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Comparison of Non-Tryptophan Fluorophores in Protein-Free Extract of Brunescent and Non-Brunescent Human Cataract

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Purpose: To investigate whether any of the fluorophores in the human lens nuclei might be responsible for human brunescent cataract formation.

Methods: Human lens nuclei (non-brunescent, from 13; brunescent, from 8) were obtained after extracapsular cataract extraction in nondiabetic patients. Protein-free extract, prepared by filtrating the water-soluble fraction of each nucleus through a centrifugal ultrafilter (molecular weight $< 5{,}000$), was analytically separated by high-performance liquid chromatography.

Results: No significant differences between non-brunescent and brunescent nuclei were observed in the concentrations (mean ± SD) of 3-hydroxykynurenine *O*-β-glucoside (0.67 ± 0.38 vs. 0.85 ± 0.62 μmol/g wet weight), 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-glucoside ($4.1 \times 10^5 \pm 2.9 \times 10^5$ vs. $6.3 \times 10^5 \pm 5.0 \times 10^5$ area unit/g wet weight), and kynurenine (0.016 ± 0.011 vs. 0.029 ± 0.021 μmol/g wet weight). A novel fluorophore that has not been identified so far was significantly present more in brunescent than in non-brunescent nuclei (brunescent: $1.5 \times 10^5 \pm 1.0 \times 10^5$ vs. non-brunescent: $2.6 \times 10^3 \pm 6.3 \times 10^3$ area unit/g wet weight, $P < .01$). Digestion of the protein-free extract with β-glucosidase eliminated the peak corresponding to the novel unidentified fluorophore.

Conclusion: The present results imply a novel protein-unbound fluorophore, presumably a β-glucoside, might possibly be involved in brunescent cataract formation. **Jpn J Ophthalmol 2000;44:198–204** © Japanese Ophtalmological Society

Key Words: Brunescent cataract, fluorophore, high-performance liquid chromatography, 3-hydroxykynurenine *O*-β-glucoside, water-soluble fraction.

Introduction

Van Heyningen[1] reported that human lenses contain several protein-unbound non-tryptophan fluorophores such as kynurenine, 3-hydroxykynurenine *O*-β-glucoside (3HKG), and an unknown glucoside, the last of which has recently been identified as 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-glucoside (AHBG) by Truscott et al[2] and Inoue and Satoh.[3] Concerning the functional role of these fluorophores with respect to the retina, they are generally expected to act as ultraviolet (UV) filters to protect the retina from UV exposure,[4,5] because these fluorophores absorb light maximally at approximately 365 nm wavelength.[5,6] The effects on the lenses per se, however, are still controversial. Some researchers[7–10] speculate that these fluorophores accelerate nuclear and/or brunescent cataract formation through their photosensitizing action because the addition of these fluorophores to the lens proteins under near-UV exposure induces cross-linking, aggregation, and browning of the lens proteins. On the other hand, other researchers[4] hypothesized that these fluorophores are not active photosensitizers because they have little photochemical and photodynamic propensities.

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In order to examine whether any of these fluorophores in the human lens nuclei might possibly be relevant to brunescent cataract formation, we compared their concentrations in brunescent and non-brunescent cataractous lens nuclei obtained after cataract extraction.

Materials and Methods

Collection of Lens Nuclei

Lens nuclei from senile cataractous patients without diabetes mellitus were obtained after extracapsular cataract extraction. Written informed consent was obtained from all patients for use of the nuclei in this study. Individual nuclei were weighed and classified into grades I to IV according to the degree of macroscopic nuclear coloration: grade I, pale yellow; grade II, yellow; grade III, dark yellow to amber; grade IV, dark brown to black. Grades I and II were rated as non-brunescent and grade IV as brunescent. Lens nuclei of grade III were excluded to avoid ambiguous classification. Thirteen non-brunescent nuclei (mean age of patients ± SD: 77.6 ± 5.8 years) and 8 brunescent nuclei (74.1 ± 9.5 years) were collected for this study. Lens nuclei were stored at −70°C until processing.

Preparation of Protein-Free Extract

Lens nuclei were homogenized individually in 1.2 mL of 0.1 M phosphate buffer (pH 4.0), and centrifuged at 10,000 *g* for 10 minutes. The supernatant was filtrated through a 5,000-molecular weight cutoff centrifugal ultrafilter (Ultrafree C3-LCC; Millipore Limited, Tokyo).

High-Performance Liquid Chromatography

The high-performance liquid chromatography (HPLC) system consisted of a pump (HLC 803-D; Tosoh, Tokyo), a sample injector (7125; Rheodyne, Rohnert Park, CA, USA), and a gradient unit (GE-4; Tosoh). Fifteen microliters of each sample were injected onto a C18 column (TSK-Gel ODS-80Ts; 4.6 mm inner diameter × 15 cm length, Tosoh). Absorbance at 360 nm and fluorescence intensity with excitation/emission at 350 nm/440 nm were measured with a UV absorption monitor (UV-8 Model II; Tosoh) and a fluorescence monitor (RF-535; Shimadzu, Tokyo). A two-channel chromato-integrator (Model D-2500; Hitachi, Tokyo) depicted the chromatograms and calculated the integrated areas of the peaks.

Two kinds of HPLC mobile phase were used: mobile phase 1, a linear gradient of 0–50% (v/v) acetonitrile in 0.1 M phosphate buffer (pH 4.0) over a period of 30 minutes at a constant flow rate of 1.0 mL/min; mobile phase 2, isocratic phosphate buffer (pH 7.0) for the initial 10 minutes and a subsequent linear gradient of 0–30% (v/v) acetonitrile in 0.1 M phosphate buffer (pH 7.0) over a period of 30 minutes at a constant flow rate of 1.0 mL/min. All chromatography was performed at room temperature.

Determination of Concentrations of Fluorophores in Each Nucleus

The protein-free extract was analyzed with HPLC eluted with the mobile phase 1. The concentrations of 3HKG, kynurenine, and AHBG were estimated from the integrated peak areas of UV absorption instead of those of fluorescence, because the fluorescence intensity of 3HKG, kynurenine, and AHBG was too weak to be employed as a quantifying parameter. The concentration of the unidentified fluorophore that we discovered in this study (see Results for detail) was estimated from the integrated peak area of fluorescence.

The concentrations of 3HKG and kynurenine were expressed as μmol/g wet weight (of nucleus), which were calculated by dividing the mols of these substances in one whole nucleus by the wet weight of each nucleus. The mols of these substances in one whole nucleus were determined by converting the mols of these substances in the analyzed volume (15 μL) into those contained in the total volume of water in each homogenate, which was estimated as 1.2 mL, the volume of the added phosphate buffer, presuming that the volume of water naturally present in the nucleus was negligible.

The mols of kynurenine in the analyzed volume of each extract were determined by comparison with authentic kynurenine (Wako Pure Chemical Industries, Osaka).

The mols of 3HKG in the analyzed volume of each extract were ascertained from the calibration curve determined as follows. In the β-glucosidase digestion study (see below for details), the mols of 3HKG in β-glucosidase-undigested sample and those of 3-hydroxykynurenine (3HK) in β-glucosidase-digested sample are theoretically equal when the same volumes were injected onto the column. The mols of 3HK were determined by comparison with authentic 3HK (Wako), thus the relationship between the mols and the integrated peak area of UV absorption of 3HKG was ascertained.

The concentrations of AHBG and the unidentified fluorophore were expressed as area unit (AU)/g wet weight, which was calculated by dividing the AU

Jpn J Ophthalmol
Vol 44: 198–204, 2000

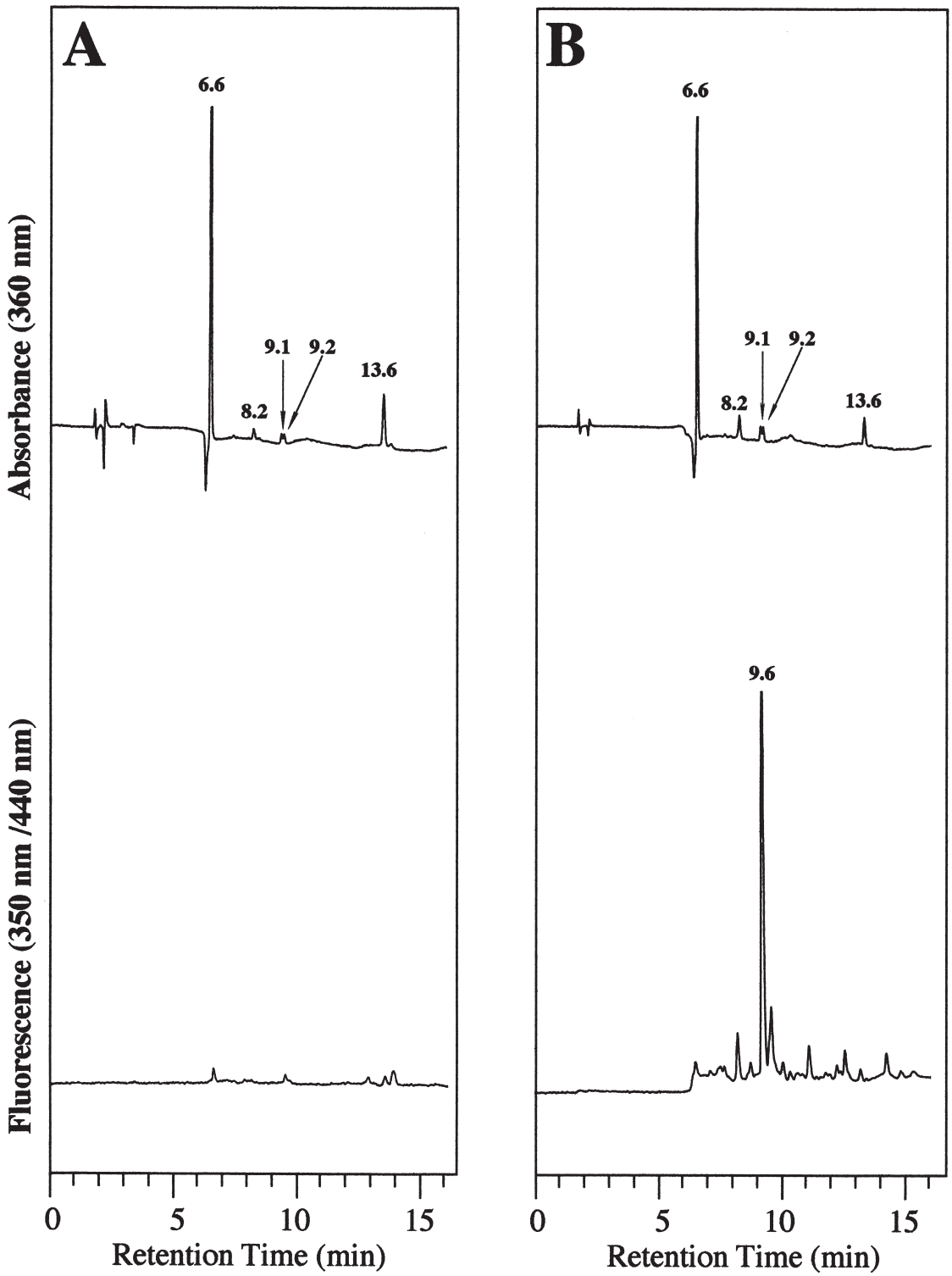

Figure 1. Typical high-performance liquid chromatography (HPLC) chromatograms of protein-free extracts from non-brunescent (**A**) and brunescent (**B**) nuclei. Analytic separation was performed using HPLC mobile phase 1 (see Materials and Methods) monitored with ultraviolet (UV) absorption at 360 nm (upper traces) and with fluorescence of excitation at 350 nm and emission at 440 nm (lower traces). The 6.6-, 8.2-, and 13.6-minute peaks of UV absorption (**A** and **B**, upper traces) were 3-hydroxykynurenine *O*-β-glucoside, kynurenine, and 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-glucoside, respectively. The 9.6-minute peak of fluorescence (**B**, lower trace) was clearly observed in every protein-free extract from brunescent lens, whereas only traces were detected in most extracts from non-brunescent nuclei (**A**, lower trace).

of these substances in the analyzed volume of each extract by the wet weight of the nucleus.

Digestion with β-Glucosidase

Protein-free extract prepared from brunescent cataractous lens nuclei was incubated either with phosphate buffer (pH 7.0) or with β-glucosidase (Wako, 3.3% w/v) at 37°C for 48 hours, the former serving as control. Because the mobile phase 1 was inappropriate for adequate separation of 3HK from 3HKG, mobile phase 2 was used. As an exception, the calibration curve of 3HKG was prepared using the mobile phase 1, as described above.

Results

As typically depicted in Figure 1, chromatograms in the mobile phase 1 of the protein-free extracts from a non-brunescent nucleus and a brunescent nucleus revealed UV absorption peaks at 6.6, 8.2, 9.1, 9.2, and 13.6 minutes in both the non-brunescent (Figure 1A, upper trace) and brunescent nuclei (Figure 1B, upper trace). The 8.2-minute and 13.6-minute peaks (Figures 1 A,B) were confirmed to represent kynurenine and AHBG, respectively, because their retention times were identical to those of commercially available authentic kynurenine and purified AHBG[3] respectively (data not shown). The 6.6-minute peak was presumed to represent 3HKG by comparison with previously reported[5,11] chromatographic profiles of the protein-free extracts. The 9.1- and 9.2-minute peaks have not been identified. The peak of 3HK was hardly detected in both the non-brunescent and brunescent nuclei. The presence or the absence of all these UV absorption peaks was independent of the degree of coloration of

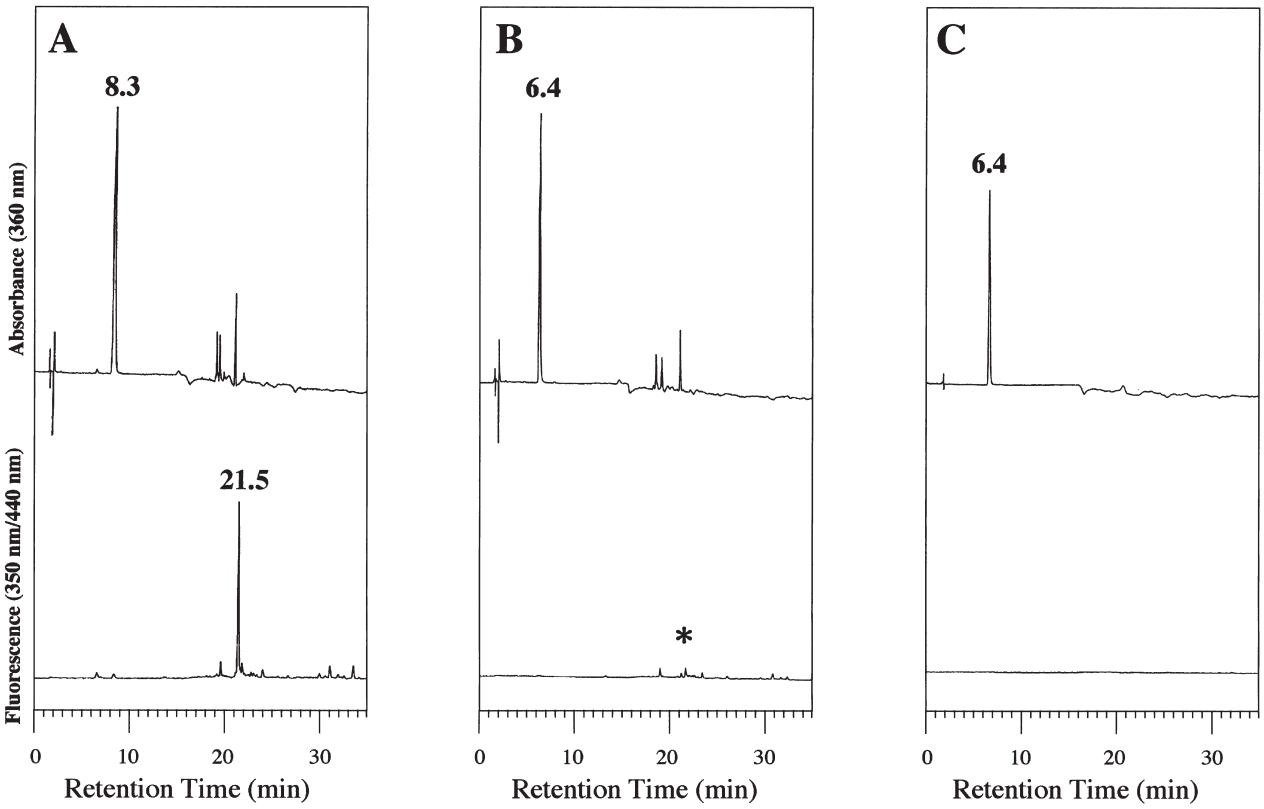

Figure 2. Disappearance of unidentified fluorophore peak by β-glucosidase digestion. Protein-free extracts were incubated with phosphate buffer as control (**A**) or with β-glucosidase (**B**). Chromatograms of authentic 3HK are shown for comparison (**C**). Analytic separation was performed using high-performance liquid chromatography mobile phase 2 to separate 3-hydroxykynurenine (3HK) from 3-hydroxykynurenine *O*-β-glucoside (3HKG) (see Materials and Methods). Elution was monitored with ultraviolet absorption at 360 nm (upper traces) and with fluorescence of excitation at 350 nm and emission at 440 nm (lower traces). With β-glucosidase treatment, the peak of the unidentified fluorophore at 21.5 minutes (**A**) disappeared (**B**, asterisk) and the peak of 3HKG at 8.3 minutes (**A**) shifted to 6.4 minutes (**B**) which is identical with the retention time of authentic 3HK (**C**).

Jpn J Ophthalmol
Vol 44: 198–204, 2000

each nucleus. On the other hand, the 9.6-minute fluorescence peak (Figure 1B, lower trace) that was nearly null in the non-brunescent nuclei (Figure 1A, lower trace) was clearly noticed in most of the brunescent nuclei. The 9.6-minute fluorescence peak did not obviously correspond to any known protein-unbound lenticular fluorophores such as 3HKG, 3HK, kynurenine, or AHBG (i.e., van Heyningen's unidentified fluorophore, see Introduction). Therefore, we refer to this substance as the unidentified fluorophore.

In the β-glucosidase digestion experiment (Figure 2), the retention time of each fluorophore was different from that in Figure 1, because mobile phase 2 was used to segregate the peaks of 3HK and 3HKG. The 8.3-minute peak in Figure 2A was thought to represent 3HKG because a digestion with β-glucosidase eliminated this peak (Figure 2B, upper trace) and instead produced a new peak at 6.4 minutes (Figure 2B, upper trace) which is equal to the retention time of the authentic 3HK peak (Figure 2C). The 21.5-minute fluorescence peak (Figure 2A, lower trace) apparently corresponds to the unidentified fluorophore that had appeared at 9.6 minutes in Figure 1A, because no other fluorophores that are known to exist in the protein-free extract of the human lens emit fluorescence comparable to the 21.5-minute peak depicted in Figure 2A, and consequently our unidentified fluorophore (Figure 1B, 9.6-minute peak) is currently the most plausible candidate. This 21.5-minute fluorescence peak was eliminated by β-glucosidase digestion (Figure 2B, asterisk).

The concentrations of 3HKG, kynurenine, AHBG, and the unidentified fluorophore were compared between non-brunescent and brunescent cata-

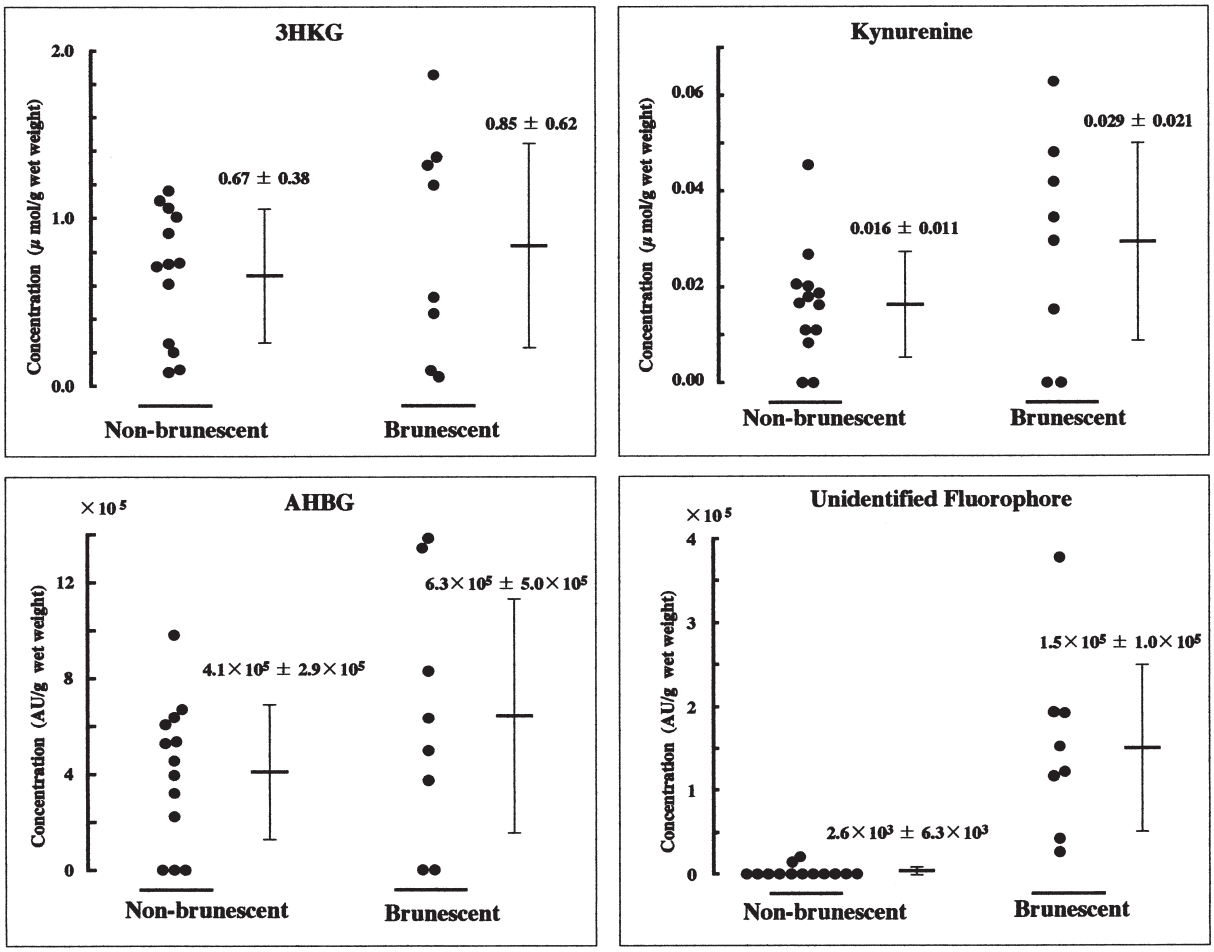

Figure 3. Comparison of concentrations of protein-unbound fluorophores between non-brunescent and brunescent nuclei. Each bar represents mean ± 1 SD. While the concentrations of 3-hydroxykynurenine *O*-β-glucoside (3HKG), 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-glucoside (AHBG), and kynurenine did not differ significantly ($P < .10$), that of the unidentified fluorophore was significantly ($P < .01$) higher in brunescent nuclei than in non-brunescent nuclei.

ractous lens nuclei. The concentration of 3HKG was 0.67 ± 0.38 μmol/g wet weight for the non-brunescent nuclei and 0.85 ± 0.62 μmol/g wet weight for the brunescent nuclei, which did not significantly differ (Figure 3, upper left). Similarly, the concentrations of AHBG (non-brunescent, $4.1 \times 10^5 \pm 2.9 \times 10^5$ AU/g wet weight; brunescent, $6.3 \times 10^5 \pm 5.0 \times 10^5$ AU/g wet weight) and kynurenine (non-brunescent, 0.016 ± 0.011 μmol/g wet weight; brunescent, 0.029 ± 0.021 μmol/g wet weight) did not differ significantly (Figure 3, lower left, upper right). In contrast, the concentration of the unidentified fluorophore was significantly higher in brunescent nuclei than in non-brunescent nuclei (non-brunescent, $2.6 \times 10^3 \pm 6.3 \times 10^3$ AU/g wet weight: brunescent, $1.0 \times 10^5 \pm 1.0 \times 10^5$ AU/g wet weight, $P < .01$) (Figure 3, lower right).

Discussion

Except for the unidentified fluorophore in the non-brunescent nuclei, the concentrations of all of the examined fluorophores showed great inter-individual variation in both the non-brunescent and brunescent nuclei (Figure 3). In some nuclei, the concentrations of all of the fluorophores were extremely low. Such low concentrations might be attributable to the loss of low molecular weight substances through the lens capsule in opacified cortices of mature and hypermature cataracts, as is reported by Khurana et al.[11]

The concentrations of 3HKG, AHBG, and kynurenine did not significantly differ between non-brunescent and brunescent nuclei, but that of the unidentified fluorophore was remarkably higher in brunescent nuclei than in non-brunescent nuclei (Figure 3). These results suggest that the unidentified fluorophore but not 3HKG, AHBG, or kynurenine might be relevant to brunescent cataract formation.

While the relevance of protein-bound non-tryptophan fluorophore to brunescent cataract formation has been widely studied,[12–16] the protein-unbound non-tryptophan fluorophores have not been thoroughly studied in relation to brunescent cataract formation. To the author's knowledge, only one report has claimed an increase in a protein-unbound previously unidentified fluorophore along with the nuclear coloration.[17] This unidentified fluorophore was later confirmed to be identical with the present unidentified fluorophore because the retention times of both the unidentified fluorophores were identical under the same HPLC conditions (data not shown).

Although it is very likely that the unidentified fluorophore is also a β-glucoside like 3HKG and AHBG, its photochemical and photodynamic properties seem quite different from the other three protein-unbound fluorophores because the unidentified fluorophore noticeably emits fluorescence while the other three fluorophores emit relatively low fluorescence. This strong fluorescence of the unidentified fluorophore might indicate its relevance to brunescent cataract formation.

Whether some of these fluorophores are active photosensitizers or not is still subject to controversy. Since the concentrations of 3HKG, AHBG, or kynurenine did not differ between brunescent and non-brunescent nuclei, they do not seem likely to be active photosensitizers for the lenses. The irrelevance of 3HKG, the major UV filter,[5] to brunescent cataract formation might also be supported by the fact that brunescent cataract usually develops after mid-life when the concentration of 3HKG decreases to its lowest level.[6]

The present study clearly revealed that a protein-unbound fluorophore, which is possibly a β-glucoside, increases more than 50-fold in brunescent nuclei compared with non-brunescent ones (Figure 3). The identification of this fluorophore as well as its role in lens coloration remains to be studied.

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Jpn J Ophthalmol
Vol 44: 198–204, 2000

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