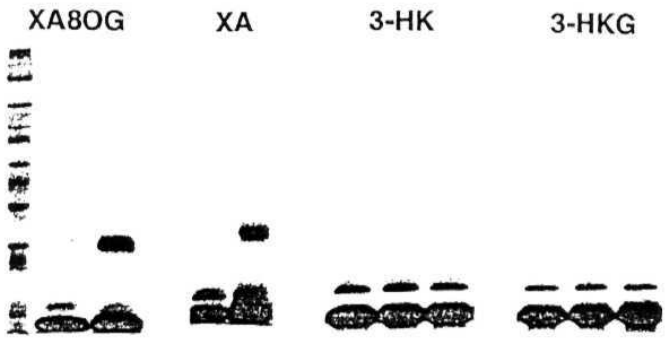

Figure 4. SDS-PAGE analysis of the photodynamic ability of 3HK, 3HKG, XA and XA8OG to cause covalent damage to the test protein RNase A. Each compound was added to the (1 mg/mL) protein solution to a final concentration of 100 μ*M*. Irradiation of each solution was at the UVA absorption maximum wavelength of the additive for 120 min. In each case, the left lane is the control, whereas the right represents the effect of the additive. Even higher concentrations (500 μ*M*) of 3HK and 3HKG (extreme right lanes) did not induce any photodamage. The extreme left lane shows molecular weight markers.

(11,29). Bova *et al.* (8) have suggested that AHBG, which is formed from 3HKG by the loss of ammonia and subsequent hydrogenation, is a UVA protectant filter. We have suggested that XA8OG is also produced in the lens from the endogenously present 3HKG through a deamination reaction, followed by cyclization, as shown in Fig. 2, and is described in detail elsewhere (17). On the other hand, XA, which is formed from 3HK *in vivo* by the action of kynurenine aminotransferase (9), is phototoxic. In the presence of light transmitted to the lens (above 300 nm) it produces both singlet oxygen and superoxide and, therefore, can photooxidize the lens and its constituents (14,15). Our present results show that its glucoside, XA8OG, is also toxic and, thus, potentially cataractogenic.

As has been argued earlier (12,13), very short lifetimes, as exhibited by 3HK and 3HKG, disfavor the intersystem crossing to the triplet state and the occurrence of subsequent sensitization or photochemistry. On the other hand, XA displays a two-component emission mode, with one lifetime around 6 ns, thus, allowing for such processes to occur in this molecule. We noted that XA8OG displays a far longer singlet excited state lifetime, allowing enough time for intersystem crossing to the triplet state and subsequent reactions from this state.

Although XA and its glucoside (XA8OG) are both potentially phototoxic, they are subjected to different photochemical mechanisms. XA8OG is more effective in producing singlet oxygen than is XA, but it does not produce significant amounts of superoxide. It appears that the covalent addition of glucose, with its multiple –OH groups, reduces the efficiency of radical production. Such a role for glycosylation has been suspected earlier (30). We also tried adding electron donors such as EDTA (1 m*M*) in an attempt to increase the superoxide-producing ability of XA8OG because the added electron donors are known to enhance this ability (12); but this had no perceptible effect in this instance. That XA acts as a photodynamic agent, causing oxidative damage to lens proteins, has been reported by Malina and Martin (9) and by Roberts *et al.* (14,15). Both XA and its glucoside photooxidize protein constituents of the lens.

Bando *et al.* (31) have shown earlier that 3HKG is present at its highest level in infant human lenses and decreases considerably with age (from about 70 μmol/g protein in a 3 year old human lens to about 17 μmol/g in a 46 year old lens). Endogenous conversion of 3HKG to AHBG (Fig. 1) could be benign, but its conversion to XA and XA8OG can result in a photooxidative stress to the lens in the UVA, radiation in which region the lens continuously receives, absorbs and transmits. We conclude that the age-dependent depletion of 3HKG and concomitant generation and accumulation of XA and XA8OG will enhance oxidative stress to the lens.

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Molecular and Cellular Assessment of Ginkgo Biloba Extract as a Possible Ophthalmic Drug

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We have investigated the biochemical and cell biological basis of the reported beneficiary effects of the leaf extracts of the plant *Ginkgo biloba*, which has been used as a possible ophthalmic drug. The antioxidant, antimicrobial, anti-apoptotic and cytoprotective properties of the standardized extract called EGb761 were assayed. Chemical stresses were induced in cells using alloxan or dexamethasone, and the effect of EGb761 on them was studied using the MTT and TUNEL assays. Its ability to modulate the activities of some antioxidant enzymes was tested in vitro. In addition, cataract was induced in rats through selenite injection, and the effect of EGb761 administration on the progression of cataract was studied using slit lamp examination. *Ginkgo biloba* was found to be an excellent antioxidant. It readily scavenges reactive oxygen and nitrogen radicals and inhibits oxidative modifications that occur to proteins in vitro. It enters intact cells and protects them from alloxan-mediated and light-mediated stress, and the nuclear DNA from single strand breaks. It also effectively inhibits chemically induced apoptosis. It does not modulate the activities of endogenous antioxidant enzymes, nor does it have any significant antimicrobial activity. Unlike some other plant extracts, it is not phototoxic. In experiments wherein selenite cataract was induced in laboratory rats, treatment with the extract significantly retards the progression of lens opacification in vivo. *Ginkgo biloba's* inherent antioxidant, antiapoptotic and cytoprotective action and potential anticataract ability appear to be some of the factors responsible for its beneficial effects.

Key words: *Ginkgo biloba*; EGb761; antioxidant; antiapoptotic agent; cytoprotective agent; potential anticataract agent.

1. Introduction

Extracts of the leaves of the Chinese tree *Ginkgo biloba* contain mixtures of bioactive ingredients used in medical practice as polyvalent therapeutics. A recent monograph has analyzed the available scientific information and has summarized four major 'concepts of action' of a standardized extract of *Ginkgo biloba*, called EGb761, namely vasoregulatory action, cognition enhancing action, stress alleviating action and gene regulatory action (De Feudis, 1998).

The main bioactive components of *Ginkgo biloba* extracts (GBEs) are biflavonoids (such as procyanidin and prodelphinidin), flavonoids (such as quercetin, kaempferol, isorhamnetin and myricetin) and their glycosides, and terpenoids (such as ginkgolides A, B, C, J and M, and bilobalide), and some small phenolic compounds. The polyphenolic flavonoids are expected to confer antioxidant properties to the extract (Marcocci et al., 1994; Küse and Dogan, 1995; Seif-El-Nasr and El-Fattah, 1995) while the terpenoids act as antagonists of platelet activating factor (Braquet, 1986; De Feudis, 1998). Some apolar compounds present in *ginkgo biloba* leaves have been reported to have antimicrobial properties (Mazzanti et al., 2000).

GBE has been tried out in ocular pharmacology. It is reported to accelerate corneal wound healing in rabbits after excimer laser keratectomy (Juarez et al., 1999); protect against retinal ischemia-reperfusion injury (Szabo et al., 1991, 1992) and retinal damage in rats (Doly et al., 1986; Besse et al., 1994; De Feudis, 1998; Ranchon et al., 1999). Chung et al. (1999) have shown that it increases ocular blood flow velocity in the ophthalmic artery of human glaucoma patients. Based on these promising results, Ritch (2000) has suggested it to be a potential drug for the treatment of glaucoma. Juarez et al. (2000) have found it to be an effective angiostatic inhibitor in experimental retinopathy of prematurity. Költringer et al. (1992) have suggested that the combination

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therapy of folic acid plus GBE has shown promise in the treatment of diabetic retinopathy. GBE has been suggested to lead to improvement in long distance visual acuity in human patients suffering from senile macular degeneration (Lebuisson et al.. 1986); however Evans (2000) has suggested that the number of volunteers being small, the beneficial effect of GBE on retarding the prevention of this disease is yet to be confirmed.

A major mode of damage to lens proteins involves oxidative reactions (Zigler and Goosey. 1981; Zigler et al.. 1985; Young, 1991). Oxidative and photic stress are thus common factors that lead to compromise in vision and pharmacological means of abating such stress would be useful. In light of the interesting possibilities and applications of GBE, we have monitored its potential as an effective cataractostatic agent.

2. Materials and Methods

We have worked with the standardized extract preparation called EGb761, made by the Beaufour-Ipsen Company of France, which contains 24% flavonol glycosides and 6 % terpene lactones, and is used widely by pharmacologists (De Feudis. 1998). The sample of EGb761 was a kind gift from Dr Katy Dricu of Beaufour-Ipsen. The rat insulinoma (RIN) cell line m.5F and the rabbit corneal cell line SIRC were obtained from the National Centre for Cell Sciences (NCCS), Pune, India and the human lens epithelial cell (HLE) line SRA01/04 was a gift from Dr Ven Reddy, Kellogg Eye Center, University of Michigan, Ann Arbor, Ml, U.S.A.

Preparation of the Extract

Stock solution of EGb761 (100 mg ml^{-1}) was prepared in water and was used to determine the antioxidant activity as well as for protein cross-linking studies. For experiments with cell lines, solution of EGb761 was made in sterile phosphate buffered saline (FBS) and filtered using a 0·2 μm filter.

ABTS Antioxidant Assay

The ABTS assay was performed based on the procedure ot Miller et al. (1993). Briefly, when azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) or ABTS is incubated with a peroxidase (such as metmyoglobin) and H_2O_2, the relatively long-lived radical cation ABTSv. is formed. In the presence of an antioxidant, the absorption of this radical cation (at 734 nm) is quenched. In a typical experiment, ABTS (30 μl, 5 mM), 50 μl of metmyoglobin (50 μM), and 820 μl of phosphate buffer (50 mM, pH 74) [of which 10 μl is replaced with EGb761 (100 mg ml^{-1} stock) when the sample is being investigated] are mixed, and the reaction is initiated by the addition of 100 μl of H_2O_2 (1 mM). The absorbance at 734 nm is measured as a function of time at 5 min. intervals for 30 min.

ESR Spin Trapping Assay

The electron spin resonance (ESR) method of trapping free radicals was also used to study the antioxidant property of EGb761. The free radicals (superoxide or $O_2^{\bullet-}$ and hydroxyl or •OH were produced using the xanthine/xanthine oxidase system that produces $O_2^{\bullet-}$ under metal-free conditions and *0H in the presence of metal ions (e.g. Fe or Co) and diethylenetriaminepentaacetate (DETAPAC). The radicals were captured in a stable and conveniently measurable manner using the 'spin trap' DMPO (5,5-dimethyl-1-pyrroline-1-oxide), which produces long-lived and stable free radicals that can be measured and quantified, using an X-band ESR spectrometer (Murali Krishna et al., 1991). The reaction mixture consisted of 0·1 mM xanthine, 50 mM DMPO, 0·1 mM DETAPAC, 0·1 mM $FeCl_3$; EDTA, 100 I! ml^{-1} catalase and 0·1 U ml^{-1} xanthine oxidase. The experiments were performed at ambient temperature, with a 100 kHz modulation of the magnetic field, 10 mW microwave power and 0·25 G modulation amplitude.

Assay for Inhibition of Photo-oxidation

The protein bovine pancreatic ribonuclease A, or RNase A, (1 mg ml^{-1}) was irradiated in the presence of the photosensitizer molecule bis(hydroperoxy)-naphthaldiimide (also called the Photo-Fenton reagent (Guptasarma et al., 1992), which generates hydroxyl radicals upon irradiation at 366 nm. The reagent was adsorbed on controlled pore glass beads and suspended in the protein solution. The mixture was taken in a fluorescence cuvette, placed in the sample compartment of a Hitachi F2500 spectrofluorimeter, and irradiated at 366 nm for 90 min, by tuning the excitation monochromator to this wavelength, with the excitation slit wide open. The light intensity falling on the sample was estimated earlier to be about 0·5 mW cm^{-2} (10^{14} photons incident per sec). The •OH produced generate oxidative cross-linking of the protein, monitored by polyacrylamide gel electrophoresis (PAGE), using 10% acrylamide for setting the gel, and in the presence of 10% sodium dodecylsulfate (SDS) and β-mercaptoethanol. Inhibition of the formation of high molecular weight products by EGb761 was assayed by adding known amounts of the substance to the reaction mixture before irradiation. An identical procedure was adopted for the photosensitized oxidation of RNase A, using riboflavin which, upon irradiation at 445 nm, yields singlet oxygen in high quantum yield (Murali Krishna et al., 1991). The damage to the protein, and protection by GBE, was monitored by SDS/PAGE.

Assay for Inhibition of Peroxynitrite-mediated Damage

The test protein bovine α-crystallin (1 mg ml^{-1}), was incubated with 1 mM peroxynitrite, with or without 10 mM bicarbonate, and a series of increasing concentrations of EGb761. Peroxynitrite was generated by reacting ice-cold solutions of $NaNO_2$ (0·6 M) with H_2O_2 (0·7 M) in acidic medium (0·6 M HCl) and rapidly quenched using NaOH (1·5 M). The reaction mixture was stored at −20°C, and the $ONOO^-$ concentrated in the upper yellow layer was collected. Its concentration was measured using a molar extinction coefficient of 1670 M^{-1} cm^{-1} at 302 nm. Protein cross-linking was studied by SDS/PAGE.

Assay for Anti-apoptotic Activity

The ability of EGb761 to prevent cells from undergoing apoptosis was studied in SIRC rabbit corneal cells. When cells were 70% confluent, apoptosis was induced using dexamethasone (1 μM) and EGb761 was added to a final concentration of 50 μg ml^{-1}. Cells were incubated at 37°C, in an atmosphere containing 5 % CO_2, and observed daily for any morphologic changes. After 72 hr of incubation, apoptotic cells were identified using an in situ cell death detection kit from Roche Diagnostics (Cat. #1684817) which is based on the principle of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling, or TUNEL (Gavrieli et al., 1992). The percentage of cells showing apoptosis in untreated cells was compared with *Ginkgo* treated cells.

Assay of Cytoprotective Ability

The cytoprotective ability of *Ginkgo biloba* was studied using the MTT assay (Hansen et al., 1989). After subjecting the cells to oxidative stress (riboflavin-induced in case of HLE cells and alloxan-mediated in case of R1N cells) with and without EGb761, cell viability was assayed. The assay is based on the ability of viable cells, but not dead cells, to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). This reduction reaction generates dark blue formozan products. After exposing the cells to stress, they were incubated in fresh medium for 18 hr at 37°C and 5% CO_2, after which 50 ul of 5 mg ml^{-1} MTT in PBS was added to 500 ul of medium in each well (cell density 1 x](P) in a 24-well plate. After 4 hr of incubation at 37°C, the medium was removed and 500 ul of 40 mM HC1/ isopropanol was added to stop MTT reduction. The blue color developed was read at 540 nm.

Assay of Antimicrobial Properties

The antibacterial activity of EGb761 was tested against the test organisms *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213) and *Pseudomonas aeruginosa* (laboratory isolate). The concentrations of the drug tested were 0·1-20 μg in regular dosage intervals. Discs containing various concentrations of the drug were placed on Mueller Hinton Agar plates inoculated with the test organisms. The plates were incubated at 37°C for 24 hr and then observed for zones of inhibition. Next, the antifungal activity of EGb761 was tested against *Curvularia lunata, Fusarium solani* and *Aspergillus flavus*. Fungal cultures were diluted to get an absorbance at 530 nm of 01 and this fungal suspension was further diluted 1:100 in RPMI-1640 medium. Graded concentrations of EGb761 ranging from 01 to 20 ug ml'^{1} were added and the tubes observed for growth after 72 hr at 30"C. Thirdly, the antiviral effect of EGb761 was tested against a laboratory isolate of Herpes Simplex virus 1 (HSV1) in SIRC cell line. Monolayers of SIRC cells, grown in a microtiter plate were infected with HSV1 in the presence of 10, 20, 30, 40 and 50 ug $ml"^{1}$ of GBE. The plates were observed for cytopathic effect after 48 hr. Finally, the anti-parasitic ability of GBE to affect the growth of the *Leishmania* parasite, *L. donovani,* was also checked, using the method of Mittra et al., 2000. We thank Dr H. K. Majumder of the Indian Institute of Chemical Biology. Kolkata, India for kind help with this assay.

Effect on the Activity of Antioxidant Enzymes

The effect of the addition of EGb761 on the activities of the five antioxidant enzymes was monitored using the standard assays for catalase (Aebi, 1984), superoxide dismutase (Misra and Fridovich, 1972), glutathione S-transferase (Habig el al., 1974), glutathione peroxidase (Flohe and Gunzalez, 1984) and glutathione reductase (Akerboom and Sies, 1981). We thank Dr Bhanu Prakash Reddy of the National Institute of Nutrition, Hyderabad, for help with the assays.

Single Cell Gel Electrophoresis

DNA damage was evaluated by single cell gel electrophoresis, also called the comet assay (Singh et al., 1988). Cultured HLE cells were trypsinized to obtain a single cell suspension. Cells were irradiated for 1 hr with broad-band visible light in the presence of 2 5 UM riboflavin, with and without EGb761. The cell suspension was then mixed with low melting agarose (2 %, in Ca- and Mg-free PBS) and layered onto frosted glass slides and allowed to gel. Prepared slides were placed in lysing solution (154 mM NaCl, 10 mM EDTA and 0·5 % N-lauryl sarcosine, pH 7·0) for 3 min. Slides were then washed in distilled water for 5 min. Electrophoresis was carried out in buffer containing 0·025 M NaCl and 1 mM EDTA at 7 V cm^{-2}, for 3 min. Finally, the slides were washed

and stained with ethidium bromide (50 μg ml^{-1}). DNA damage was visualized using a fluorescence microscope equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm. DNA migration, nuclear diameter and tail intensity were analyzed.

Induction of Cataract in vivo and the Effect of EGb761

(1) Selenite cataract model. Selenite cataract was induced using a modification of the method of Devamanoharan et al. (1991). Twenty-six Wistar rat pups (average weight 13·5 g) were randomly grouped as follows: control group (n — 8), *ginkgo* group (n = 9), *ginkgo*-selenite group (n = 9). Starting from postnatal day 8, the control group was administered i.p. injections of 0·1 ml of sterile physiological saline and the other two groups were administered 50 mg of EGb761 in 0·1 ml of saline. The injections were repeated at 24 hr intervals until postnatal day 20. On post-natal day 10, 30 min after the i.p. injections, sodium selenite dissolved in physiological saline was administered to the control and *ginkgo*-selenite group subcutaneously in the shoulder to give a dose of 3·2 mg kg^{-1} body weight. After their eyes had opened (postnatal day J 5–17), the lenses were observed for cataract with penlight examination, or a slit lamp. The extent of cataract was quantified (Hiraoka and Clark, 1995), using the scale of 1-6. When the animals were 6 weeks old, they were killed and the lenses excised. Animal experiments were performed following the NIH Guidelines on the Care and Use of Laboratory Animals in Research and the ARVO Statement for the Use ol Animals in Ophthalmic and Vision Research.

(2) *Phase separation model.* The effect; of EGb761 on the phase separation temperature of fish lens cytoplasm was determined as per Hiraoka et al. (1996). The whole lens of fish was dissected out, cut into small pieces, homogenized at 37°C and centrifuged at 20 000 rpm for 1 hr to remove membranes. The resulting homogenate was used to determine the phase separation temperature. A stock solution of EGb761 was prepared in 01 M phosphate buffer. pH 7·0 and one part of this stock solution was mixed with five parts of fish lens homogenate to yield a final extract concentration of 1 5 mg ml^{-1}. In the control, the extract was replaced with buffer. The sample tube was placed in a fluorescence spectrophotometer (Hitachi F2500) with a temperature-controlled circulating water bath and the scatter at 600/600 nm was recorded over the range ol +20 to —6°C. The transition from transparent to opaque was observed as a sharp increase in scatter, which occurred over a narrow temperature range.

The effect of GBE on thermally induced self-aggregation of lens proteins was also studied, in a similar manner. Scatter was recorded over the range of 25–67°C.

3. Results

Inherent Antioxidant Ability of GBE

While there are reports on the possible antioxidant nature of GBR in cell suspensions, tissues, organs and whole animals, the actual molecular effects have not been reported in detail, barring the early pioneering study ol Marcocci et al, (1994) on the antioxidant ability of the standardized extract preparation EGb761. We extend the antioxidant analysis of GBE here, using ESR spin trap assay, and the ABTS assay (Miller et al., 1 993). Addition of EGb761 (1 mg ml^{-1}) led to a rapid drop in the ABTS absorption at 734 nm within minutes, showing EGb761 to be an efficient antioxidant.

We then looked into the ability of EGb761 to quench individual oxyradicals. Fig. Ka) shows that EGb761 is able to quench the ESR signals due to superoxide radicals at concentrations beyond 40 μg ml^{-1}. Fig. l(b) shows its ability to quench hydroxyl radicals (•OH). It is seen that higher concentrations of EGb761 are needed to do so. We next tried these ESR spin trap experiments using isolated samples of the terpenoids ginkgolide and bilobalide, and found neither of them reduced these oxyradicals.

Protection by GBE from Oxidative Damage to Proteins and DNA

Figure 2(a) shows the electrophoresis patterns of the test protein RNase A subjected to oxidative stress by the Photo-Fenton reagent, which generates hydroxyl radicals. EGb761 is seen to be able to inhibit such oxidative damage effectively. In similar experiments, we used riboflavin as the sensitizer which, upon irradiation with 445 nm light, produces reactive oxygen species, predominantly singlet oxygen ($^1O_2$). GBE was able to quench $^1O_2$ and protect the protein from damage. Fig. 2(b) shows that GBE is also able to effectively inhibit peroxynitrite-mediated covalent modifications in the test protein bovine α-crystallin. Peroxynitrite (ONOO") reacts with proteins and produces oxidative cross-links as well as degradation products. In the presence of bicarbonate, it efficiently nitrates trp and tyr residues in proteins and also generates the dityrosine cross-link. GBE is seen to inhibit both the reaction pathways.

Fig. 3 shows the ability of GBE to protect oxidative strand-breaks in the nuclear DNA in intact human lens epithelial cells. When single cells are electrophoresed, the intact DNA in the nuclei is seen as a compact disk or a 'moon'; when strand break and thus chain scission occurs by oxidative damage, it is visualized as a trailing 'comet' in the electrophoregram. Addition ol

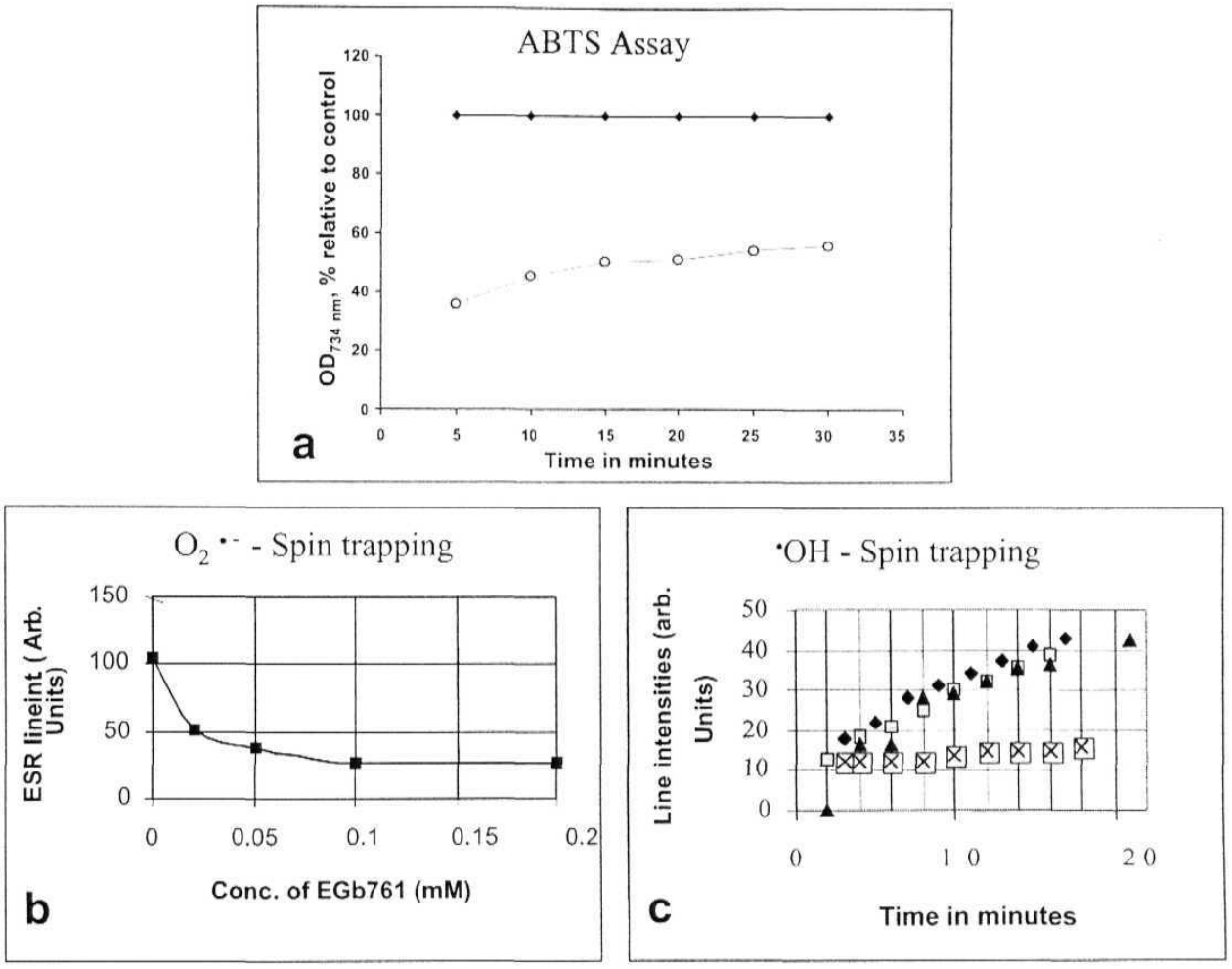

FIG. 1. (a) ABTS Assay: *Ginkgo biloba* has antioxidant property. The formation of $ABTS^{\bullet+}$ in the absence (◆) and presence of EGb761 (0). (b) *Ginkgo biloba* effectively scavenges superoxide. Assay of the ability of various concentrations of EGb761 in quenching superoxide radicals, measured by ESR spin trapping. (c) *Ginkgo biloba* quenches hydroxyl radicals. Assay of the ability of various concentrations of EGb761 in quenching hydroxyl radicals, monitored by ESR spin trapping. Symbols ◆, □, ▲, and × refer to 0, 2, 5 and 25 mM EGb761, assuming an average molecular weight of 400.

GBE is seen to inhibit the damage to DNA, reduce the trail and restore the 'moon'.

Cytoprotective Ability of GBE

This ability of GBE to be able to enter cells and protect the nuclear DNA led us to study the cyto-protective properties of the extract. We subjected HLE to riboflavin-induced stress in the absence and presence of EGb761, and assayed the viability of cells using the MTT metabolism method. Fig. 4(a) shows that EGb761 protects the cells from permanent damage and keeps them viable. Fig. 4(b) shows that this protective ability is more general. When the R1N cell line is stressed with the diabetogenic agent alloxan for 1 hr, about half the number of cells are killed. Pre-treatment of the cells with GBE effectively protects the cells from alloxan insult. But, interestingly, when *ginkgo* is added 4, or 8 hr *after* the removal of the medium, almost 100% of the cells can be rescued. Even when it is added as late as 12 hr, 75 % of the cells can be rescued and made viable, as judged by the MTT assay. It has been suggested that treatment with diabetogenic agents such as alloxan imposes, *inter alia*, an oxidative stress on cells (Öllinger and Brunk, 1995). The protective action of *ginkgo* seen here may thus arise, in part, due to its antioxidative ability.

Anti-apoptotic Ability of GBE

Apoptosis in cells can be identified by the characteristic nuclear disintegration and chromatin condensation. Upon TUNEL staining, the nucleus of apoptotic cells stains brown while normal cells appear purple when counterstained with hematoxylin. As Fig. 5 shows, dexamethasone could induce apoptosis in 40% of cells, while cells treated with 50 $\mu g\ ml^{-1}$ ot EGb761 showed normal cell morphology comparable to untreated control, evincing the protective action of the extract.

Effect on Selenite Cataract Induced in Animals in vivo

Fig. 6 shows that a single subcutaneous injection of selenite to rat pups on postnatal day 10 leads to the

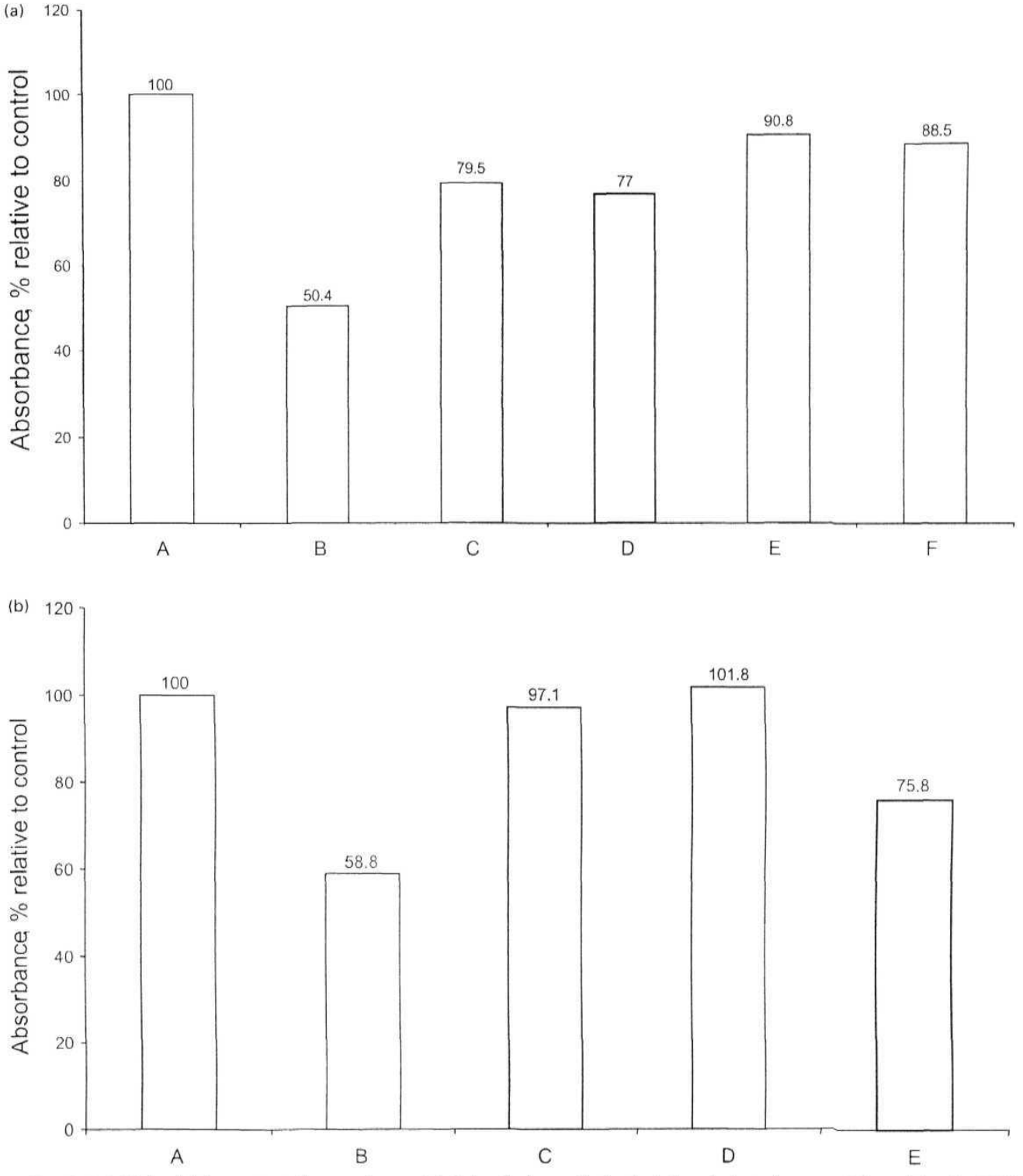

FIG. 4. (a) *Ginkgo biloba* protects human lens epithelial cells from riboflavin-induced photodamage, followed by the MTT assay. A: control. B: after photo-damage by 20 μM riboflavin; C, D, E and F: are photo-damaged by riboflavin in the presence of 5, 10, 15 and 20 μg ml^{-1} of EGb761. (b) *Ginkgo biloba* protects RIN cells from alloxan-mediated damage, monitored by the MTT assay. A: untreated control cells; B: after treatment with 6 mM alloxan; C, D and E: 4 μg ml^{-1} of EGb761 added to the treated cells after 4, 8 and 12 hr, respectively after alloxan treatment. Cytoprotection of about 75% is seen even when GBE is added after 12 hr of insult.

wavelength where the protein does not absorb but GBE does, and monitoring the covalent damage to the protein by gel electrophoresis. No modifications were seen in the gel, indicating that GBE is not phototoxic, i.e. it does not act as a sensitizer producing any reactive species upon excitation.

4. Discussion

A major mode of action of GBE is through its antioxidant ability. The flavonoids are responsible for it, since we found that ginkgolide and bilobalide did not quench oxyradicals. Chen et al. (1999) also found

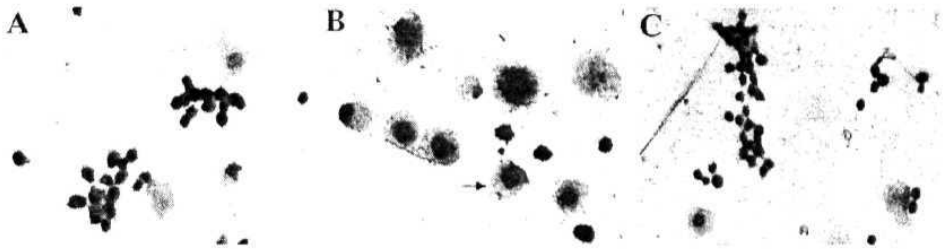

FIG. 5. *Ginkgo biloba* is an antiapoptotic agent. TUNEL assay of apoptosis of the corneal cell line SIRC by 1 μM dexamethasone and the anti-apoptotic action of 50 μg ml^{-1} EGb761. (A) shows the control cells, (B) shows apoptosis induced by dexamethasone, and (C) the protection offered by added EGb761.

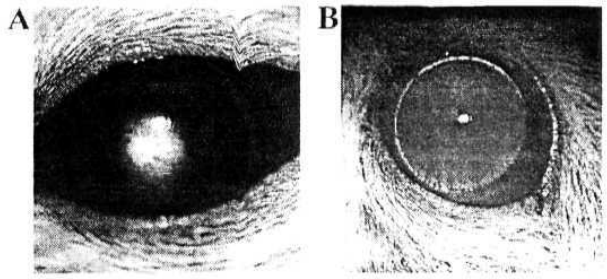

FIG. 6. *Ginkgo biloba* retards the progression of selenite cataract. Generation of cataract in rat pups in vivo by the administration of sodium selenite (A) and the retardation of cataract progression by EGb761 (B).

that these terpenoids in GBE did not scavenge hydroxyl radicals. GBE is also able to permeate cell membranes and exert its action on the nuclear DNA and also cytoplasmic components, as the comet, MTT and TUNEL assays reveal. The role of the non-flavonoid components appears to be important in these instances. GBE is cytoprotective, and is able to rescue cells from photo-induced, alloxan-mediated and apoptotic modes of assault. While the protection from light-induced assault may well be largely due to its antioxidant character, the ability of GBE to protect against apoptosis appears to involve both the flavonoid and terpene components of the extract. Ahlemeyer et al. (1999) have shown that bilobalide is the most potent anti-apoptotic factor in GBE. Ginkgolide B also shows this activity, while ginkgolide A is inactive in this regard. Yao et al. (2001) have recently shown that a terpene- and flavonoid-free extract of GBE, called HE208, failed to protect neuronal cells from beta-amyloid-induced cytotoxicity and cell death, and that the flavonoids and terpenoids present in GBE act in combination. Liebgott et al. (2000) have further shown that the terpenes and the flavonoids act complementarily in offering protection to the heart during ischemia and reperfusion. From these it appears that the beneficial action of GBE is derived from both its flavonoid and terpenoid components.

We did not find EGb761 to display any antimicrobial activity. This is in contrast to the report by Mazzanti et al. (2000), who found some antibacterial activity in some semipurified fractions (methanolic extract) of *ginkgo biloba* leaves. We also investigated the possibility of *ginkgo* extract in extending the storage time of donor corneas. The addition of EGb761 to the corneal preservation solution (the MK medium) did not increase the shelf life of donor corneas any more than the parent medium alone.

However, *ginkgo biloba* appears to be of value to the lens since our results show that it has cataracto-static ability. The fact that i.p. injection of GBE led to a retardation in the progression of selenite cataract in experimental animals is consistent with the hypothesis that components of the extract are able to reach the eye through the blood–aqueous barrier. That molecules of the flavonoid type are able to do so was shown earlier with tea extracts, which contain similar flavonoids (Zigman et al., 1999; Thiagarajan et al., 2001). The cataractostatic action of GBE seems related to its antioxidant property, since the cataract initiated in the animals was through selenite, which is a model for age-related lens opacification. It is not effective against the phase separation and precipitation that occurs on cooling of the lens crystallins, nor is it effective against the heat denaturation-induced aggregation of the lens proteins; this is to be expected since neither of these phenomena involves any oxidative or other covalent chemical modifications. The effect of GBE on other forms of cataract, e.g., diabetic or steroid mediated, is yet to be determined. But its ability to retard other ocular conditions that are brought about by oxidative stress, such as senile macular degeneration (Lebuisson et al., 1986; Evans, 2000), appears promising from our results on oxidative cataract. That GBE is able to protect cultured retinal pigment epithelial cells from oxidative stress was shown some time ago by Chader (personal communication). The photoprotective action of *ginkgo biloba*, seen in Fig. 2(a) with proteins in vitro, and reported by Ranchon et al. (1999) on photoreceptors in vivo must also result from its radical scavenging ability. The neuroprotective ability of GBE has been listed in some detail by DeFeudis (1998). We believe that this property would form the basis of the recommendation of GBE by Ritch (2000) as a drug against glaucoma.

Finally, the issue of toxicology and safety of GBE doses to humans needs to be addressed. DeFeudis (1998) has summarized these points and has

concluded that a large body of clinical experience shows a remarkably low rate of adverse drug events associated with the use of EGb761-containing products. Rhee et al. (2001), commenting about the use of complementary and alternative medicine for glaucoma, have worried about possible toxicities of GBE and other herbal remedies. Le Bars and Kastelan (2000) have summarized the efficacy and safety of GBEs, and suggest that a dose of 120 mg EGb761 per day is acceptable. While this is reassuring, the material available over the counter in drug stores and nature food shops is not the standardized extract EGb761, but leaf powder, capsules and caplets. These contain alkylphenols (e.g., ginkgolic acids, ginkgol, bilobol), which EGb761 does not, and might have potential allergenic and toxic properties. Baron-Ruppert and Luepke (2001) have evaluated the toxicity of these alkylphenols, using the chicken egg test, and find that ginkgolic acids are lethal to chick embryos at very low levels (LD50: 3·5 mg per egg, 64 ppm). On the other hand, the biflavone fraction (that contains 1 % or less of ginkgolic acids) has a LD50 value of 250 mg per egg, or 4540 ppm. Westendorf and Regan (2000) report that ginkgolic acids might cause DNA strand breaks in rat hepatocytes. There has also been the suggestion that GBE may contain constituents with immunotoxic properties, as monitored by the popliteal lymph node assay (Koch et al., 2000), though it is not clear yet what these constituents are. Thus, while the flavonoids themselves are safe, these alkylphenols are best avoided.

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