Properties of Partially Purified Esterase in Human Crystalline Lens and Variation in Its Enzyme Activity during Aging and with Advance of Senile Cataract

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Two fractions of esterase were partially purified from the soluble fraction of normal human lens. The apparent molecular masses of these enzymes were approximately 200 kDa (esterase-I) and 30 kDa (esterase-II). The optimal pH of esterase-I and esterase-II was 6.0 and 7.5, and their respective optimal temperature were 43 °C and 46 °C. The K_m values of esterase-I and esterase-II for 4-methylumbelliferyl-palmitate were 0.14 and 0.11 μM , respectively. The activity of these enzymes was inhibited by EDTA. The fraction with esterase activity also displayed lipase activity, although it is unknown whether the two enzymes are identical.

Variation in the activity of these esterases was examined as a function of age for normal lens and as functions of age and coloration for senile cataractous lenses. The normal lenses maintained high enzyme activity up to the 60 age group and their enzyme activity then fell abruptly. In the senile cataractous lenses, enzyme activity was very low as compared to that of normal lenses of similar age. This shows that cataract formation may have a deleterious effect on the catalytic activity of esterase in the lens.

Key words esterase; human lens; senile cataract; age; coloration

It is well known in ophthalmology that senile cataract is the main cause of blindness. Thus, many investigators have searched for the cause of cataract formation and attempted to clarify its mechanism. It was recently reported that arachidonic acid (AA) and its metabolites by the pathways of cyclooxygenase and lipoxygenase perturbed the normalcy of eye tissues and induced some damage.¹⁾ We reported earlier that AA and its metabolite induced cataract in an *in vitro* experiment.²⁾ However, no one yet knows whether AA comes from endogenous lens material or exogenous materials. I think it is necessary to investigate the properties of esterase, lipase and/or phospholipase in the crystalline lens in order to clarify the source of AA. Although those enzymes in various tissues of human and animals have been investigated by many researchers, there are very few reports concerning them in the crystalline lens. In 1971 Rana reported some enzymatic properties of carboxylesterase in the chick lens and its isozymes.³⁾ Then, Arnold *et al.* reported the existence of esterase in normal human lens and its characteristics using α -naphthyl acetate as a substrate.⁴⁾ The latter suggested that the esterase of mammalian tissues is ubiquitously distributed in tissues and has been shown to exhibit an extensive heterogeneity. I recently found the two types of esterases in normal human crystalline lens by using 4-methylumbelliferyl-palmitate as a substrate. To the best of my knowledge, this is the first event of two types of esterases being found in the mammalian lens.

This paper reports properties of the two partially purified two esterases (EC. 3.1.1.1) from the normal human lens. Variation in the esterase activity during aging and with advance of senile cataract was also reported.

MATERIALS AND METHODS

Human Lens Tissue Normal human and senile cataractous lenses were kindly donated by Dr. Horwitz,

Jules Stein Eye Institute, University of California, Los Angeles. Briefly, normal human lenses were obtained from autopsied eye that were removed 4 to 8 h following death. Upon removal, the eyes were stored at -80°C until use. Senile cataractous lenses were also obtained during routine cataract surgery at the same institute.

Materials 4-Methylumbelliferone (4-MU) and 4-methylumbelliferyl-palmitate (4-MU-palmitate) as a substrate for esterase were purchased from Wako Pure Chemical Industries, Japan. Other chemicals were analytical grade or highest purity available

Preparation of Partially Purified Esterase from Human Lens Ten normal human lenses with decapsuled (between 40 and 50 age groups) were quickly homogenized in 10 ml of 50 mM $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$, pH 7.0, in an ice bath and then centrifuged at $25000 \times g$ for 30 min at 4°C . The supernatant was applied on a Sephacryl S-300 superfine column (3.0×90 cm) with 150 mM $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$, pH 7.0, containing 150 mM NaCl. The fractions with esterase activity were pooled. Then, the sample was dialyzed against 1000 ml of 20 mM Tris-HCl buffer, pH 7.0, for 12 h, and the resulting solution in the dialysis bag was submitted to ion exchange chromatography on a DEAE-Sepharose CL6B column (2.0×7 cm) by linear gradient elution using 0 to 0.8 M NaCl in 20 mM Tris-HCl buffer, pH 7.0. Finally, the fraction with esterase activity was pooled and used as the partially purified enzyme.

Assay of Enzyme Activity The enzyme activity was assayed by using 4-MU-palmitate (10 μM at final concentration) solved in ethanol as substrate according to the method of Jiang *et al.*⁵⁾ Briefly, 50 μl of the substrate solution was mixed quickly with 200 μl of enzyme solution and 750 μl of 0.2 M sodium phosphate-0.1 M citric acid buffer solution, pH 7.0, and the mixture was incubated for 30 min at 37°C . After the reaction was done, the fluorescence at $\text{Em} = 450$ nm ($\text{Ex} = 329$ nm) of liberated 4-MU was measured immediately using a Shimadzu

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RF-5000 spectrofluorophotometer with free 4-MU as standard. One unit (U) of enzyme activity was defined as that amount causing transformation of $1.0\ \mu\text{mol}$ of substrate/min under optimal conditions of measurement. The specific activity was represented as mU/mg protein.

Determination of Optimal pH and Optimal Temperature

The optimal pH of the partially purified enzyme obtained from lenses of the 50 age group was measured in $0.2\ \text{M}$ sodium phosphate– $0.1\ \text{M}$ citric acid buffer with different pH in the range from pH 3.0 to pH 8.0 and $0.1\ \text{M}$ potassium phosphate– $0.05\ \text{M}$ sodium borate buffer, pH 8.5 and pH 9.0, under conditions of 30 min incubation at 38°C .

The optimal temperature was determined in $0.2\ \text{M}$ sodium phosphate– $0.1\ \text{M}$ citric acid buffer under conditions of 30 min incubation at respective optimal pH.

Protein Assay The protein concentration was measured by the method of Bradford.⁶⁾ Bovine serum albumin, $1.39\ \text{mg/ml}$, was used as a standard.

RESULTS

Partial Purification of Esterase from Human Lens First, the esterase separation was done from ten normal human lenses (between the 40 and 50 age groups) as follows. The soluble fraction of the human lens was gel filtration

chromatographed on a Sephacryl S-300 column (Fig. 1A), and two fractions, fraction-1 and fraction-2 with esterase activity were obtained. The molecular weight of these fractions was approximately 200 and $30\ \text{kDa}$, respectively, as estimated from the peak position of the gel filtration chromatogram. A gel filtration standard (Bio-Rad Laboratories, Inc.) was used for calibration of the gel filtration column. The two fractions were subsequently chromatographed on a DEAE-Sephacryl CL6B column (Figs. 1B-1 and 1B-2). Finally, the yield of partially purified esterase-I (from fraction-1) and esterase-II (from fraction-2) was $3.1\ \text{mg}$ (as protein) and $1.2\ \text{mg}$ from 10 human lenses, respectively. This fact shows that the overall purification was 80-fold with a yield of 18%, when calculated as the total activity.

Optimal pH, Optimal Temperature and Heat Stability

The respective optimal pH of esterase-I and esterase-II on 4-MU-palmitate was 6.0 and 7.5, respectively (Fig. 2A). The optimal temperatures of the two enzymes were 43 and 46°C , respectively (Fig. 2B); this fact shows that properties of two enzymes were slightly different. Furthermore, their heat stability was determined; both showed high enzyme activity up to 50°C for 1 h, but thereafter the activity suddenly decreased and was almost undetectable at 60°C or above. Esterase-I was slightly unstable as compared to esterase-II (data not shown).

Fig. 1. Elution Patterns by Sephacryl S-300 Gel Filtration Chromatography (A) and by DEAE-Sephacryl CL6B Ion-Exchange Chromatography (B-1 and B-2)

The details of column chromatography are given in Materials and Methods. Enzyme activity was measured with 4-MU-palmitate as the substrate as also described in Materials and Methods. ■, enzyme activity; ●, absorbance at 280 nm. (A) Sephacryl S-300 column chromatogram of soluble fraction of normal human lens. Fractions 25–27 and fractions 31–33 with esterase activity were collected as fraction-1 and fraction-2, respectively. (B-1 and B-2) DEAE-Sephacryl CL6B Ion-exchange column chromatograms of fraction-1 and fraction-2. Fractions 56 and 57, and fractions 52–54 with esterase activity were pooled as esterase-I and esterase-II, respectively.

Fig. 2. Optimal pH and Optimal Temperature of Esterase-I and Esterase-II

Details of the determination for optimal pH and optimal temperature of two enzymes are given in Materials and Methods. This experiment was done using the lenses between the 40 and 50 age groups. Each dot of various pH and temperatures represents the mean of two lenses. —●—, esterase-I; ---■---, esterase-II. The enzyme activity was measured in 0.2 M phosphate-0.1 M citric acid (pH 3.0–8.0) and 0.1 M potassium phosphate-0.05 M borate buffer (pH 8.5 and 9.0). Other conditions are given in Materials and Methods.

Fig. 3. Variations in Specific Activity of Esterase in Normal Human Lens during Age and in Senile Cataractous Lenses during Age and with Advance of Coloration

Details of the determination method for specific activity of esterase are described in Materials and Methods. Each dot represents the mean of two lenses of similar age. (A) esterase-I; (B) esterase-II. ○, normal human lens; ●, white senile cataractous lens; ■, yellow senile cataractous lens; ▲, brown senile cataractous lens. I could not obtain senile cataractous lenses below the 30 age group (for white cataractous lens), below the 40 age group (for yellow cataractous lens) or below the 60 age group (for brown lens).

Specific Activity and Michaeli's Constant of Esterase-I and Esterase-II for 4-MU-palmitate First, the specific activities of esterase-I and esterase-II for 4-MU-palmitate were determined under respective optimal conditions: they were 0.16 and 0.06 mU/mg protein, respectively. Then, the substrate dependence on activity was determined. The K_m values estimated from the double reciprocal plots and V_{max} values of esterase-I and esterase-II were 0.14 and 0.11 μ M, and 1.02 and 1.23 nmol/min, respectively.

Variation in Enzyme Activity in Normal Human Lenses in Aging and in Senile Cataractous Lenses with Various Stages of Cataract Related to Age and Coloration In this experiment, the crude enzyme that was separated only by gel filtration chromatography on a Sephacryl S-300 column was used to determine the variation in the specific activity of esterase-I and esterase-II in normal lenses as a function of age and in senile cataractous

lenses both as a function of age and with the advance of cataract, coloration from white to brown (Figs. 3A and B). In the normal lenses, the esterase-I activity was the maximum level between the 40 and 50 age groups, and then it fell gradually to the 70 age group; thereafter enzyme activity dropped abruptly with age. The esterase-II activity fell gradually with age up to the 60 age group, then went down abruptly as shown in Fig. 3B. Concerning senile cataractous lenses, the two enzymes showed similar variation in their esterase activity. Briefly, that in the white senile cataractous lens was approximately one-fourth or less that in normal lens of similar age between the 40 and the 70, and the activity decreased gradually with age. The esterase activity in yellow senile cataractous lenses was very low, approximately one-tenth that in normal lens even in the 50 age group; in the over the 70, the activity was scarcely detectable. Esterase activity in the brown lens

in contrast, was undetectable in all age groups available. This fact shows that the advance of cataract may inhibit the esterase activity in the lens.

DISCUSSION

The current study demonstrates that in the normal human lens two types of esterase are active, although they have different physical properties of optimal pH, optimal temperature and K_m values, as examined in the present study. The enzyme activity showed maximum level in the lenses between 40 and 50-years of age. At over 70-years of age their enzyme activity decreased suddenly and in the 80 age group the activity was only slightly detectable. The enzyme activities of esterase-I and esterase-II were inhibited by EDTA (approximately 50% inhibition). This fact suggests that esterase-I and esterase-II may need at least bivalent metal ion. I also found lipase activity in the same fraction with esterase activity. However, in this connection, no further investigation were carried out in this study, and in my next study I will answer this by using a purified enzyme.

The activity of several types of enzymes in the lens reportedly reduced with aging and this phenomenon was generally observed in the lens at 50- or 60-years of age.⁷⁾ These findings suggest that the aging process over 50-years of age or above may suppress some enzymatic activity in the lens, although this phenomenon may be one of the symptoms of aging.

Arnold *et al.*⁴⁾ reported the existence of esterase in normal human lens in 1974 and were first to characterized the enzyme. At that time, they investigated only as total enzyme activity. As the result of the detailed analysis in the present study, I found two types of esterase in normal human lens and examined their properties. Compared with the esterase reported by Arnold *et al.*,⁴⁾ the esterase I found was a remarkable different in K_m value. Although this may be due to the difference in substrate, I have no other explanation for this discrepancy. To the best of my knowledge, this is the first report of the existence of two types of esterase in the human lens and of correlation between the variation in esterase activity in the lens and aging or coloration of senile cataractous lenses.

In the senile cataractous lens, the esterase activity was much lower than that in the normal lens. Furthermore, the present study demonstrated that the esterase activity in the deeper colored lens was inhibited strongly as compared to the white lens. Similar phenomena are observed in some enzymes in the lens.⁸⁾ Generally speaking, it is well known that the activities of various enzymes decrease with cataract progression. From these facts, I confirmed that cataract formation may have a deleterious effect on the enzyme action of esterase in the lens.

There are many reports concerning esterase in various human tissues. It was reported that the physiological role of the enzyme is remarkably diverse, for example deamidation,⁹⁾ bile-salt-stimulation,¹⁰⁾ pancreatic fatty

acid ethyl ester syntheses,¹¹⁾ and others. The physiological role of esterase in the lens is not yet clear. However, the present study suggests the relationship between lowering of esterase in the lens and senile cataract formation as follows. AA and/or its metabolites have been suspected to be one of the major triggers of senile cataract formation,^{1,2)} hence, many investigators have tried to elucidate whether AA comes from endogenous or exogenous lens materials. No one has been able to answer this so far however. I believe that the current experiment gives us a hint, indicating that AA may come from exogenous lens material. The reason for this is that the esterase or lipase activity in the human lens decreases and becomes very low with age and cataract progression, and that such reduction in enzyme activity may render impossible the enzymatic release of AA from endogenous material and allow progression of senile cataract. I feel the role of the esterase in the human lens should be investigated further to determine the mechanisms of senile cataract.

Finally, I must to mention that the data on each group in the present study is the mean of only two or three lenses, because it is very difficult to obtain normal and cataractous human lenses of identified age. I know that biochemical changes in human lenses reflect a great deal of individual variation. However, I firmly believe that these data are useful for further research on senile cataract formation.

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