NADH-dependent Dehydroascorbate Reductase in the Rabbit Lens

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The present investigation demonstrates the existence of NADH-dependent dehydroascorbate (DHA) reductase activity in the soluble fraction of the rabbit lens. This DHA reductase was specific for NADH, and its apparent K_m values for DHA and NADH were 5.7 mM and 4.0 μM, respectively. In a gel filtration of the lens soluble fraction on a Sephadex G-75 superfine column, the NADH-dependent DHA reductase activity was eluted at the oligomeric β_{L1}-crystallin fraction, which may also contain λ-crystallin (a rabbit-specific crystallin). Furthermore, about 80% of protein fractions separated from the β_{L1}-crystallin fraction by DEAE-cellulose ion-exchange column chromatography exhibited DHA reductase activity. In the SDS-PAGE analysis of the protein fractions with DHA reductase activity, 32–33, 27 and 25 kDa protein subunits were commonly identified. These results suggest that oligomers of β-crystallin and/or λ-crystallin subunits may be associated with the DHA reductase activity. The present paper also discusses that the function of the reductase may be to enhance the antiphotoxidation capacity of the lens.

Key words : Rabbit lens, Dehydroascorbate reductase, NADH-specific, Enzyme/Crystallin, Antiphotoxidation

INTRODUCTION

The eye lens, a transparent organ, is maintained in a very high reductive state, and also characterized by the presence of very high concentrations of soluble structural proteins called crystallins (α , β and γ-crystallins, etc.) [7]. Oxidation of the lens constituents is an early event in the development of cataracts and, through this oxidation, lens insoluble protein aggregates are formed [20]. The lens is usually protected from oxidative stress by the antioxidation systems such as ascorbate redox cycle [1] and glutathione redox cycle [16]. Therefore, the lens oxidation may be closely associated with a decrease in the activities of the antioxidation systems.

The lens of diurnal animals including human contains ascorbate at high concentrations, in excess of 1 mM [8, 24]. It has been reported that ascorbate reduces oxidants and free radicals very effectively in vitro [4], and protects the rat lens from oxidants in organ culture [23]. Vitamin C-deficient guinea pig develops cataracts following injection of various quinoids [14], and dietary ascorbate appears to decrease ultraviolet (UV) light-induced damage of lens proteins in guinea pig [5]. The high ascorbate concentration may be beneficial in protecting the lens against oxidative and photoxidative damage if its high level is maintained. In addition, oxidation products of ascorbate are supposed to be cataractogenic, since it has been reported that its oxidation products cause crosslinking of bovine lens proteins [13], and that opacity occurs in the rabbit lens exposed with a high concentration (1 mM) of dehydroascorbate (DHA), an oxidized form of ascorbate [17]. From these facts, it is suggested that ascorbate regeneration is essential for the lens transparency. When ascorbate is oxidized, ascorbate free radical

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(AFR) is first formed, and then DHA, which decomposes rapidly at physiological pH, is generated spontaneously by dismutation of AFR [4, 21]. In the lens, ascorbate is regenerated from AFR by NADH-dependent AFR reductase [1, 11], and also from DHA by non-enzymatic reaction with sulphydryl groups such as glutathione and the like [26]. Glutathione or NADPH-dependent DHA reductase activity is detected in various animal tissues [6, 25], but not in the lens.

The present investigation was undertaken to investigate for the potential existence of NADH-dependent DHA reductase in the rabbit lens soluble fraction. This enzyme activity was found in the β_{L1}-crystallin fraction. The relation of the DHA reductase activity to β_{L1}-crystallins (oligomeric forms of β-crystallin [18]) and its function in the lens are also discussed. So far as we know, the present paper is the first report of NADH-specific DHA reductase activity in tissues.

MATERIALS AND METHODS

Materials and Chemicals

Rabbit lenses were obtained from freshly enucleated eyes of Japanese albino rabbits (about 6‐20 months old) sacrificed with overdoses of anesthetics. The lenses were weighed, and kept frozen at $-80\,℃$ until used. All animal procedures were in accordance with the ARVO resolution on animals and opthalmic research.

The lenses were homogenized in ten times their weight of 0.1 M KCl, 10 mM K-phosphate, pH 7.2 in a glass homogenizer in ice, and the soluble fraction was separated from the insoluble fraction by centrifugation at 15,000 g for 1 hour at $4\,℃$.

Sephadex G-75 superfine was obtained from Pharmacia Biotechnology (Uppsala, Sweden), and DEAE-cellulose (DE52) from Whatman International Ltd. (Kent, England). Dehydroascorbic acid was purchased from Aldrich Chemical Company (Milwaukee, USA), NADH (disodium salt) from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and NADPH from Oriental Yeast Company, Ltd. (Tokyo, Japan). Other reagents and chemicals used were of the purest grade available from commercial sources.

Assay for DHA Reductase Activity

DHA reductase activity was determined at $27\,℃$ by a conventional spectrophotometer, measuring the decrease in absorbance at 340 nm due to NADH oxidation in the presence of DHA. The reaction mixture contained 1 ml of 50 mM K-phosphate, pH 7.2 plus lens extracts ($25‐500\,\mu$l), $10\,\mu$l of 10 mM NADH and $10\,\mu$l of 50 mM DHA. Reactions were started by the addition of DHA. Control experiments were performed with omission of DHA or lens extracts. In some experiments, the concentrations of NADH or DHA were varied, and NADH was replaced by NADPH. The reaction rate, expressed in nmol NAD (P) H oxidized/min, was estimated by employing 6.2×10^3 $M^{-1}cm^{-1}$ as the molar extinction coefficient of NADH and NADPH in phosphate buffer at 340 nm, as described previously [1]. Protein concentration was assayed by the bicinchoninic acid (BCA) method [19] using bovine serum albumin as the standard.

Column Chromatography

All chromatography was carried out at 0 ‐4℃. The soluble fraction of the rabbit lens was subjected to a Sephadex G-75 superfine column (2.6 cm in diameter $\times$ 95 cm in length) equilibrated with 0.1 M KCl, 10 mM K-phosphate, pH 7.2. Fractions were collected in 5 ml aliquotes at a flow rate of about 15 ml/hr, and DHA reductase activity and absorbance at 280 nm of the eluates were measured. The fractions containing DHA reductase activity were pooled, concentrated to about 5 ml by ultrafiltration, and dialyzed twice against 500 ml of 10 mM K-phosphate, pH 7.2. The prepared enzyme concentrate was subjected to a DEAE-cellulose column (1.6 cm in diameter $\times$ 34 cm in length) equilibrated with 10 mM K-phosphate buffer. The column was washed with 100 ml of 10 mM K-phosphate buffer at a flow rate of about 30 ml/hr, and then eluted with 400 ml of a linear gradient from 0 to 0.4 M KCl in the same buffer. The eluted fractions collected in 5 ml aliquotes were measured for DHA reductase activity, absorbance at 280 nm, and KCl concentration. KCl concentration was determined by assaying potassium with flame photometry.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

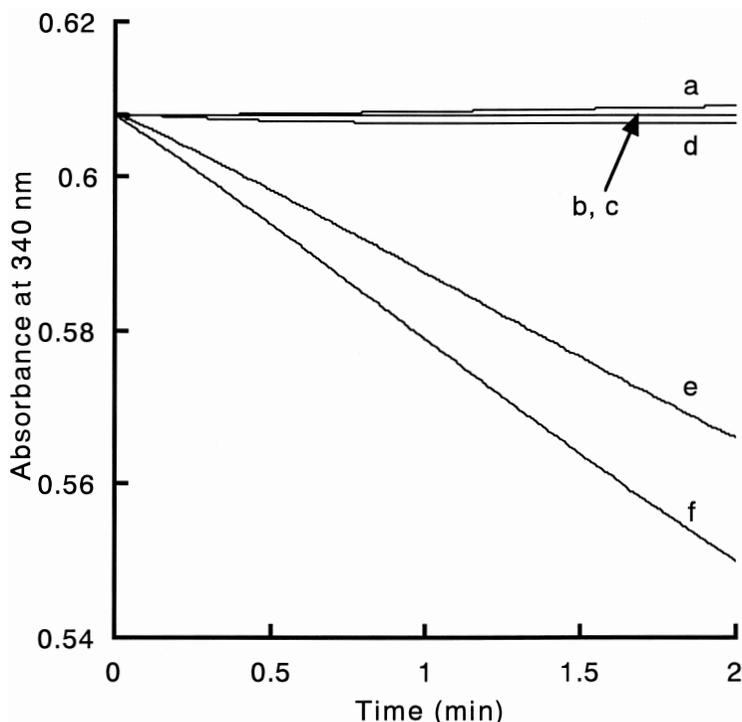Electrophoresis was run at room temperature. SDS-PAGE of the active fractions isolat-

Fig. 1 NADH oxidation catalyzed by the rabbit lens soluble fraction in the presence of DHA.
The reaction mixtures (a final volume of 1.02 ml) in 50 mM K-phosphate, pH 7.2, contained 100 μM NADH, and either: (a) 500 μM DHA; (b) no addition of DHA and the soluble fraction; (c) 25 μl of the soluble fraction; (d) 50 μl of the soluble fraction; (e) 25 μl of the soluble fraction +500 μM DHA; (f) 50 μl of the soluble fraction +500 μM DHA. Protein concentration of the soluble fraction was 30.4 mg/ml.

ed by the ion-exchange column chromatography was carried out on slab gels of 5–20% acrylamide according to the method of Laemmli [10], and the gels was stained with Coomassie Brilliant Blue.

RESULTS

DHA Reductase Activity in the Rabbit Lens Soluble Fraction

NADH oxidation was catalyzed by the soluble fraction of the rabbit lens in the presence of DHA, as shown in Fig. 1. The NADH oxidation rate was proportional to the amount of the lens soluble fraction. This NADH oxidation was observed using either authentic DHA or DHA prepared from ascorbate oxidation by ascorbate oxidase. In this investigation, the authentic DHA compound was used for the analysis of DHA reductase activity, since the preparation of high concentrations of DHA solution was simple. The NADH-dependent DHA reductase activity was not detected in the soluble fractions of the lenses of bullfrog, guinea pig (Hartley), rat (Wistar), swine, bovine and human (data not shown).

Kinetic Properties and NADH Specificity of the Rabbit Lens DHA Reductase Activity

Table 1 shows that DHA reductase activity in the rabbit lens soluble fraction was specific for NADH. Replacement of NADH by NADPH resulted in a 93% decrease in the activity. The apparent K_m values of the DHA reductase activity for NADH and DHA were calculated to be 4.0 μM and 5.7 mM, respectively, as shown in the same table.

Table 1 Kinetic constants and NADH specificity of DHA reductase activity in the rabbit lens soluble fraction.

Apparent K_m for NADH	4.0 μM
Apparent K_m for DHA	5.7 mM
Activity ratio (NADPH/NADH)	0.07

The K_m values for NADH and DHA were derived from Lineweaver-Burk plotting of the DHA reductase activity at 500 μM DHA and 100 μM NADH, respectively. The activity ratio was calculated from the means of three experiments replacing 100 μM NADH with 100 μM NADPH.
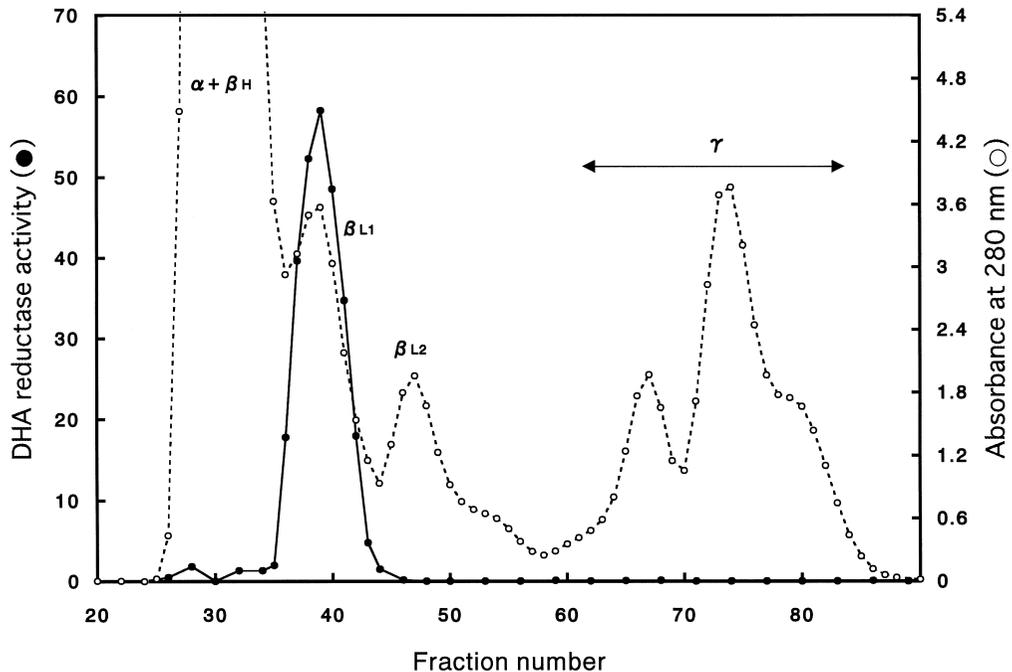

Fig. 2 Gel filtration of the rabbit lens soluble fraction on a Sephadex G-75 superfine column (2.6 ϕ × 95 cm).
Soluble fraction containing 516 mg protein was subjected to the column. DHA reductase activity (●) and absorbance (○) at 280 nm were measured in the eluates. $\alpha + \beta_H$, β_{L1}, β_{L2} and γ : eluting positions of lens specific crystallins.

Separation of the Rabbit Lens DHA Reductase Activity by Column Chromatography

Figure 2 shows that the rabbit lens DHA reductase activity was eluted at the β_{L1}-crystallin fraction by a gel filtration of the soluble fraction on a Sephadex G-75 superfine column. The reductase activity was little found in any other fractions.

For the further isolation of the DHA reductase, the β_{L1}-crystallin fraction with DHA reductase activity was subsequently applied to DEAE-cellulose ion-exchange column chromatography. The elution profiles of DHA reductase activities and absorbances at 280 nm are shown in Fig. 3. Most of protein fractions separated by the ion-exchange column chromatography exhibited DHA reductase activity. Only two protein fractions, which might be more acidic, had no DHA reductase activity. The protein fractions with DHA reductase activity are estimated to be about 80% of the β_{L1}-crystallin fraction and about 10% of total protein by a rough calculation based on the values of absorbance at 280 nm. In the SDS-PAGE analysis of Fractions I–IV (marked in
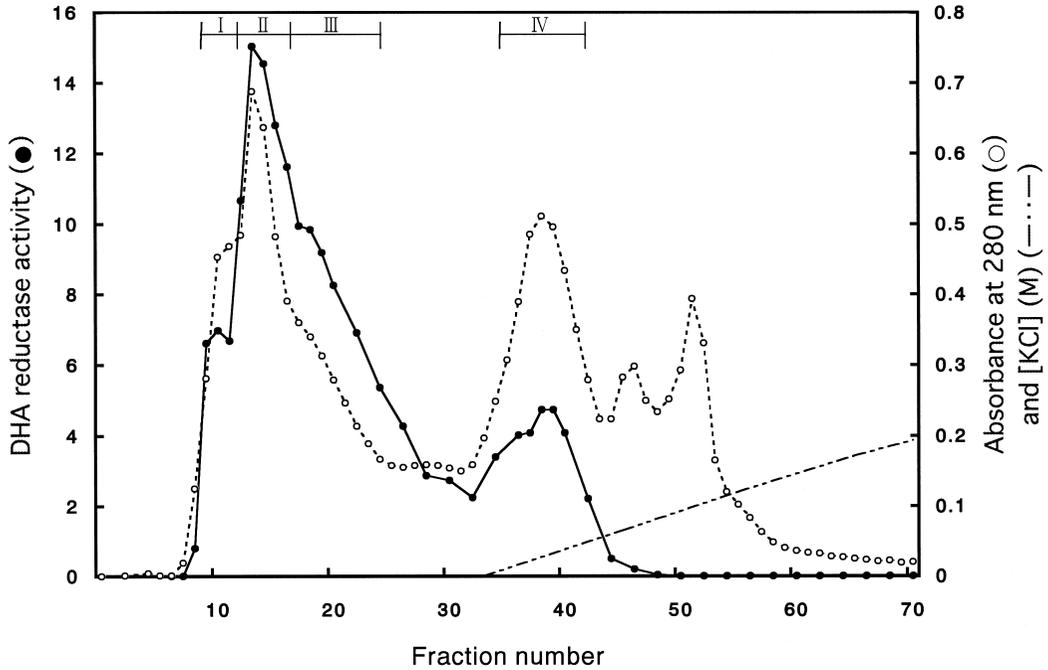
Fig. 3 DEAE-cellulose ion-exchange column (1.6 ϕ × 34 cm) chromatography of the rabbit lens β_{L1}-crystallin fraction with DHA reductase activity shown in Fig. 2.
The β_{L1}-crystallin fraction containing 52 mg protein was applied to the column. DHA reductase activity (●), absorbance (○) at 280 nm and KCl concentration (– – – –) were measured.
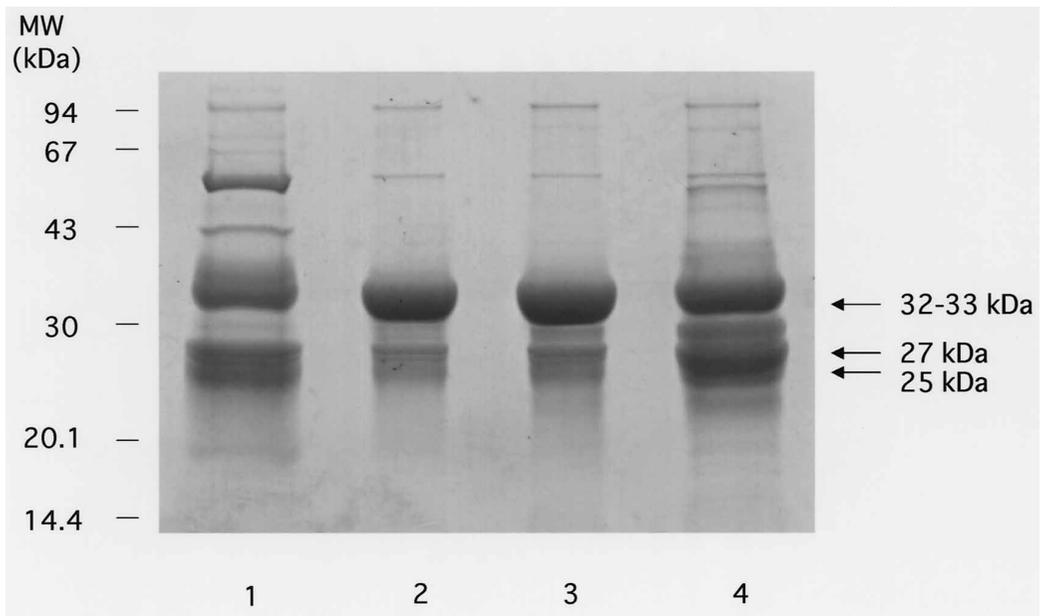

Fig. 4 SDS-PAGE of DHA reductase fractions, Fractions I–IV marked in Fig. 3, separated by DEAE-cellulose ion-exchange column chromatography of the rabbit lens β_{L1}-crystallin fraction.
Lane 1: Fraction I (13 μg protein); Lane 2: Fraction II (8 μg protein); Lane 3: Fraction III (13 μg protein); Lane 4: Fraction IV (20 μg protein). The gel was stained for protein with Coomassie Brilliant Blue.

Table 2 Vertebrate taxon-specific crystallins related to NAD(P)H/NAD(P)$^+$-dependent enzymes (Quoted from Wistow (1995) [27])

Crystallin	Related enzyme	Activity	Cofactor	Species
ε	lactate dehydrogenase (LDH) B	LDH activity	NADH	birds, crocodiles
ζ	NADPH: quinone oxidoreductase	quinone reductase activity	NADPH	guinea pig, camels, hystricomorphs
η	aldehyde dehydrogenase	retinal dehydrogenase activity	NAD$^+$	elephant shrews
λ	hydroxyacyl-CoA dehydrogenase	?	NADH?	rabbits, hares
μ	ornithine cyclodeaminase glutamyl-tRNA reductase	?	NADPH	marsupials
π	glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	GAPDH activity	NAD$^+$	geckos (*Phelsuma*)
ρ	aldo-keto reductase	?	NADPH	frogs (*Rana*)

Fig. 3) containing the DHA reductase activity separated by the ion-exchange column chromatography, 32–33, 27 and 25 kDa protein subunits were commonly identified, as shown in Fig. 4. 32–33 kDa protein may be a subunit of λ-crystallin [12, 30], and 27 and 25 kDa proteins may be β-crystallin subunits [18, 30]. Thus, it is suggested that oligomers of β- and/or λ-crystallin subunits are associated with the NADH-dependent DHA reductase activity.

DISCUSSION

Thioltransferase (glutaredoxin) (a cytosolic enzyme) and protein disulfide isomerase (a microsomal enzyme) have been reported to have glutathione-dependent DHA reductase activity in mammalian tissues such as liver, thymus and placenta [25]. Thioltransferase has been also found in bovine ocular tissues including the lens [28], although its DHA reductase activity has not yet been determined in the ocular tissues. In addition, it has been identified in the rat liver cytosol that 3-a-hydroxysteroid dehydrogenase has NADPH-dependent DHA reductase activity [6]. In the present investigation, we first found NADH-specific DHA reductase activity in the soluble fraction of the rabbit lens, whereas no NADH-dependent reductase activity was detected in the lenses of bullfrog, guinea pig, rat, swine, bovine and human. The apparent Km of

this enzyme activity for DHA (Table 1) is at a comparable level to those [6, 25] of the above glutathione and NADPH-dependent DHA reductase activities.

Since the lenses of rabbit as well as other animals contain glutathione at a high concentration of 2–10 mM [9], DHA in the lenses can be usually reduced nonenzymatically to ascorbate by glutathione [26]. NADH-dependent DHA reductase may be involved in a peculiar function in the rabbit lens. The human lens contains a particular near UV light absorbing compound, 3-hydroxykynurenine glucoside at a high concentration of 1–2 mM [22], and the lenses of animals such as bullfrog, guinea pig and rabbit seem to use a high concentration of reduced pyridine nucleotide, NADH or NADPH as a near UV light filter [29]. The high levels of pyridine nucleotide in the lenses seem to be achieved by its binding to enzyme/crystallins. As shown in Table 2, various taxon-specific crystallins are discovered in the vertebrate lenses to be related to NAD(P)H/NAD(P)$^+$-dependent enzymes [27]. Our recent investigation [11] has shown that the soluble fraction of the bullfrog, guinea pig, rabbit and human lenses with the function of a near UV light filter contains much higher AFR reductase activity than that of the rat, swine and bovine lenses lacking such function, and suggested that the soluble AFR reductase activity enhances

the antiphotoxidation capacity of ascorbate through the rapid and efficient ascorbate regeneration. But, the soluble AFR reductase activity in the rabbit and guinea pig lenses is about one-third that in the bullfrog and human lenses [2, 3, 11], and the rate of ascorbate regeneration by AFR reductase is thought to be slower in the lens of the former species than of the latter species. It has been reported that in the guinea pig lens, ζ-crystallin (about 10% of total protein, a taxon-specific enzyme/crystallin) is an active NADPH-depedent quinone reductase [15]. A mutation in the gene for this enzyme/crystallin is associated with an autosomal dominant congenital cataract in guinea pig, and the mutant crystallin fails to bind coenzyme and appears to lack this quinone reductase activity [15]. Therefore, NADH-dependent DHA reductase in the rabbit lens and NADPH-dependent quinone reductase in the guinea pig lens are presumed to be necessary in addition to the slower ascorbate regeneration by AFR reductase, in order to protect the lens from the photoxidation and to regulate the high level of reduced pyridine nucleotide in the lenses. However, further investigation is needed to elucidate the true antiphotoxidative role of the DHA reductase and quinone reductase in the animal lenses.

β-crystallins are a major class of lens proteins made up of several related basic and acidic subunits, and form dimers (β_{L2}), oligomers (β_{L1}) and higher aggregates (β_H) [18, 30]. Our present study shows that the rabbit lens NADH-dependent DHA reductase activity is found in the oligomeric β_{L1}-crystallin fraction (Fig. 2). This fraction had little AFR reductase activity, which was detected eluting just after the peak of β_{L2}-crystallin fraction (our unpublished observation). In Fig. 3 of the present study, most of protein fractions separated from the rabbit lens β_{L1}-crystallin fraction by the anion-exchange column chromatography are observed to exhibit the DHA reductase activity. The protein fractions with DHA reductase activity are estimated to be about 10% of total protein , as mentioned in Results. 32–33, 27 and 25 kDa protein subunits are commonly identified from the separated DHA reductase fractions (Fig. 4). These results suggest that the rabbit lens DHA reductase may be one of enzyme/crystallins.

Since the 27 and 25 kDa proteins may be β-crystallin subunits [18, 30], oligomeric β-crystallins may be associated with the DHA reductase activity. However, even if this hypothesis holds true, some unidentified cofactor(s) may be necessary for the enzyme activity, because the enzyme activity is not detected in the lens of animals other than the rabbit, nor in β_H- and β_{L2}-crystallin fractions of the rabbit lens. The rabbit lens β_{L1}-crystallin fraction may also contain λ-crystallin (4–8% of total lens protein, a rabbit-specific enzyme/crystallin), and the 32–33 kDa protein identified in the present study may be its crystallin subunit [12, 30]. It has been reported that λ-crystallin shows 30% homology to hydroxyacyl-CoA dehydrogenase and contains a putative NADH-binding site [12], but its enzyme activity is as yet unidentified. The NADH-dependent DHA reductase activity may be also associated with oligomeric λ-crystallin. Thus, it is of interest whether β-crystallin forms oligomers with λ-crystallin and this molecular association brings about the DHA reductase activity. These possibilities will be examined in the near future through a further purification of the DHA reductase.

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