Journal of Fluorescence, Vol. 9, No. 3, 1999

A Comparative Study of the Effect of Exogenous and Endogenous Photostabilizers on Lens Crystallin Photodegradation

Alina Kamińska,[1] Małgorzata Kowalska,[1] and Aleksander Balter[2]

Received December 1, 1998; accepted December 11, 1998

The purpose of the present study was to determine *in vitro* the effect of sodium azide, ethanol, trans-β-carotene, and the reduced form of glutathione on phototransformations in the lens crystallin. These photostabilizers show a specific affinity for different kinds of free radicals. The water-soluble protein from the cortical part of the eye was irradiated with doses of UV C ranging from 0 to 4.07 J/cm^2. Changes in the structure of the crystallins have been monitored by steady-state absorption and fluorescence spectroscopy. Irradiation of dialyzed samples of these proteins at a wavelength of 254 nm (1.13 ± 0.02 mW/cm^2) caused photooxidation of aromatic residues; the crystallin solutions became opaque and turbid. The samples displayed increasing attenuance at a wavelength of 280 nm as photodamage proceeded. The fluorescence of tryptophan at 333 nm systematically decreased and a new band between 400 and 500 nm appeared during the UV C irradiation. Our results show that the antioxidants can protect proteins from UV C-induced photodegradation and the protective effect is significantly dependent on their concentration in the protein solution. There are no dramatic differences in the rates of exogenous and endogenous scavenging of generated free radicals for all concentrations used, with rate constants varying by a factor no greater than 2. The mechanism and the rate of scavenging or quenching are dependent on the nature of the radical species and the photostabilizer structure. Although this study provides evidence for free radical scavenging and protein protection, extrapolations to possible antioxidant effects *in vivo* must be made cautiously.

KEY WORDS: Lens crystallins; glutathione; sodium azide; ethanol; trans-β-carotene; photodegradation; UV irradiation; attenuance; intrinsic fluorescence.

INTRODUCTION

The principal component of the vertebrate eye lens is a group of water-soluble proteins, generally known as crystallins. The crystallins can be divided into two superfamilies: polymeric α-crystallins and oligomeric β- and γ-crystallins, which exhibit a sequence identity of about 30% and a different state of oligomerization. β-crystallins can form homodimers or heterooligomers with molecular masses ranging from 45 to 200 kDa, whereas γ-crystallins (≈20 kDa) are exclusively monomeric. The three-dimensional structure of β- and γ-crystallins has been determined in detail from X-ray analysis [1,2]. The three-dimensional structure of α-crystallin is currently unknown, but various models have been proposed [3,4].

Besides, it has been found that α-crystallin can function as a molecular chaperone in that it prevents the thermal- or near-UV-induced aggregation of a number of enzymes and the lens β- and γ-crystallins [5,6]. Their

[1] Institute of Chemistry, N. Copernicus University, Gagarina 7, 87-100 Toruń, Poland.

[2] Institute of Physics, N. Copernicus University, Grudziądzka 5, 87-100 Toruń, Poland.

1053-0509/99/0900-0213$16.00/0

relative proportions vary with age and location within the lens as a result of both differential synthesis during development and selective degradation.

In diabetes and cataractogenesis and with aging, the crystallins undergo different physicochemical changes, including increased protein aggregation, the formation of insoluble proteins, increased pigmentation in the nucleus, the production of a yellow to brown coloration, and the appearance of blue fluorescence [7,8]. Although the nature of many of the age- and cataract-related changes is still unknown, a number of experimental studies indirectly support the idea that UV irradiation may be cataractogenic [9,10].

One of the major consequences of UV irradiation of organic matter under aerobic conditions is the production of the active states of molecular oxygen, namely, singlet oxygen, superoxide anion, hydroxyl radical, and hydrogen peroxide. Tryptophan, histidine, phenylalanine, cysteine, and methionine are especially vulnerable to oxidation [11].

The lens is chronically exposed to UV irradiation throughout life. Photochemical damage of the lens proteins may involve a series of reactions including charge transfer, conversion of tryptophan to kynurenine derivatives, and oxidation of the sulfydryl group. As a consequence, the protein molecules can be fragmented, aggregated, or cross-linked and this can lead to loss of biological function, increased susceptibility to proteolysis, and heat denaturation [12–14]. Near-UV irradiation of the lens protein causes some of these structural changes as well as direct or sensitized photooxidation of the tryptophan residues to kynurenine derivatives and yields a pigmented and fluorescent oxidation product that may be similar to those identified in cataracts or aged lenses [15,16].

Although the involvement of free radical-mediated damage to crystallin upon irradiation has been demonstrated in epidemiological studies and in animal experiments, the role of different active species of oxygen generated by UV irradiation of the lens protein and the detailed nature of this change in the protein structure remain obscure [17,18].

One of the principal aims of protein photodegradation studies is finding an efficient and safe method of protecting their structure against photooxidation. A normal lens contains inhibitors, such as superoxide dismutase and glutathione, which can scavenge and quench reactive free radicals. We decided to compare the photochemical transformation of lens crystallins in the presence of selected endogenous and exogenous photostabilizers upon exposure to UV C radiation at 254 nm using the spectroscopic techniques of steady-state absorption and fluorescence spectroscopy.

MATERIALS AND METHODS

Preparation of the Samples

The lenses were isolated from 3- to 5-year-old animals, decapsulated, pooled, and homogenized in 50 ml of deionized, cold water (18 mΩ). The water-soluble proteins were obtained according to the modified method of Bloemendal *et al.* [19]. The total protein concentration was determined by the method of Lowry *et al.*, using bovine serum albumin as a standard [20]. Glutathione (reduced form), sodium azide, ethanol, and trans-β-carotene were purchased from Sigma Chemical Co. (St. Louis, Mo). Mixtures contained photostabilizers in the concentration range from 10^{-4} to 10^{-6} *M*. All solutions were filtered using membrane filters (Sigma Chemical Co.).

Illumination Conditions

Each solution was irradiated in a quartz cuvette (2 ml) by a mercury lamp (Philips TUV-30, The Netherlands) which emitted mainly light at 253.7 nm. The distance between the samples and the light source was about 10 cm. The light intensity at this position was 1.13 ± 0.02 mW/cm^2. The intensity of the incident light was measured using an IL1400A radiometer (International Light, USA). Various doses of UV C radiation were obtained by varying the irradiation time (0–60 min, i.e., 0–4.07 J/cm^2). The sample temperature did not exceed 22°C.

Spectroscopic Measurements

UV-VIS absorption spectra were recorded with a Shimadzu spectrophotometer (Model UV-1601PC). Data collection and plotting were accomplished by the UVPC program and the computer data station supplied by the manufacturer.

Intrinsic fluorescence studies were performed with a Perkin–Elmer LS-50B spectrofluorimeter equipped with a differential-corrected spectral unit. The rate of photolysis was followed by monitoring the decrease in tryptophan fluorescence at the wavelength of the emission maximum for a range of excitation wavelengths from 270 to 350 nm. Fluorescence of *N*-formylkynurenine (N-FK) or other tryptophan derivatives (with the emission maximum around 452 nm) was obtained under excitation at 320–360 nm. Spectra were collected using the Perkin–

Elmer Data Manager in the PECSS (Perkin–Elmer Computer Spectroscopy Software) program to obtain the differential excitation and emission scans.

RESULTS AND DISCUSSION

The photochemical transformation of lens crystallins in the presence of selected photostabilizers was studied for the water-soluble protein fraction from the cortical part of the eye lens during UV irradiation at a 254-nm wavelength for periods of 2 to 60 min. The authors emphasize that a rather high dose of UV C irradiation was used in these studies, much higher than that which might reach the lens *in vivo*.

The lens can be photodamaged by a single large dose of UV irradiation but chronic low-dose exposure is also harmful. Several studies have shown that the major reactive oxygen species formed under these conditions are singlet oxygen ($^1O_2$) and hydroxyl radical ($\cdot OH$) [21–23].

The absorption of UV irradiation at 254 nm by crystallins is due to the presence of aromatic amino acids such as phenylalanine, tyrosine, and tryptophan as well as cysteine. It should be mentioned here that irradiation at 254 nm can also cause the breaking of peptide bonds in the range of 180 to 200 nm. McLaren has observed that the energy of UV radiation at 254 nm is sufficient to rupture a peptide bond [24].

The defense system of the lens, such as endogenous antioxidants [glutathione (GSH) and superoxide dismutase] which can protect lens components against photooxidation, is insufficient. We chose endogenous and exogenous photostabilizers with a high chemical reactivity toward the active forms of oxygen.

The attenuance or each solution was measured in the range between 200 and 600 nm, while the emission spectra were recorded in the range of 290 to 500 nm(Figs. 1 and 2).

Under UV C irradiation (λ = 254 nm), the attenuance spectra of the crystallin solutions with and without all photostabilizers used changed markedly (Fig. 3). Prolonged UV C irradiation caused a gradual increase in attenuance, while the absorption band at 280 nm became less distinct. This suggests the occurrence of photochemical transformations, cross-linking, and a decrease in tryptophan content caused by active species of oxygen generated in the protein solution. The addition of antioxidants appears to prevent or reduce the degree of protein photodegradation caused by UV C irradiation.

The most promising protective effect was observed for water-soluble antioxidants such as GSH and sodium azide (Fig. 3). GSH is an endogenous tripeptide, which

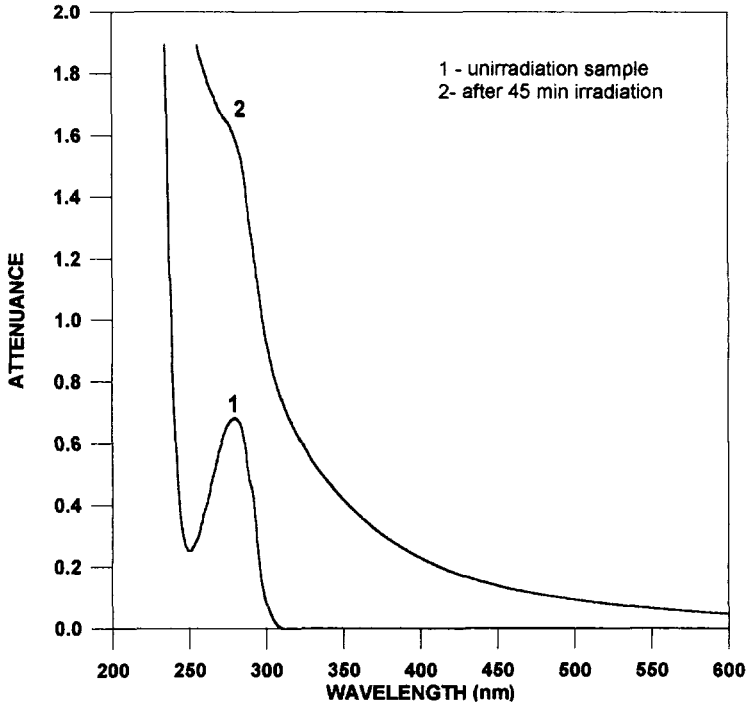

Fig. 1. UV-VIS spectra of lens crystallins before (1) and after (2) UV C irradiation at 254 nm for 45 min.

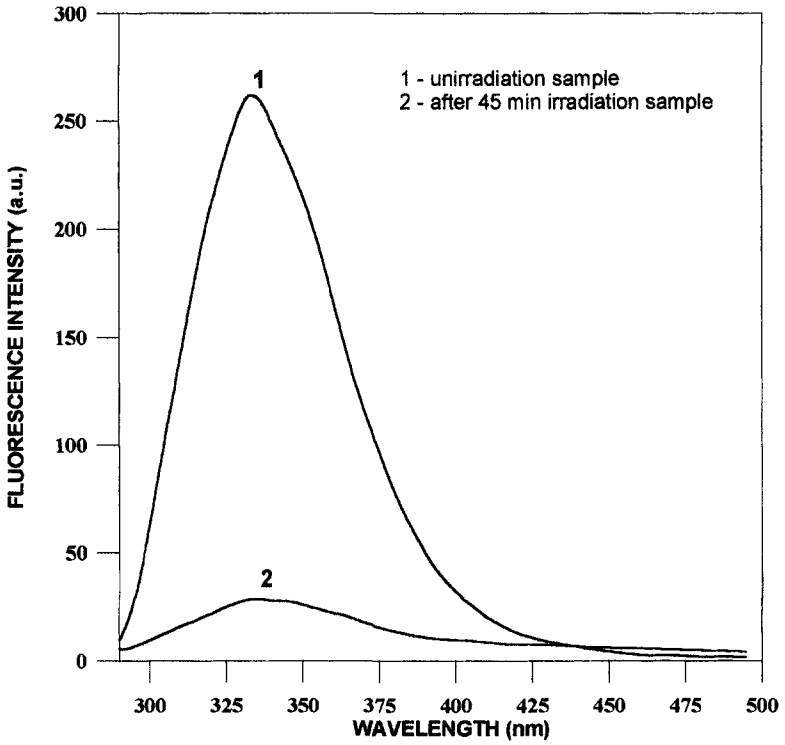

Fig. 2. Fluorescence spectra of lens crystallins before (1) and after (2) UV C irradiation at 254 nm for 45 min. Excitation wavelength, 280 nm.

plays a pivotal role in lens defense against photooxidative damage. It can act as a direct free radical scavenger and this is thought to be the main protective effect against UV irradiation with different wavelengths. This is consistent with a rate constant of $2.0 \times 10^6\ M^{-1}\ s^{-1}$ for singlet oxygen and $8.0 \times 10^9\ M^{-1}\ s^{-1}$ for hydroxyl radical at a physiological pH [25]. GSH quenches radicals by hydrogen atom donation and the resulting much less reactive thinyl radicals decay bimolecularly or via an oxygen-dependent mechanism, in both cases forming glutathione disulfide (GSSG) [26,27].

Oxidation of the protein sulfydryl group has been considered one of the most likely causes for cross-linking, aggregation, and insolubilization of the lens proteins.

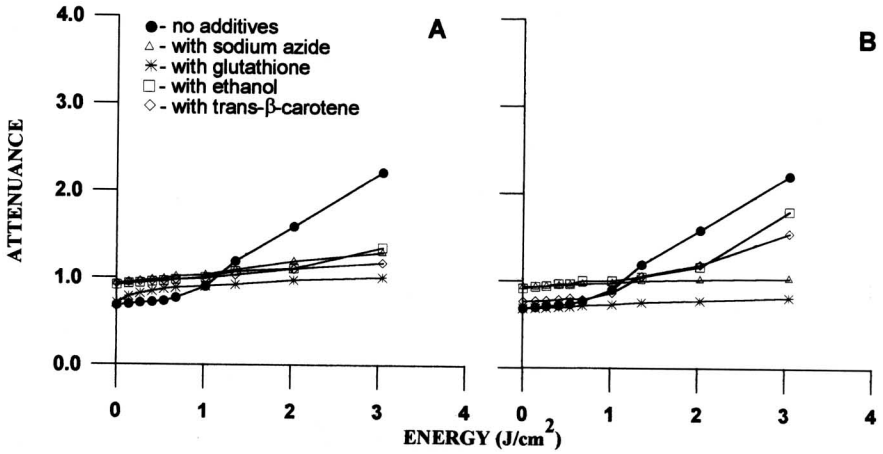

Fig. 3. Changes in the attenuance of the lens crystallin solution at 280 nm under UV C irradiation at 254 nm. The concentration of photostabilizers was (A) $10^{-6}\ M$ and (B) $10^{-4}\ M$.

When the reduced form of GSH was added to the protein solution, the protein thiol was lost more rapidly then the protein sulfydryl.

The data presented here agree with results of other investigators showing that GSH can very efficiently prevent protein photooxidation by the autooxidation of cystine residues [28,29]. The UV light-induced sample turbidity is most due to crystallin aggregation and/or cross-linking. These two photostabilizers significantly retarded the photodegradation of the proteins; even after 2 h of irradiation the mixtures remained transparent. GSH in reduced form and sodium azide inhibit protein cross-linking and aggregation caused by UV irradiation. Based upon its inhibition of cross-linking and ability to retard this process, we concluded that sodium azide quenched singlet oxygen and hydroxyl radical with a very high efficiency, however, details of this process are unknown.

Trans-β-carotene and ethanol retarded the photochemical transformation to a lesser degree; these two photostabilizers only delayed the onset of turbidity (manifested by the background attenuance) caused by UV light. Trans-β-carotene, a precursor for vitamin A and the most important member of the carotenoid family of antioxidants, is claimed to have anticataract properties [30,31]. Indeed, epidemiological data also suggest that trans-β-carotene can reduce the overall oxidative risk, serve as a singlet oxygen quencher, and prevent cataract formation or molecular photodegradation [32,33]. Trans-β-carotene is capable of quenching excited triplet states and singlet oxygen.

Visible turbidity of the protein solutions was observed after either 30 or 60 min of UV C irradiation of the crystallin samples with and without mixtures of trans-β-carotene in ethanol, respectively. Added solutions of trans-β-carotene in ethanol inhibited photoaggregation of the crystallins to a lesser degree than GSH and sodium azide. Besides, the use of trans-β-carotene as an antioxidant is limited due to its insolubility in water. Longer times of irradiation at 254 nm caused precipitation of the coagulated proteins in all mixtures.

The fluorescence of all crystallins is due predominantly to tryptophan residues, however, all of the aromatic residues are excited to some extent at 270-to 300-nm wavelengths, yet the tyrosine and phenylalanine bands are not visible in the native crystallins.

In general, the emission spectra of the proteins depend strongly on the wavelength used to excite fluorescence, thus revealing the heterogeneity of fluorophores. The question arises as to the magnitude of such heterogeneity for tryptophan residues contained in the crystallins and its dependence on irradiation with and without photostabilizers added. We used excitation wavelengths of 270 to 300 nm, however, no significant heterogeneity was detected (unpublished data).

Nonirradiated aqueous solutions of the fresh crystallins showed a single strong band at 333 nm characteristic of tryptophan residues. This indicates that the tryptophan residues are located in a largely apolar environment [34].

The emission spectra of the protein samples obtained for the 280-nm excitation wavelength exhibited a progressive decrease in intensity after short-wavelength UV irradiation (Fig. 4). This decrease in emission intensity is due to the photransformation of tryptophan residues of crystallins, which can occur by either direct absorption of the 254-nm light or a sensitized reaction [35,36]. A

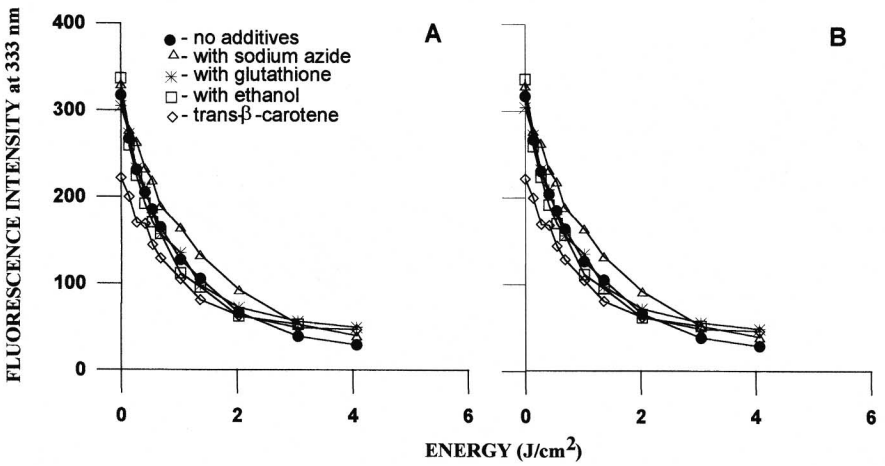

Fig. 4. Changes in the tryptophan fluorescence at 333 nm obtained for excitation at 280 nm in lens crystallin solutions under UV C irradiation at 254 nm. The concentration of photostabilizers was (A) 10^{-6} *M* and (B) 10^{-4} *M*.

new band at around 400–500 nm appears as a result of UV C irradiation. This band is not present in the nonirradiated control samples.

Although several photoproducts of tryptophan are known to form, considering the position of the new band, the corresponding excitation spectrum, and earlier observations, the band at 452 nm is assigned to the aerobic photoproduct of tryptophan, *N*-formylkynurenine (N-FK), or other derivatives [37–39]. Independent monitoring of the tryptophan (excitation at 280 nm; Fig. 4) and N-FK (excitation at 360 nm; Fig. 5) emission levels showed a significant decrease in the fluorescence intensity of tryptophan, with a concomitant increase in N-FK-type emission as irradiation proceeds.

The same general features were obtained in crystallin mixtures with all photostabilizers, but with some interesting differences. Phototransformation of tryptophan residues was observed in the pure crystallin solution as well as in the samples with exogenous and endogenous antioxidants, however, the rate of tryptophan transformation was distinctly higher in a solution without photostabilizers. Added antioxidants are seen to inhibit photolysis and the changes are less pronounced (Fig. 6). In general, all antioxidants appeared to be rather efficient photodegradation inhibitors; the initial decrease in tryptophan fluorescence (Fig. 4) probably resulted from an interaction of the stabilizer with the protein residues.

It has been shown that ethanol, trans-β-carotene, and azide ions quench singlet oxygen with a very high efficiency. Smaller changes in the presence of inhibitors such as GSH and sodium azide necessarily indicate a predominant role of singlet oxygen in this photoprocess. The possibility of formation of ·OH radicals, which are also quenched by NaN_3 and ethanol, cannot be excluded, however, this remains unclear. In general, the reaction rate constant of hydroxyl radical is rather large; it can react with a number of cellular components and reveals only a low specificity.

Active species of oxygen generated in these photoreactions can easily penetrate and interact with protein molecules, causing destabilization of their structure. The singlet state of oxygen and hydroxyl radical could initiate photodegradation of proteins and oxidation of some of the amino acids.

The above results, i.e., differences in the attenuance spectra and changes in fluorescence intensity after UV irradiation, different onset times of turbidity of the samples, and formed solid precipitation of crystallins, suggest that these photochemical reactions in crystallins are inhibited by all the photostabilizers used. In the work presented here, we observed a photoprotective effect of endogenous and exogenous compounds in the concentration range from 10^{-4} to 10^{-6} *M*; the toxicity at these concentrations is usually very low.

With aging, the balance between the endogenous antioxidant photoprotective system and oxygen radicals becomes more sensitive to physiological stress. Moreover, the age-related decrease in antioxidant activity is not sufficient to compensate for an increase in the rate of photodegradation of the protein lens. Probably, this is one of the reasons for the appearance of the senile cataract. It appears that the onset of senile cataract may be significantly delayed by supporting the endogenous photoprotective system with exogenous antioxidants. Probably, a combination of photostabilizers would be more effective than individual compounds.

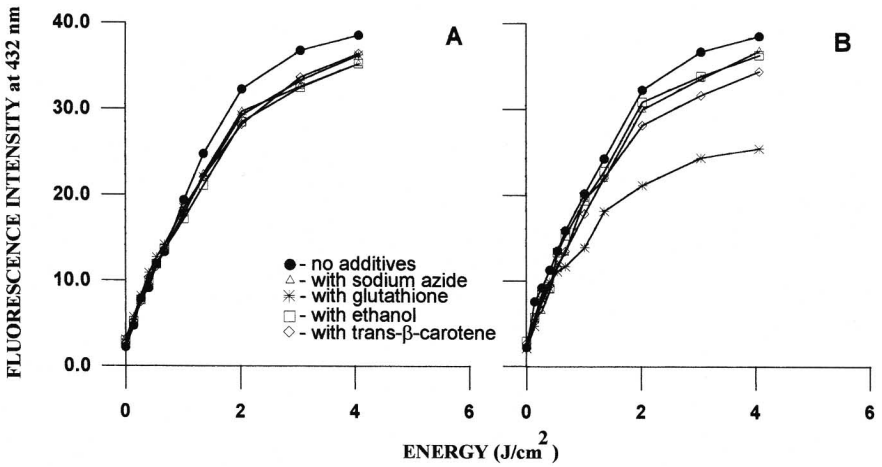

Fig. 5. Changes in the N-FK fluorescence at 432 nm obtained for excitation at 360 nm in lens crystallin solutions under UV C irradiation at 254 nm. The concentration of photostabilizers was (A) 10^{-6} *M* and (B) 10^{-4} *M*.

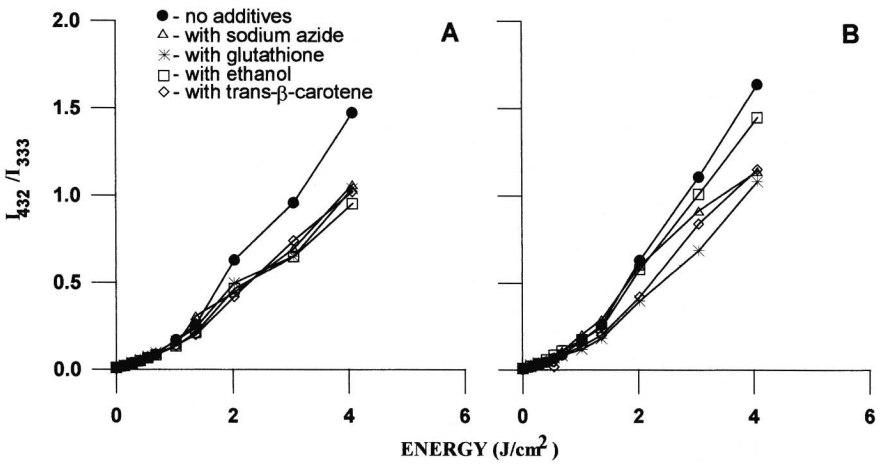

Fig. 6. The ratio I_{432}/I_{333} of fluorescence intensities at 432 nm (N-FK) and 333 nm (tryptophan) of lens crystallin solutions under UV C irradiation at 254 nm. The concentration of photostabilizers was (A) 10^{-6} *M* and (B) 10^{-4} *M*.

ACKNOWLEDGMENTS

This work was supported by State Committee for Scientific Research Grant 4P05A 06214. The fluorescence spectra were recorded with a Perkin–Elmer LS-50B spectrofluorimeter purchased within the framework of the TEMPUS SJEP 11415 project.

REFERENCES

1. V. Nalini, B. Bax, H. Driessen, D. S. Moss, P. F. Lindley, and C. Slingsby (1994) *J. Mol. Biol.* **236**, 1250.
2. H. Driessen, B. Bax, C. Slingsby, P. F. Lindley, D. Mahadevan, D. S. Moss, and I. Tickle (1991) *Acta Crystallogr. Sect. B* **47**, 987.
3. G. Wistow (1993) *Exp. Eye Res.* **56**, 729–732.
4. R. C. Augusteyn and J. Kortez (1987) *FEBS Lett.* **22**, 1–5.
5. R. F. Borkman and J. McLaughlin (1995) *Photochem. Photobiol.* **62**, 104–105.
6. J.-S. Lee, J.-H. Liao, S.-H. Wu, and S.-H. Chiou (1997) *J. Protein Chem.* **4**, 283–289.
7. K. Mandal, S. K. Bose, and B. Chakrabarti (1986) *Photochem. Photobiol.* **43**, 515–523.
8. Ch. Mohan Rao, D. Balasubramantan, and B. Chakrabarti (1987) *Photochem. Photobiol.* **46**, 511–515.
9. R. J. W. Truscott and R. C. Augusteyn (1977) *Biochim. Biophys. Acta* **492**, 43–52.
10. A. Balter (1991) *Lens Eye Tox. Res.* **8**, 195–215.
11. K. Uchida, N. Enomoto, K. Itakura, and S. Kawakishi (1989) *Agr. Biol. Chem.* **53**, 3285–3292.
12. J. N. Liang, S. K. Bose, and B. Chakrabarti (1985) *Photochem. Photobiol.* **40**, 461–469.
13. U. P. Andley, P. Sutherland, J. N. Liang, and B. Chakrabarti (1984) *Photochem. Photobiol.* **40**, 343–349.
14. S. K. Bose, K. Mandal, and B. Chakrabarti (1985) *Biochem. Biophys. Res. Commun.* **128**, 1322–1328.
15. D. Y. Li, R. F. Borkman, R. H. Wang, and J. Dillon (1990) *Exp. Eye Res.* **51**, 663–669.
16. J. D. Goosey, J. S. Zigler, and B. Chakrabarti (1985) *Exp. Eye Res.* **40**, 461–469.
17. B. J. Ortwerth and P. R. Olsen (1994) *Photochem. Photobiol.* **60**, 53–60.
18. B. J. Ortwerth, M. Linetsky, and P. R. Olesen (1995) *Photochem. Photobiol.* **62**, 454–462.
19. H. O. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall (1951) *J. Biol. Chem.* **193**, 265–275.
20. H. Bloemendal (1981) *Molecular and Cellular Biology of the Eye*, John Wiley and Sons, New York.
21. M. Linetsky and B. J. Ortwerth (1995) *Photochem. Photobiol.* **62**, 87–93.
22. M. Linetsky and B. J. Ortwerth (1996) *Photochem. Photobiol.* **63**, 649–655.
23. M. Linetsky and B. J. Ortwerth. (1997) *Photochem. Photobiol.* **65**, 522–529.
24. S. Lerman (1980) *Radiant Energy and the Eye*, Macmillan, New York.
25. B. Halliwell and J. M. Gutteridge (1990) *Methods Enzymol.* **186**, 1–85.
26. B. J. Ortwerth, A. Coots, H. J. James, and M. Linetsky (1998) *Arch. Biochem. Biophys.* **351**, 189–196.
27. M. Linetsky, N. Ranson, and B. J. Ortwerth (1998) *Arch. Biochem. Biophys.* **351**, 180–188.
28. T. P. Hum and R.C. Augusteyn (1987) *Curr. Eye Res.* **6**, 1103.
29. J. A. Thomas, B. Poland, and R. Honzatko (1995) *Biochim. Biophys. Acta* **319**, 1–9.
30. B. P. Lim, A. Nagao, J. Terao, K. Tanaka, T. Suzuki, and K. Takama. (1992) *Biochim. Biophys. Acta* **1126**, 178–184.
31. Ch. S. Foote, Y. C. Chang, and R. W. Denny (1970) *J. Am. Chem. Soc.* **92**, 5218–5219.
32. A. Taylor (1993) *J. Am. Coll. Nutr.* **12**, 138–146.
33. S. T. Mayne (1996) *FASEB J.* **10**, 690–701.
34. R.Ugarte, A. M. Edwards, M. S. Diez, A. Valenzuela, and E. Silva (1992) *J. Photochem. Photobiol. B Biol.* **13**, 161–168.
35. J. Dillon (1991) *J. Photochem. Photobiol. B Biol.* **10**, 23–40.
36. S. Lerman and R. Borkman (1977) *Science* **197**, 1287–1288.
37. J. N. Liang, S. Bose, J. Thomson, and B. Chakrabarti (1988) *Photochem. Photobiol.* **47**, 583–591.
38. K. Mandal, M. Kono, S. K. Bose, J. Thomson, and B. Chakrabarti (1988) *Photochem. Photobiol.* **4**, 583–591.
39. J. N. Liang, S. K. Bose, and B. Chakrabarti (1985) *Photochem. Photobiol.* **40**, 461–469.