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Isolation and characterization of a blue fluorophore from human eye lens crystallins: In vitro formation from Maillard reaction with ascorbate and ribose

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A blue fluorophore, named LM-1 was isolated from human eye lens crystallins. The fluorescence property of LM-1 (excitation/emission, 366/440 nm) is similar to the fluorescence originating during non-enzymatic glycation (Maillard reaction) of proteins with the reducing sugars. LM-1 accumulates linearly with age in highly cross-linked water insoluble crystallins and is present at higher levels in cataractous lenses. The fluorophore could be synthesized by incubation of bovine serum albumin (BSA) with ribose, but not with glucose or fructose. Incubation of bovine lens crystallins with ascorbic acid (ASA) and its oxidative products, dehydroascorbic acid (DHA) and 2,3-diketogulonic acid (DKG) in presence of oxygen resulted in LM-1 formation. When oxygen was removed from the system, only DHA and DKG could synthesize LM-1, but not ASA, suggesting that ASA oxidation is obligatory for LM-1 synthesis. Modification of lysine residues on BSA prior to incubation with ribose resulted in corresponding decrease in LM-1 formation. Since ASA concentration is unusually high in lens and has been found to be a powerful glycating agent of crystallins and since LM-1 does not form with hexoses, it is likely that ASA is the major precursor of LM-1.

Introduction

The aging of human lens is characterized by an increase in insoluble proteins, associated with yellow coloration and non-tryptophan fluorescence [1–7]. These changes are enhanced in senile and diabetic cataractous lenses. The proteins of the lens are long-lived and, thus, accumulate modifications. Modifications by photooxidation has been shown to cause protein conformational changes, aggregation and oxidation of aromatic amino acids [8–11]. Fluorescent oxidation products of tryptophan, kynurenines and carbolines have been isolated from human lens [12,13]. Alternatively, lenticular fluorescence may also originate from non-enzymatic glycation and the Maillard reaction. Reducing sugars like glucose or glucose 6-phosphate when incubated with crystallins react with ε-amino groups of lysine to form an initial Amadori compound, which by subsequent reactions forms fluorescent advanced glycosylation end products with absorption over 300 nm [14]. The fluorophores thus formed have been found to have similar spectral properties as those present in cataractous lenses [14,15]. Further, in diabetic [16,17] and galactosemic cataract [18], an increase in protein-bound non-tryptophan fluorescence and a concomitant increase in high molecular protein content have been observed, suggesting a role for sugars in cataractogenesis.

A number of fluorescent molecules attributable to the advanced Maillard reaction have been found in the human lens. Oimomi et al. [19] reported a fluorescent peak (peak L_1) in cataractous human lenses chromatographed by HPLC with a similar peak isolated from bovine serum albumin incubated with glucose. Recently a fluorescent product of glucose (MFP-1) identical to pentosidine was isolated by Dyer et al. [20] and

Abbreviations: BSA, bovine serum albumin; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; CH_3CN, acetonitrile; HFBA, heptafluorobutyric acid; TFA, trifluoroacetic acid; ASA, ascorbic acid; DHA, dehydroascorbic acid; DKG, 2,3-diketogulonic acid; RM-BSA, reductively methylated bovine serum albumin.

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found to be present in human lenses in aging and diabetic cataractous lenses [21]. Futhermore, low levels of pentosidine, a protein crosslink isolated from human collagen rich extracellular matrix that could be synthesized by the reaction of ribose with lysine and arginine residues were found in human lenses [22]. Finally ascorbate (ASA) and its oxidative products have been shown to react with lens crystallins and form crosslinks and non-tryptophan fluorescence in a Maillard-type reaction [23,24].

Although the formation of fluorophores in aging lenses and in cataractogenesis is well established, their biochemical nature remains highly speculative. We have now initiated a systematic investigation into the nature of blue fluorophores of the human lens and report below the isolation and characterization of a blue fluorophore with excitation/emission maxima at 366 nm and 440 nm, respectively, and its in vitro synthesis from ribose and ascorbate.

Materials and Methods

Materials

Human cataractous lenses were obtained from the Division of Surgical Pathology at University Hospitals of Cleveland, and normal lenses were supplied by Cleveland Eye Bank and National Disease Research Interchange, Philadelphia. Cataractous lenses were routinely classified into Type I-IV and brunescent based on pigmentation using the Pirie classification [25]. Bovine serum albumin (essentially fatty acid free, cat. No. A-6003), pronase E (proteinase type XXV), carboxypeptidase Y (from Baker's yeast) and fructose were purchased from Sigma Chemicals Aminopeptidase M (from hog kidney) was obtained from Boehringer Mannheim Biochemicals. Glucose, ribose, ascorbic acid (sodium salt) and dehydroascorbic acid were from Aldrich Chemicals. All other chemicals were of analytical grade.

Preparation of lens protein extracts

Lenses were decapsulated and homogenized in 0.05 M phosphate buffer (pH 7.4) (3.0 ml per lens) in a Con-Torque glass homogenizer (Eberbach Corporation, Ann Arbor, MI) for 3 min. The homogenate was centrifuged at $20000 \times g$ for 30 min at 4°C. The supernatant (water soluble) and pellet (water insoluble) were separated. The pellet was suspended in 2.0 ml buffer and centrifuged as described above. The two supernatant fractions were pooled and dialyzed against 3.0 l of water for 24 h using Spectrapor dialysis membrane (Spectrum Medical Industries, Los Angeles, CA) of 3500 MWCO. The dialyzate was lyophilized . The water insoluble (WI) and the lyophilized powder of water soluble (WS) were treated with 3.0 ml chloroform-methanol (2:1) mixture for 3 h with stirring and centrifuged at $3500 \times g$ for 30 min. The pellet was then treated with 5.0 ml diethyl ether for 10 min and centrifuged at $3500 \times g$ for 30 min. The residue obtained was then dried in a desiccator kept at 4°C. In case of pooled cataractous lenses (100 lenses), protein extraction was done in ten batches of ten lenses each in 30.0 ml buffer. Extraction procedure was same as above. Total weight of water insoluble fraction obtained was 1.4 g.

Proteolytic digestion of water soluble and insoluble crystallin fractions

Lens proteins were treated with pronase E (2% w/w) in 0.05 M phosphate buffer (pH 7.4) containing 0.02% sodium azide. The buffer was filtered through 0.2 micron Acrodisc filters (Gelman Sciences, Ann Arbor, MI) and incubated at 37°C for 24 h with shaking. Digestion was continued with another addition of pronase E (2% w/w) for 16 h. The digest was treated with aminopeptidase M (0.5% w/w) and carboxypeptidase Y (0.5% w/w) for 16 and 8 h, respectively. The digest was centrifuged at $3500 \times g$ for 30 min to remove floating debris. Corresponding enzyme blanks were run simultaneously. Amino acid estimation in enzyme digests were done by the ninhydrin method of Moore and Stein [26] using L-leucine as standard. The combination of enzymes used and the sequence of their addition resulted in 70–75% digestion.

Bio-Gel P-2 chromatography

The digests from cataractous lenses was lyophilized and the residue was washed twice with water. The residue was taken up into 15.0 ml 0.2 M acetic acid and subjected to gel filtration on a Bio-Gel P-2 column (110.0×1.5 cm, bed volume 186 ml, eluent 0.2 M acetic acid). 5 ml samples in three batches were applied and 5.0 ml fractions were collected at a flow rate of 10.0 ml per h. For individual lenses, the lyophilized digests were taken up in 2.0 ml 0.2 M acetic acid and chromatographed on a 40.0×1.0 cm column at a flow rate of 5.0 ml per h and fractions of 1.0 ml were collected. The eluent was monitored at 210 nm and fluorescence at 370/440 nm. Corresponding enzyme blanks were run under similar conditions. Fractions 18 to 36 and 7 to 12 were pooled in case of cataractous lenses and individual lenses respectively and designated as high molecular weight fraction (HMW), and fractions 36 to 72 and 13 to 23, respectively and designated as low molecular weight fraction (LMW).

High-performance liquid chromatography (HPLC)

The lyophilized LMW fraction from Bio-Gel column was taken into 7 mM phosphoric acid (2.25 ml in pooled cataractous lenses and 0.2 ml in case of individual lenses) and a 50 μl sample was injected into a C_{18} reverse-phase analytical column (Vydac, 4.6 mm $\times$ 25

cm, 10 micron, The Separation Groups, Hesperia, CA). A Waters HPLC (Waters Chromatography Div., Milford, MA) with model 510 pumps, U6K or automatic injector (model 712 WISP) and a 680 controller were used. The column was eluted for 40 min with 7 mM phosphoric acid at a flow rate of 1.0 ml per min and the tightly bound materials were washed with 50% acetonitrile (CH_3CN) in water and 0.1% heptafluorobutyric acid (HFBA) for 45 min (0–100%). The eluent from the column was monitored with an on-line fluorescence detector (Varian, fluorochrom or Waters 470 scanning fluorescence detector) at 440 nm (excitation at 370 nm). The chromatograms were recorded with a Waters Data Module (Model 740) at a chart speed of 0.2 cm per min. For routine analysis, enzyme digested WS and WI fractions were acid hydrolyzed with 6 M HCl at 110°C for 20 h and acid was evaporated in a Speed Vac concentrator (Savant Instruments, Hicksville, NY). The residue was taken into 7 mM phosphoric acid containing per ml 142.5 μmol leucine equivalent amino acids by ninhydrin method. Protein corresponding to 7.12 and 4.27 μmol leucine equivalent for WI and WS fractions, respectively, was injected into HPLC as described above. For routine analysis, the HPLC program was shortened. The column was eluted with 7 mM H_3PO_4 for 25 min and the tightly-bound material was washed with a gradient (0–100%) of 50% CH_3CN in water and 0.1% HFBA over 25 min. LM-1 peak was estimated quantitatively on the basis of peak height. samples with known peak height were run periodically to check for variations in fluorometer sensitivity.

Incubation of BSA with sugars

BSA (100 mg/ml) and 100 mM either glucose or fructose or ribose were dissolved in phosphate-buffered saline (PBS), filtered through 0.2 micron Acrodisc filters (Gelman Sciences, Ann Arbor, MI) in laminar flow hood and incubated for a maximum of 22 days at 37°C under dark. Aliquots of 0.7 ml were withdrawn on day 0, 7, 15 and 22 days and dialyzed against 2 × 3000 ml of water for 48 h. The dialyzate was lyophilized and subjected to acid hydrolysis with 6 M HCl at 110°C for 20 h. Hydrolyzate corresponding to 3.0 mg protein was analyzed for LM-1 by HPLC.

Incubation of BSA with ascorbic acid (ASA) or dehydroascorbic acid (DHA)

BSA (100 mg/ml) and ASA or DHA at a concentration of 25 mM were dissolved in 0.2 M phosphate buffer (pH 7.4) and filtered through 0.2 μm Acrodisc filters under sterile environment and incubated for 7 days at 37°C. BSA was also incubated with either DHA or ribose in presence or absence of oxygen. Nitrogen was flushed through the incubation mixture under vacuum. Incubation was done for 7 days and processed as desribed above. Blank samples without sugars were run simultaneously. Whenever necessary, presence of LM-1 in the HPLC run with phosphoric acid was confirmed by collecting and lyophilizing the peak and rechromatography in 5% CH_3CN in water +0.1% HFBA using a C_{18} reverse-phase column and a gradient from 5–50% CH_3CN + 0.1% HFBA in water over 45 min. LM-1 eluted between 32–35 min.

Detection of LM-1 in bovine lens crystallins incubated with ASA, DHA or Diketogulonic acid (DKG)

The preparation of these samples was done as described by Prabhakaram and Ortwerth [27]. In brief, crystallins (10 mg/ml) in 0.1 M phosphate buffer (pH 7.0) was incubated with each of 20 mM ASA, DHA or DKG and 0.1 M dithylenetriaminepentaacetic acid at 37°C for a maximum of 3 weeks in presence or absence of oxygen. Aliquots were withdrawn at weekly intervals. Samples were dialyzed against distilled water and lyophilized. The lyophilized powder was hydrolyzed with 6 M HCl. Samples corresponding to 5 μmol leucine equivalent in 100 μl were analyzed for LM-1 as described before. In addition, eluent from 8.0 to 13.0 min was collected and dried. The dried pellet was reconstituted in 120 μl 0.02 M acetate buffer (pH 4.47) and 100 μl was injected to sulfopropyl cation exchange column (Waters, Protein Pak, SP 5W, 7.5 × 75 mm). The column was eluted with a gradient of 0–0.3 M NaCl in 0.02 M acetate buffer (pH 4.47) over 40 min at a flow rate of 1.0 ml per min. LM-1 eluted between 21–22 min. HPLC was run under similar instruments setting as described for reverse phase column. This latter procedure was found to be necessary for accurate determination of LM-1.

Purification of LM-1 from BSA-ribose incubated mixture

5 g BSA (100 mg/ml) was incubated with 150 mM ribose in 0.2 M phosphate buffer containing 0.025% sodium azide for 7 days at 37°C. The deep brownish yellow mixture was dialyzed against 3 l of 0.05 M phosphate buffer (pH 7.4) for 48 h changing buffer every 16 h. The dialyzate was lyophilized and hydrolyzed with 6 M HCl for 20 h at 110°C. Acid hydrolyzate was dried and taken into 20.0 ml 7 mM phosphoric acid and 0.12 ml fractions were injected into a C_{18} semi-preparative HPLC column (Vydac, 1.0 × 25 cm, 10 μm, flow rate 2.0 ml/min). The column was eluted with phosphoric acid for 25 min and washed with 50% CH_3CN in water +0.1% HFBA. The eluent was directed to an on-line Waters fluorescent detector (model 470, excitation/emission, 370/440 nm). Peak LM-1 (retention time 16–19 min) was collected by repeated injections and lyophilized. The yellow pellet obtained was washed twice with water by lyophilization and finally taken in 0.5% CH_3CN in water +0.1% HFBA and further purified by repeated injection on a C_{18}

analytical column with a gradient of CH_3CN from 5–50% over 45 min. HFBA was exchanged with trifluoroacetic acid (TFA) by HPLC on a C_{18} analytical column with a gradient of from 0–50% CH_3CN in water +0.1% TFA over 45 min. The final product was judged pure by virtue of a single fluorescent and ninhydrin positive spot on thin layer chromatography, single HPLC peak under various chromatographic conditions and a baseline resolved absorption spectrum.

Role of lysine residues in LM-1 formation

Lysine residues in BSA were blocked by reductive methylation (RM) using the method of Jentoft and Dearborn [28]. BSA (1 mg/ml), 2 mM formaldehyde and 20 mM sodium cyanoborohydride in 10 mM Hepes buffer (pH 7.5) were incubated at 22°C for 2 h and dialyzed against 0.05 M phosphate buffer (pH 7.4), for 36 h. The extent of lysine modification was determined by the method of Kakade and Leiner using trinitrobenzene sulfonic acid [29]. The RM-BSA and unmodified BSA at a concentration of 3 mg/ml were incubated with 25 mM ribose in PBS for 10 days. Aliquots were withdrawn on day 0, 3, 6 and 10 and dialyzed against 0.025 M phosphate buffer (pH 7.6) for 36 h, changing the buffer after 24 h. LM-1 was determined by HPLC as described before.

Spectroscopy

Absorption spectra were recorded with Hewlett-Packard 8452A diode array spectrophotometer connected to an IBM PC/AT computer. Fluorescence spectra were recorded with a J4-8202 Aminco-Bowman spectrophotofluorometer (SLM instruments, Urbana, IL).

Results

Gel permeation chromatography of WI enzyme digest of pooled cataractous lenses on a Bio-Gel P-2 column is shown in Fig. 1. Approx. 80% fluorescence at 440 nm (excitation 370 nm) was present in highly

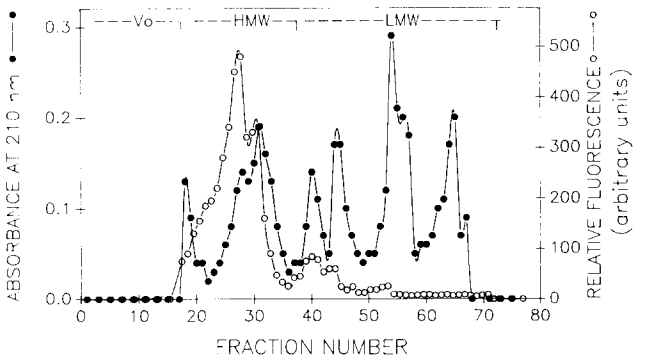

Fig. 1. Bio-Gel P-2 chromatography of cataractous lens water insoluble enzyme digest as described in Materials and methods section. Column was eluted with 0.2 M acetic acid and fractions of 5.0 ml were collected at a flow rate of 10.0 ml per h.

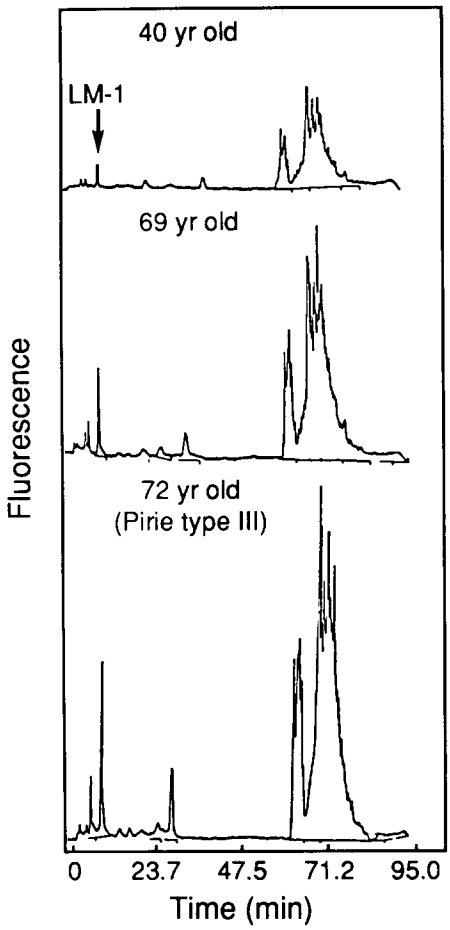

Fig. 2. HPLC of LMW fraction from Bio-Gel P-2 chromatography of water insoluble crystallin digest on a C_{18} reverse phase column as described in Materials and Methods. Fluorescence was monitored at excitation/emission wavelength, 370/440 nm.

cross-linked high molecular weight (HMW) fraction. Because the HMW fraction was highly resistant to further digestion, the LMW fraction was used for further studies. HPLC of the LMW fraction revealed a discrete fluorescent peak with a retention time between 9.5–11.5 min. The peak intensity increased in a 69-year-old lens compared with a 40-year-old lens and was further enhanced in a cataractous lens, when individual water insoluble lens fractions were separated on a Bio-Gel P-2 column and subjected to HPLC (Fig. 2). The peak was named LM-1 (lens Maillard product-1). The fluorophore had excitation and emission maxima at 366 and 440 nm, respectively. LM-1 obtained from pooled cataractous lenses was subjected to acid hydrolysis at 110°C for 20 h and injected again on HPLC under the same conditions. The peak retention time, fluorescence intensity and spectrum remained identical, thus indicating that LM-1 was acid stable. We exploited this property for quantitative measurement of LM-1 in aging and cataractous human lenses. A linear increase in the level of LM-1 as a function of age was observed in water insoluble (WI) crystallins (peak

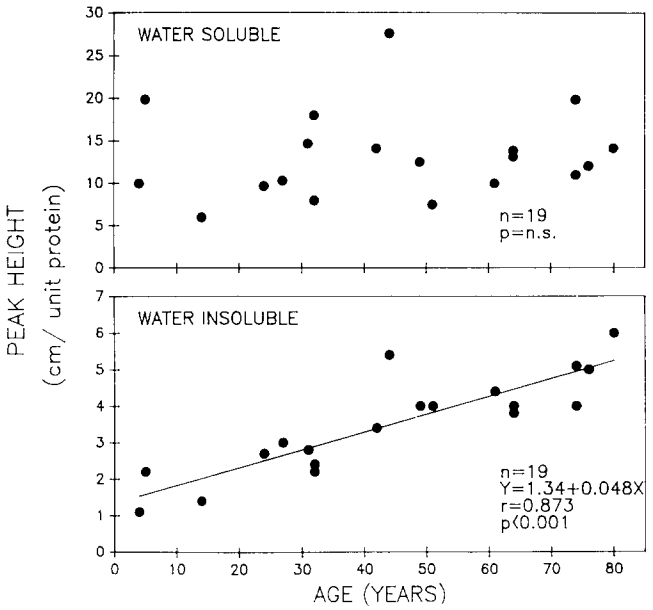

Fig. 3. Level of fluorophore LM-1 as a function of age in water soluble and water insoluble crystallins of normal lenses expressed per unit protein. Water soluble and insoluble crystallins acid hydrolysates corresponding to 4.27 and 7.12 μmol leucine equivalent of amino acids (which correspond to 0.55 and 0.92 mg protein, taking molecular weight of crystallin subunit as 20000) were injected to C_{18} reverse phase column. LM-1 levels in WS were adjusted to protein concentration of 7.12 μmol leucine equivalent (1 unit protein) for comparative purpose.

height in cm/unit protein; mean ± S.D.: young, 2.5 ± 0.3; old, 4.2 ± 0.5) In contrast, 6–7-times higher levels were present in water soluble (WS) crystallins with, however, no relationship to age (young, 15.5 ± 4.4; old, 14.5 ± 3.6; Fig. 3). In WS and WI fractions of cataractous lenses, a significant increase in LM-1 was noted in moderately and highly pigmented Pirie Type III and IV lenses ($P < 0.05$), but surprisingly not in brunescent lenses compared to age-matched control subjects (Table I). LM-1 was also elevated significantly ($P < 0.005$) in WI but not in WS crystallins from diabetic lenses.

Based on the observation of Oimomi et al. [19] that a similar peak (peak L_1) could be obtained from bovine serum albumin (BSA) incubated with glucose and based

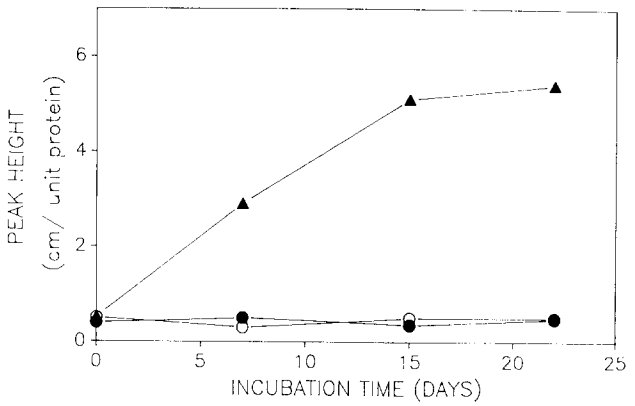

Fig. 4. Formation of LM-1 in BSA incubated with reducing sugars (●, glucose; ○, fructose and ▲ ribose. Acid hydrolysates corresponding to 3.0 mg protein were analyzed by reverse-phase HPLC.

on the observation made in our laboratory that the fluorescent protein cross-link, pentosidine can form from protein incubated with ribose [30], we investigated the role of reducing sugars in LM-1 formation. A time-related increase in LM-1 formation was observed in bovine serum albumin incubated with ribose reaching plateau level after 15 days of incubation. However, glucose or fructose incubated samples did not show LM-1 formation up to 22 days of incubation (Fig. 4). We further examined the ability of ascorbate to form LM-1 since it is known to have a tendency to fractionate and release CO_2 during Maillard reaction with proteins. LM-1 was found in bovine lens crystallins upon incubation with ascorbate, dehydroascorbate or 2,3-diketogulonate in presence of oxygen (Fig. 5). However, when oxygen was depleted from the system, LM-1 formed only from dehydroascorbate and 2,3-diketogulonate, thus indicating that for the synthesis of LM-1 from ascorbate to occur, oxidation is obligatory.

A number of studies were undertaken to ensure that LM-1 obtained from human lenses is identical with synthetic LM-1 from ribose and ascorbate. The HPLC retention times under different conditions as well as the fluorescence spectra were rigorously identical between LM-1 obtained from human lens and BSA-ribose

TABLE I

Levels of fluorophore LM-1 in cataractous lenses classified according to Pirie

Data are expressed as mean ± S.D. Lenses used are more than 55 years old; n = number of samples analyzed. 1 unit of protein corresponds to 0.92 mg protein. WS, water soluble crystallins; WI, water insoluble crystallins; Nor, Normal lenses; TY, Pirie type; BRUN, brunescent; DIAB, diabetic. Statistical significance was calculated using non-paired Student's t-test. * $P < 0.05$, ** $P < 0.005$ when compared with normal lenses

	Peak height (cm/unit protein)						
	Nor	TY.I	TY.II	TY.III	TY.IV	BRUN	DIAB
WS	13.3 ± 3.2	9.2 ± 3.5 *	12.2 ± 2.7	17.7 ± 6.0	18.8 ± 9.4	9.2 ± 3.4 *	9.9 ± 5.9
n	7	6	7	9	7	7	7
WI	4.6 ± 0.7	4.4 ± 1.1	4.6 ± 1.3	6.8 ± 2.3 *	7.1 ± 2.8 *	5.2 ± 1.8	8.3 ± 2.6 **
n	7	8	9	9	7	8	7

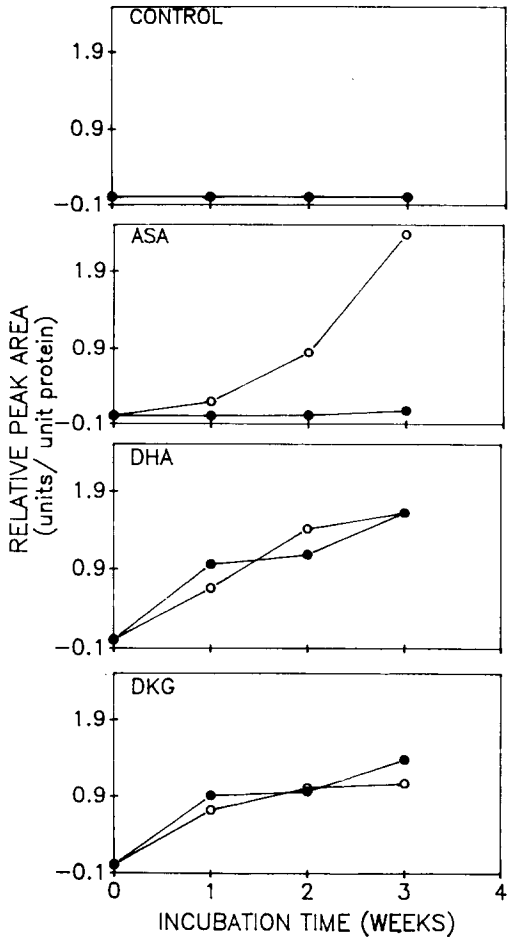

Fig. 5. Formation of LM-1 in bovine lens crystallins incubated with ascorbic acid (ASA), dehydroascorbic acid (DHA) and 2,3-diketogulonic acid (DKG) in presence (○) or absence (●) of oxygen. Acid hydrolyzed protein samples were injected into a C_{18} column and the eluent which had LM-1 was rechromatographed on cation-exchange HPLC.

(Figs. 6 and 8) or BSA-ascorbate (data not shown). The absorption spectrum of the lens fluorophore showed maxima that matched those of the synthetic compound. In addition, thin-layer chromatography (TLC) of purified LM-1 fractions from cataractous lenses and BSA-ribose on a silica gel plate (solvent system, butanol/acetic acid/water/pyridine in 4:2:2:2 ratio, run for 5.5 h) showed a blue fluorescent spot with the same R_F value (0.32) which, when mixed together, moved as a single spot suggesting that they are the same compound (Fig. 7). In cataractous lens, in addition to the fluorophore LM-1, a yellow-green fluorophore which was less polar than LM-1 was observed. The presence of this fluorophore interfered with absorption spectrum of LM-1 (between 250 and 320 nm, Fig. 6B). However, it did not interfere with the characteristic long wavelength fluorescence of LM-1. Effect of pH on fluorescence spectra showed almost complete quenching of fluorescence at pH 9.0, probably due to zwitterionic state of LM-1 (Fig. 8). A shift in excitation spectra at pH 11.0 from 366 to 385 nm suggests the presence of a hydroxyl group on the aromatic ring of the compound as previously observed for pyridinoline, a fluorescent crosslink of collagen [31]. LM-1 samples from both cataractous lenses and BSA-ribose showed similar fluorescence spectra at different pH values, which is an additional proof for their similarity. No effect on the level of LM-1 formation was observed when BSA-ribose was incubated in the absence of oxygen (data not shown), which indicated that the formation of LM-1 from ribose does not involve an oxidation step. Furthermore, sodium borohydride reduction of LM-1 isolated from BSA-ribose before or after acid hydrolysis revealed no change in fluorescence intensity or recovery, indicating that LM-1 is not an artifact from acid hydrolyzed Maillard product and that it does not contain reducible system of conjugated electrons (data not shown). Further, a 53% decrease in LM-1 formation

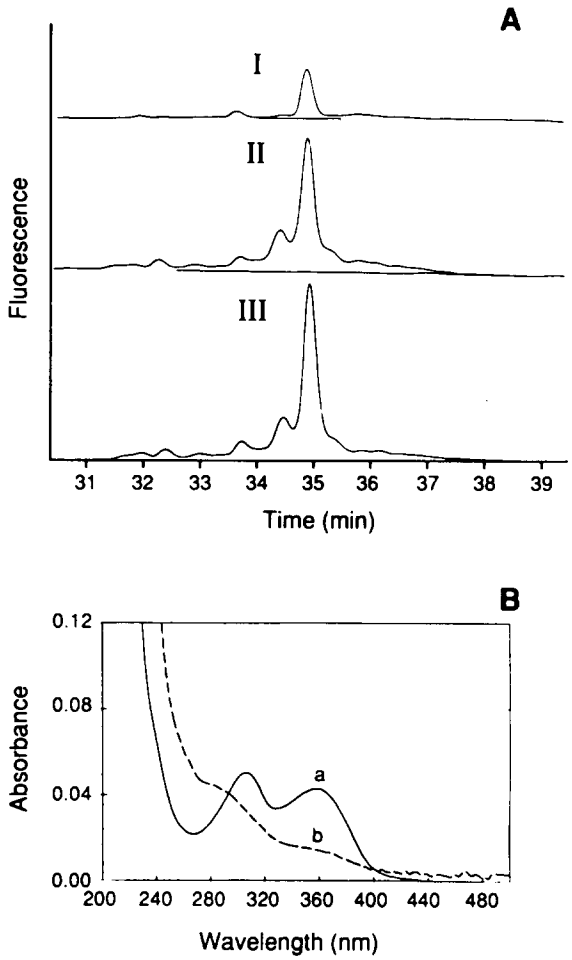

Fig. 6. (A) Rechromatography of LM-1 obtained from phosphoric acid on a C_{18} reverse-phase column using acetonitrile and water system as described in methods. I, cataractous lens; II, BSA incubated with ribose; and III, mixture of I and II. Chromatogram is expanded between 30–40 min. (B) Absorption spectrum of (a) purified LM-1 from BSA incubated with ribose and (b) from cataractous lens.

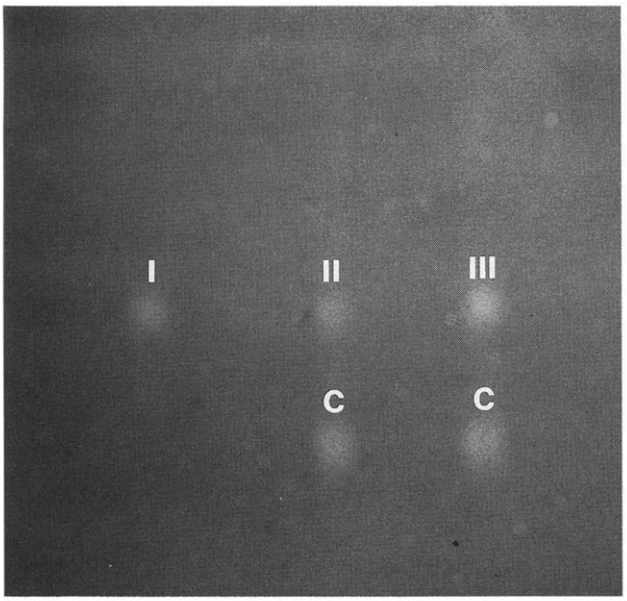

Fig. 7. Thin-layer chromatography of LM-1 on a silica plate using butanol/acetic acid/water/pyridine (4:2:2:2 ratio, run time 5.5 h). Fluorescent spots were visualized by long wavelength ultraviolet light. I, LM-1 from BSA-ribose; II, LM-1 from cataractous lens; III, mixture of I and II; C, green fluorescent compound which is a contaminant.

was observed when lysines on bovine serum albumin were modified up to 65% by reductive methylation prior to incubation with ribose. A concomitant decrease in fluorescence (370/440 nm) was also observed with reductively methylated bovine serum albumin (Fig. 9). These data strongly indicate that LM-1 formation occurs through Maillard-type of reaction.

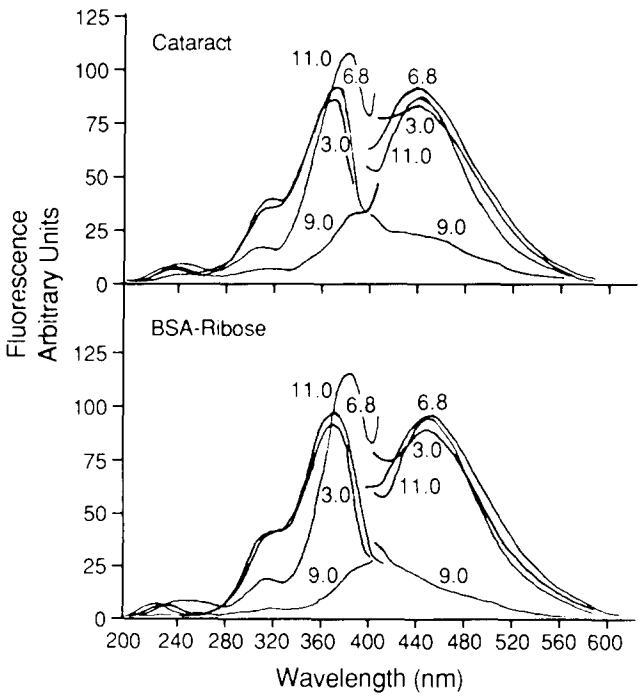

Fig. 8. Effect of pH on the fluorescence spectra of LM-1 purified from cataractous lens and BSA-ribose. Purified samples were taken in water and pH was adjusted with dilute HCl or NaOH. Excitation spectra were recorded at emission 440 nm and emission spectra were recorded at excitation 366 nm.

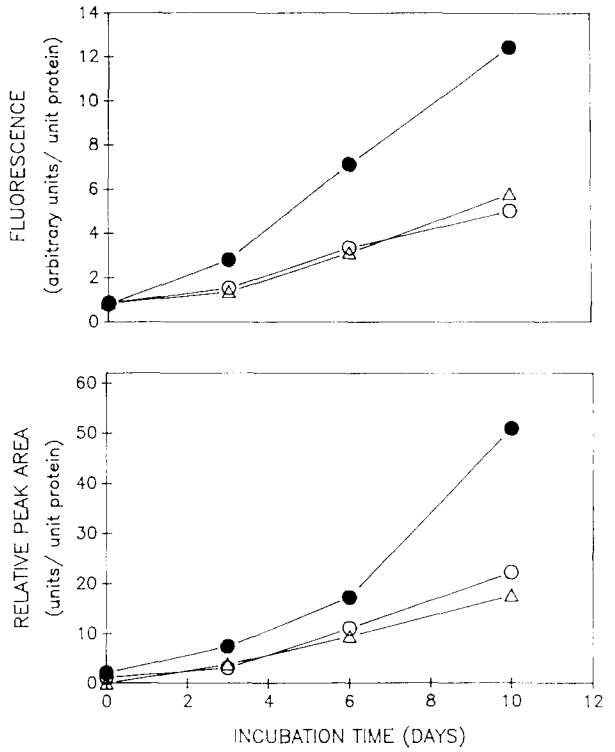

Fig. 9. Effect of reductive methylation of BSA on the fluorescence (370/440 nm, top panel) and LM-1 level (bottom panel) in ribose incubated samples. ●, control; ○, reductive methylated for 0.5 h; △, reductive methylated for 2 h.

Discussion

Increased pigmentation in lens crystallins accompanies an enhanced non-tryptophan fluorescence and high molecular protein formation suggesting a possible role for fluorophores in cataractogenesis. A systematic approach, initiated by exhaustive digestion with proteolytic enzymes and chromatographic steps led us to isolate the fluorophore LM-1. Although LM-1 was obtained under similar conditions as those for peak L_1 of Oimomi et al. [19], we did not detect LM-1 in BSA incubated with glucose unlike peak L_1. This observation may be due to the higher reactivity of ribose compared with glucose or fructose. Further, Liang and Rossi [32] have demonstrated that glucose and ribose react with crystallins to form Maillard compounds with similar fluorescence, although the rate of synthesis was slower with glucose, suggesting that autooxidation or fragmentation of hexoses into pentoses occurs. Since we have not tested for LM-1 formation in BSA incubated with glucose beyond 22 days, synthesis from glucose can not be ruled out. In fact recent work

showed that pentosidine can also be synthesized from glucose [20,33].

The linear increase of LM-1 in water insoluble crystallins and its overall higher levels in water soluble crystallins suggests that it accumulates in the water insoluble crystallins as a result of protein insolubilization during aging. This process seems to be accelerated in cataractous lenses, since higher levels of LM-1 were observed in highly pigmented cataractous lenses. However, the lower level in brunescent lenses is somewhat unexpected and points to possibility of involvement of other Maillard pathways since high levels of pentosidine were found in these lenses [22].

Characterization of LM-1 by spectroscopy suggests presence of a lysyl-pyridinium moiety as in pentosidine (data not shown). Since pentosidine could also be synthesized by incubation of crystallins with ribose and ascorbic acid [22], it is conceivable that the biosynthetic pathway of these compounds is related. It is likely that ascorbate undergoes fragmentation either to a 5-carbon compound either by decarboxylation or autooxidative degradation [34] before LM-1 synthesis. Thus, ascorbate emerges as a precursor not only of pentosidine but also LM-1. This may not be too unexpected since lens ascorbate concentrations are very high compared to other tissues [35] and can reach up to 2 mM and has been known to decrease in cataractogenesis [36]. Furthermore, Ortwerth and co-workers [37] have shown that ascorbate and its oxidative products like dehydroascorbate and 2,3-diketogulonate can undergo Maillard reaction with lens crystallins to form high mol wt proteins and non-tryptophan fluorescence. Thus, these data strongly suggest that ascorbate plays an important role in lens aging and cataractogenesis.

If ascorbate is such a potent Maillard reactant, how does the lens protect itself from its toxicity? In the lens ascorbate is mostly present in its reduced state which seems to be tightly regulated [36]. Rapid reduction of dehydroascorbate to ascorbate in the lens epithelium [38] supports this view. Any disturbance in the redox system of ascorbate might result in its conversion to dehydroascorbate or 2,3-diketogulonate which can lead to formation of LM-1. One of the most important defense systems against oxidation of ascorbate is probably the glutathione system. In lens aging and cataractogenesis, the concentration of reduced glutathione decreases [3] and the ability to restore glutathione diminishes because of a loss of glutathione reductase [39]. In addition, glutathione per se has been shown to prevent non-enzymatic glycation by ascorbate [40] and its autooxidation in vitro [41]. Recently an enzyme, ascorbate free radical reductase was isolated from human lens. The enzyme requires NADH for its activity and a loss of this enzyme was found be associated with an increase in protein insolubilization [42]. The ability of the enzyme to convert ASA free radical back to ascorbate might prevent the formation of LM-1 from dehydroascorbate or 2,3-diketogulonate. Alternatively, dehydroascorbate reductase, which has been described in liver and kidney [43,44] might be present in lens and keep ascorbate in its reduced form, thus, preventing the accumulation of dehydroascorbate. Breakdown of these defense systems might explain accelerated synthesis of LM-1 in cataractous lenses.

Fluorophore LM-1 does not appear to be a major contributor to lens pathology since it accounts for only 2% of total fluorescence (370/440 nm) in water insoluble fraction of cataractous lenses. Nevertheless, while purifying LM-1 from BSA-ribose or BSA-ascorbate incubated mixture, several fluorophores of different retention time but with almost similar absorption spectra were observed. Thus, LM-1 may be a member of a large family of structurally-related fluorophores, and its complete structure elucidation might help in understanding their synthesis by Maillard reaction and their role in aging and cataractogenic processes in lens and other tissues. Due to extensive protein crosslinking capability of ascorbate [23,24,40], and the finding in the present study that LM-1 could form from ascorbate and its oxidative products, estimation of LM-1 may serve as a useful marker for cumulative damage induced by ascorbate.

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