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Astaxanthin Protects against Oxidative Stress and Calcium-Induced Porcine Lens Protein Degradation

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Astaxanthin (ASTX), a carotenoid with potent antioxidant properties, exists naturally in various plants, algae, and seafoods. In this study, we investigated the in vitro ability of ASTX to protect porcine lens crystallins from oxidative damage by iron-mediated hydroxyl radicals or by calcium ion-activated protease (calpain), in addition to the possible underlying biochemical mechanisms. ASTX (1 mM) was capable of protecting lens crystallins from being oxidized, as measured by changes in tryptophan fluorescence, in the presence of a Fenton reaction solution containing 0.2 mM Fe^{2+} and 2 mM H_2O_2. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis analysis demonstrated that β_{high}-crystallin was the most vulnerable protein under these conditions of free radical exposure. The proteolysis of lens crystallins induced by calcium ion-activated calpain was also inhibited by ASTX (0.03−1 mM) as determined by daily measurement of the light-scattering intensity at 405 nm for five consecutive days. ASTX at 1 mM was as potent as a concentration of 0.1 mM calpain inhibitor E64 in protecting the oxidative damage/hydrolysis of porcine crystallins. At a concentration of 1 mM, ASTX provided better protection than the endogenous antioxidant glutathione in terms of suppressing calcium-induced turbidity of lens proteins. Thin-layer chromatography analysis indicated that ASTX interacted with calcium ions to form complexes, which we believe interfere with the hydrolysis of lens crystallins by calcium-activated calpain. This in vitro study shows that ASTX is capable of protecting porcine lens proteins from oxidative insults and degradation by calcium-induced calpain.

KEYWORDS: Astaxanthin; calcium-induced turbidity; calcium complex; lens proteins; oxidative stress

INTRODUCTION

It has been suggested that in the daily diet vitamins and trace minerals possessing antioxidant properties can help to reduce cataract risk and that certain foods or supplements may be of benefit in terms of providing prevention (*1*−*5*). Indeed, as early as 1988, zeaxanthin was reported to be predominant over lutein in the foveal region of the human eye (*6*). It was suggested that the xanthophylls, lutein and zeaxanthin, were protective against age-related cataracts in humans (*7*−*9*). Higher concentrations

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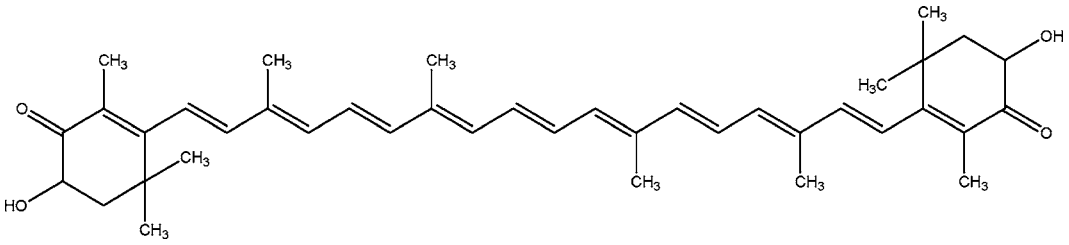

Figure 1. Structure of ASTX.

of carotenoids were found in the epithelial and cortical layer than in the nucleus of the human lens (*10*−*12*). The two most common hypotheses for the protective role of these carotenoids are based on their ability to filter out the phototoxic short wavelength visible light and their capacity to efficiently quench light-induced free radicals such as singlet oxygen (*12*).

Astaxanthin (ASTX; **Figure 1**), one of the most common xanthophylls, can be found in the red pigment of crustacean shells (for example, crab and shrimp), salmon, and asteroideans (*13*, *14*). Many reports demonstrate that ASTX is a more powerful antioxidant than other carotenoids, or vitamin E, and that it may confer numerous health benefits (*15*). Several prior studies have demonstrated that ASTX displays wide-ranging biological activity, including antioxidant (*16*−*18*), antihepatotoxicity (*19*), antitumor (*20*), anti-*Helicobacter pylori* (*21*, *22*), and antiinflammatory effects (*14*). In contrast to α-carotene, ASTX, an oxygenated carotenoid (xanthophylls), possesses no provitamin A activity. Furthermore, it was recently reported that supplementing the diet with ASTX provided significant protection against the development of cataracts in Atlantic salmon (*23*).

Cataractous lenses are characterized by morphological changes including the appearance of lens opacification resulting from aggregation of lens proteins (*24*, *25*). Crystallins, especially α-crystallins, the major proteins of the ocular lens, play a prominent role in the maintenance of the transparency and refractive properties of the lenses (*26*−*28*). The development of lens opacity caused by free radical formation (*29*), thermal (*30*, *31*) and osmotic impacts (*32*, *33*), ultraviolet radiation (*34*−*36*), oxidative stress (*37*−*41*), and calcium accumulation (*25*, *42*−*44*) involves biochemical processes such as conformational changes, proteolysis, and denaturation of the lens proteins. It was found that UV irradiation and aging result in the decrease of tryptophan fluorescence intensity of α-crystallin and γ-crystallin, indicative of the structural changes of these lens proteins through protein modification (*45*, *46*). Additionally, the structural changes of α-crystallin in turn resulted in the reduction of chaperone-like activity (*45*).

A more recent study demonstrated that ASTX may provide protection against UV insults to lens epithelial cells (*45*). Therefore, it is of interest to study the role of ASTX in protecting lens proteins from various oxidative insults. In this study, we have investigated the in vivo protection afforded by ASTX toward porcine lens proteins stressed with either iron-mediated hydroxyl radicals (*46*) or Ca^{2+}-mediated activation of calpains (*47*).

MATERIALS AND METHODS

Materials. ASTX [(3*S*,3′*S*)-3,3-dihydroxy-β,β-carotene-4,4′-dione], ferrous sulfate, hydrogen peroxide, and reduced/oxidized glutathione were purchased from Sigma (St. Louis, MO). Tris-HCl, ethylenedinitrilotetracetatic acid (EDTA), β-mercaptoethanol, sodium azide, and calcium chloride were purchased from Merck (Darmstadt, Germany). Double-distilled water was used to prepare all solutions. The materials used for running sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) were purchased from Invitrogen (Carlsbad, CA).

Porcine Lens Protein Preparation. Porcine (*Sus scrofa* var. *domestica*) lenses purchased from a local farm were homogenized in a pH 8.0 buffer containing 50 mM Tris-HCl, 0.1 M NaCl, 5 mM EDTA, 0.01% β-mercaptoethanol, and 0.02% sodium azide, as described in a prior study (*36*). After centrifugation at 27000*g* for 30 min, the supernatants were collected and the lens protein concentration was subsequently determined using the Bradford dye-binding method (Bio-Rad Laboratories, Hercules, CA). The lens protein preparations were further used for the oxidative study under the hydroxyl radical insults.

Porcine Lens Proteins Exposed to Various Concentrations of Hydroxyl Radicals. Crude porcine lens proteins were incubated with various Fenton solutions (OX-2, OX-0.5, and OX-0.2) modified based on the method of Huang et al. (*48*). The final concentrations of Fe^{2+}/H_2O_2 in Fenton solutions of OX-2, OX-0.5, and OX-0.2 were 2 mM Fe^{2+}/20 mM H_2O_2, 0.5 mM Fe^{2+}/5 mM H_2O_2, and 0.2 mM Fe^{2+}/2 mM H_2O_2, respectively. The incubations with or without ASTX (1 mM) were carried out at 37 °C for 1 h. Tryptophan fluorescence measurements of the lens samples were then used to assay the degree of free radical damage in the absence and in the presence of ASTX. The fluorescence emission spectra were taken at room temperature and recorded with a Hitachi F4010 fluorescence spectrophotometer excited with 295 nm light. The emission spectra were recorded from 300 to 400 nm using a light slit of 5 nm for both excitation and emission modes. Spectra of normal lens proteins and ASTX were used as baselines for the calculation of the tryptophan fluorescence intensity change. The intensity change at different emission wavelengths for each experiment was the average change of three repeated incubations, and then, the plots of the intensity change vs the wavelength were determined. SDS−PAGE was also employed to analyze the vulnerability of lens crystallin components under various stresses in the presence or absence of ASTX.

Porcine Lens Proteins Exposed to Calpain under Excess Calcium Ions. Calpain, a cysteine proteinase, hydrolyzes a variety of endogenous proteins including lens proteins. To evaluate the effects of ASTX in preventing lens proteins from hydrolysis by calcium-activated calpain, a modification of the methods of previous studies was used (*47*, *49*). Microtiter plates (96 wells, Costar, MA) were used to incubate the hydrolysis. Twenty microliters of various concentrations of ASTX (0, 0.03, 0.1, 0.3, and 1 mM), 140 μL of lens proteins (50 mg/mL), and 20 μL of physiological grade KCl solution (120 mM) were placed in the wells. To each well was then added calcium ions to a final concentration of 1 mM, and incubations were carried out at 37 °C. Additionally, incubation in the presence of the calpain inhibitor E64 (100 μM) or glutathione (1 mM), an endogenous antioxidant, was also carried out for comparison. The turbidity developed in each well during incubation was subsequently measured daily for five consecutive days in terms of light-scattering intensity at 405 nm. On the fifth day of the incubation, the lens protein in each treatment was further analyzed by SDS−PAGE.

Interactions between ASTX and Calcium Ions. The interactions between ASTX and calcium ions were identified using a silica-coated thin-layer chromatographic (TLC) technique (*50*, *51*). The interaction was performed by adding calcium ions to the ASTX solution in molar ratios of 1:1, 2:1, and 10:1. TLC plates were eluted with a solvent system of dichlormethane/methanol in the ratio of 9/1 (v/v). Chromatograms developed with this solvent system revealed bright orange spots. The R_f value for each spot was also determined.

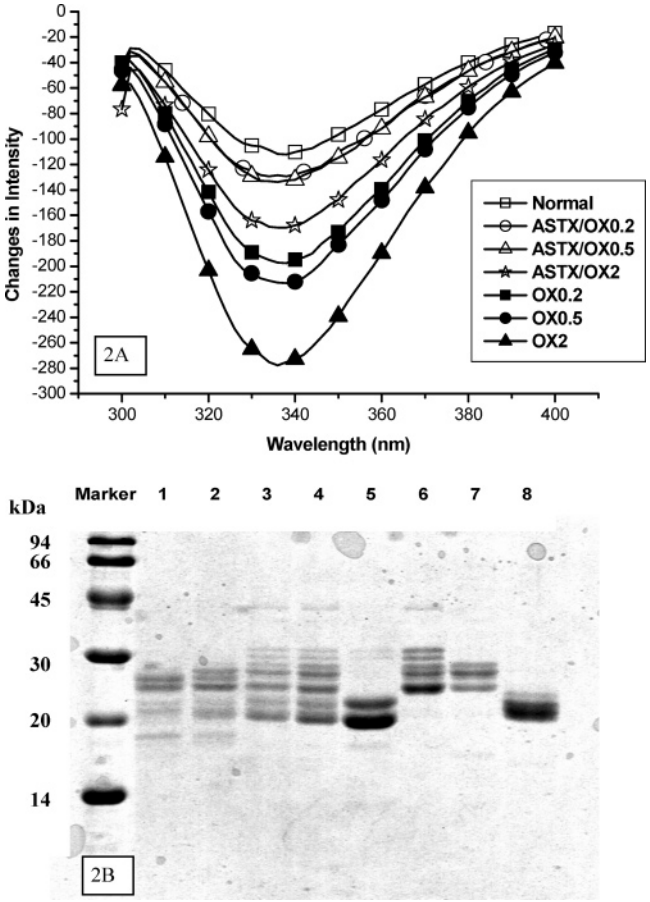

Figure 2. Porcine crystallins subjected to oxidative stress (OX) by various strengths of Fenton solutions with/without the protection of ASTX. (**A**) Negative changes indicate the loss of fluorescence intensity for tryptophan (FIT) following 1 h incubations. FIT of normal lens proteins (without OX exposure) changed slightly over the time. The magnitude of FIT loss increased with the increased strength of OX. The protective activity of ASTX was concentration-dependent. (**B**) The SDS–PAGE of the soluble porcine lens proteins. Lane 1, normal lens proteins + OX-0.2; lane 2, normal lens proteins + OX-2 + 1 mM ASTX; lane 3, normal lens proteins + OX-0.2 + 1 mM ASTX; lane 4, normal lens proteins; lane 5, porcine α-crystallin; lane 6, porcine β_{high}-crystallin; lane 7, porcine β_{low}-crystallin; and lane 8, porcine γ-crystallin.

RESULTS

ASTX Prevents Porcine Lens Proteins from Oxidative Damage by Hydroxyl Radicals. The mean changes in tryptophan fluorescence emission spectra of porcine lens proteins subjected to oxidative stress at various concentrations of hydroxyl radicals in the presence or absence of ASTX are shown in **Figure 2A**. This study found that the reductions in tryptophan fluorescence intensity in the absence of ASTX were clearly observable, especially when exposed to the 2 mM Fenton solution, indicating that ASTX has significant antioxidant activity to protect proteins from this harsh oxidative insult. The fluorescence spectra also showed that no wavelength shift for the maximum emission was observed, suggesting that some buried tryptophan residues might have become exposed during the exposure to the hydroxyl radicals; however, the tertiary structure somehow remained folded.

To analyze which lens protein components were most vulnerable under the stress of hydroxyl radicals, SDS−PAGE analysis was performed. The SDS−PAGE results as shown in **Figure 2B** revealed that β_{high}- and γ-crystallins were more vulnerable to oxidative stress than β_{low}- or α-crystallin. Under the protection of ASTX, these lens proteins were more resistant to oxidative insults.

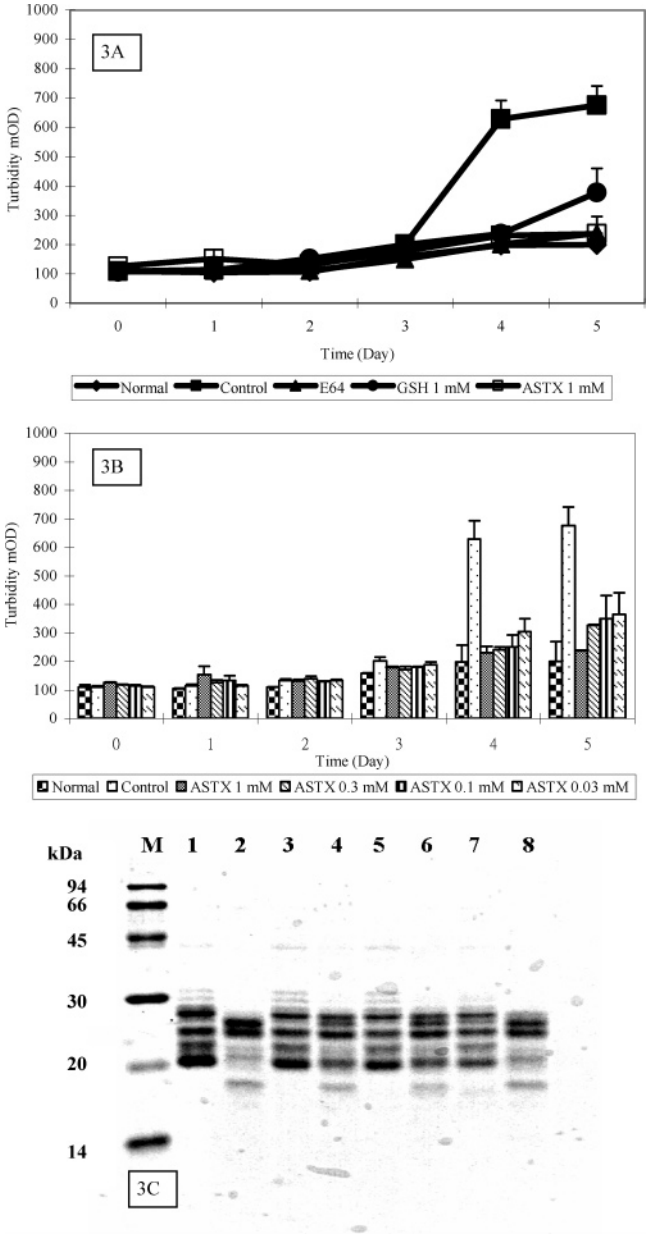

Figure 3. Effects of ASTX, the calpain inhibitor E64, and glutathione (GSH) on the protection of porcine lens proteins (50 mg/mL) from denaturation and aggregation caused by calcium-activated endogeneous calpain. All samples except the normal group had calcium added to a final concentration of 1 mM. Panels **A** and **B** were based on the intensity of the light scattering measured at 405 nm. The light intensity (turbidity value) was the mean ± *SD for 3*–4 identically prepared wells. Panel **C** was based on SDS–PAGE analysis of the soluble proteins. Lane 1, normal lens proteins; lane 2, control without any additions; lane 3, control with the addition of calpain inhibitor E64; lane 4, control with the addition of 1 mM GSH; and lanes 5–8, control with the addition of 1 M and 0.1, 0.3, and 0.03 mM ASTX, respectively.

Porcine Lens Protein Exposure to Excess Calcium. The effects of ASTX on calcium-induced turbidity due to endogenous lens calpain are illustrated in **Figure 3A**, which shows that after 3 days in the absence of glutathione, calpain inhibitor E64, or ASTX, the proteins began to denature. The result also revealed that 1 mM ASTX was as potent as 0.1 mM the calpain

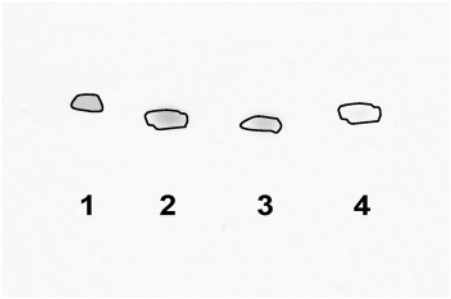

Figure 4. Silica-coated thin layer plates of ASTX/Ca^{2+} complex. Lane 1, ASTX and calcium ions at 1:2 (R_f = 0.66); lane 2, ASTX and calcium ions at 1:1 (R_f = 0.64); lane 3, ASTX (R_f = 0.63); and lane 4, ASTX and calcium ions at 1:10 (R_f = 0.65).

inhibitor E64 in protecting lens proteins from degradation. Dose−response data for ASTX in providing the protection of proteins from oxidative damage are shown in **Figure 3B**, which demonstrates that the effective doses for the protection of lens proteins from oxidative degradation by ASTX can be as low as 0.03 mM even after 5 days of incubation. In contrast to this, without the protection of ASTX, protein degradation became obvious after 3 days of incubation. The results of the SDS−PAGE analyses of lens protein proteolysis by endogenous calpain activated by calcium for 5 days in the presence and the absence of glutathione or ASTX are illustrated in **Figure 3C**. We found that 1 mM ASTX was as active as the calpain inhibitor, E64, in protecting lens protein from proteolysis. These results are consistent with those from the analyses using the light-scattering technique.

Interaction between ASTX and Calcium Ions. Because calpain requires activation to function as a protease and because ASTX was capable of preventing the occurrence of lens protein hydrolysis, ASTX may have likely interacted with either or both of them before the hydrolysis taking place. **Figure 4** illustrates the results of the interactions between ASTX and calcium ion obtained by TLC. We found that the R_f values for the molar ratio of ASTX/calcium ion being 1/1, 1/2, and 1/10 were 0.66, 0.64, and 0.65, respectively, while for ASTX alone it was 0.63. This result indicated that ASTX must have reacted with Ca^{2+} to form a complex leading to the decrease of free Ca^{2+}, which then resulted in less possibility of activating calpain to hydrolyze lens proteins.

DISCUSSION

Pure carotenoids even in a crystalline state are unstable when exposed to air and are rapidly broken down if samples are stored in the presence of traces of oxygen. The most important moiety of the ASTX molecule is the polyene chain (*52*). The 13 conjugated double bonds, in contrast to the seven in β-carotene, gives it significantly greater antioxidant capacity, and its long chain conjugated polyene structure makes it highly reactive to singlet oxygen and free radicals.

Our tryptophan fluorescence study indicated that ASTX is able to retard lens crystallin oxidation under high concentrations of metal-mediated radicals for up to an hour. Although all amino acid residues in the protein chain are susceptible to modification by the hydroxyl radical, among them tryptophan is the most vulnerable amino acid. Previous studies of two lens proteins had demonstrated that the tryptophan residues of crystallins are readily modified in the Fenton oxidation reaction (*53*) and in the chemical-produced hydroxyl radical (*46*); however, no aggregation caused by protein covalent bonding was observed. The latter study also showed that loss of the fluorescence intensity, due to the formation of N-formylkynurenine via the oxidation of tryptophan, was inhibited by a hydroxyl radical scavenger, mannitol, at 1 mM (*48*). Our result of 1 mM ASTX being capable of preventing the loss of tryptophan fluorescence intensity was consistent with that 1 mM mannitol being able to inhibit the damage caused by hydroxyl free radicals.

That antioxidant activities of ASTX are concentration-dependent was further supported by the subsequent electrophoresis analysis. The severity of degraded lens crystallins was also related to the presence of ASTX and the relative concentrations of metal-mediated radicals. ASTX at 1 mM proved to be an effective concentration to protect the proteins from the damage by free radicals generated from a relatively low concentration of Fenton solution, OX-0.2 (0.2 mM Fe^{2+}/2 mM H_2O_2). Although 1 mM ASTX did not provide complete protection against the oxidative insults from the higher concentration of Fenton solution, OX-2 (2 mM Fe^{2+}/20 mM H_2O_2), the lens protein degradations (patterns) of the sample incubated in ASTX (1 mM)/OX-2 were almost the same (patterns) as those incubated in OX-0.2 alone, as shown in **Figure 2B**, indicating that β_{high}-crystallin was more vulnerable to free radicals, whereas both α- and γ-crystallins were partially degraded. This differentiation in the destruction among the crystallins is consistent with the previous study that β-crystallin was more susceptible to hydrogen peroxide than both α- or γ-crystallin (*54*). It was found that the degradations of both α- and γ-crystallins were from the NH_2 termini as found in the calpain-induced lens protein degradation (*55*). In fact, it is well-known that α-crystallin is a major lens protein with a chaperone-like activity; more recently, it was found that bovine α-crystallin also showed antioxidant and free radical-scavenging properties in a series of in vitro studies (*56*). In this study, besides ASTX, α-crystallin may have provided additional antioxidative protection against oxidative insults to other crystallins. However, in the presence of hydrogen peroxide, the increased expression of calpain II in rat lens epithelial cells was observed (*57*) and the loss of α-crystallin's chaperone-like activity was observed when incubated with calpain II (*58*). Therefore, if ASTX also affects the progression of calcium-activated proteolysis mediated by calpain, the chaperone activity of α-crystallin would also be expected to influence ASTX's overall protective activity.

Under conditions of adequate free concentrations, calcium plays an important role in calpain activation. Calcium at 1 mM is an appropriate concentration to be used to activate calpain for the hydrolysis of lens protein in in vitro cataract model studies. Inhibition of the occurrence of calcium-induced turbidity in lens proteins was observed in the presence of ASTX (0.03−1 mM). The possible mechanism for the antiproteolytic activity exerted by ASTX was likely the formation of ASTX/Ca^{2+} complexes as evidenced by the results observed in the TLC experiment, in which more polar spots were observed for the mixtures of ASTX and Ca^{2+} prepared by mixing various molar ratios ASTX and Ca^{2+} (1:1, 1:2, and 1:10). The formation of ASTX/Ca^{2+} complexes would be expected to leave inadequate Ca^{2+} for the activation of calpain. The complexation of ASTX/Ca^{2+} can also be used to explain the observed results shown in **Figure 3C**, in which ASTX (1 mM) showed better protection than glutathione (1 mM), an endogenous antioxidant, in suppressing calcium-activated calpain hydrolysis of lens proteins. This complex may also have the ability to enhance the resistance of lens proteins to degradation/proteolysis besides reducing the available free calcium ions for activating calpain. Our finding of calcium−ASTX complexation may also provide a plausible explanation for a previous study indicating that the reduced

serum calcium levels resulted from the daily consumption of ASTX (*59*). Like corticosteroids, the 3′hydroxyl/2′oxo structure in each cyclohexene ring of ASTX may be a site for Ca^{2+} to bind. The possibility of an unwelcome calcium metabolic disturbance due to the ability of excessive overconsumption of ASTX to sequester calcium ion requires investigation.

Taken together, these studies demonstrate that ASTX provides appreciable protection for vulnerable tryptophan residues against oxidative stress and also for β_{high}-crystallin as well. In this in vitro cataract model study induced by calcium, ASTX at a concentration of 1 mM was capable of inhibiting calpain-induced proteolysis, which was as active as that observed with the calpain inhibitor E64 at a concentration of 0.1 mM. In addition, at a much lower concentration (0.03 mM), ASTX was still able to significantly retard protein degradation. The complex formation of calcium ions with ASTX led mainly to less free calcium ions available for the activation of calpain, which was evidenced as a decrease in lens protein turbidity and proteolysis. The xanthophylls ASTX may play a beneficial role in eye health. An in vivo study for the effectiveness of ASTX in protecting eyes from various stressors is currently underway in this laboratory.

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