Published in final edited form as:

Protein Expr Purif. 2010 February ; 69(2): 147–152. doi:10.1016/j.pep.2009.08.001.

Expression and Purification of his-tagged Recombinant Mouse ζ -Crystallin

Mukoma F. Simpanya, Victor R. Leverenz, and Frank J. Giblin

Eye Research Institute, Oakland University, Rochester, MI

Abstract

ζ -crystallin is an NADPH-binding protein consisting of four identical 35 kD subunits. The protein possesses quinone oxidoreductase activity, and is present in large amounts in the lenses of camelids, certain hystricomorphic rodents, and the Japanese tree frog, and in lower catalytic amounts in certain tissues of various species. In this study, recombinant methods were used to produce substantial quantities of his-tagged recombinant mouse ζ -crystallin, which was then purified to homogeneity. The yield of pure recombinant mouse ζ -crystallin was 5 times that obtained previously for purification of recombinant guinea pig ζ -crystallin. The quinone oxidoreductase activity of purified his-tagged recombinant mouse ζ -crystallin was comparable to that of purified native guinea pig lens ζ -crystallin, and to that previously reported for recombinant guinea pig ζ -crystallin. The method permits production of substantial amounts of recombinant ζ -crystallin for conducting studies on the biological role of this interesting protein, which exists in such high concentration in the lenses of certain species.

Keywords

Protein expression; purification; his-tagged recombinant mouse ζ -cystallin; *E. coli* BL21-DE3; ion exchange; affinity chromatography; guinea pig lens

INTRODUCTION

ζ -crystallin is an NADPH-binding protein existing as a homotetramer of 35 kD subunits. The protein is contained in major amounts in the lenses of camelids, certain hystricomorphic rodents and the Japanese tree frog [1–3]. It is also present in lower, catalytic amounts in the lens and other tissues of various species, including human [4,5]. ζ -crystallin comprises 10% of the total protein of the lens of the guinea pig [6], a species that possesses two promoters for ζ -crystallin, one for high level expression in the lens, and the other for expression at enzymatic amounts in other tissues such as the liver [7]. Similar to other taxon-specific lens crystallins such as ϵ -, δ - and τ -crystallins, ζ -crystallin possesses enzyme activity, as an NADPH-dependent oxidoreductase with quinone oxidoreductase activity [6,8]. The physiological significance of ζ -crystallin and its quinone oxidoreductase activity is unclear, particularly in the lens. A mutation in the guinea pig ζ -crystallin gene has been linked with congenital cataract [9].

*Corresponding Author: Frank J. Giblin, Ph.D., Corresponding Author's Institution: Oakland University, Address: Eye Research Institute, 409 Dodge Hall, Rochester, Michigan 48309-4480, giblin@oakland.edu (Frank J. Giblin), Corresponding Author telephone: (248) 370-2395, Corresponding Author fax number: (248) 370-4322.

Disclosures: MF Simpanya, none; VR Leverenz, none; FJ Giblin, none

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Possible physiological roles for ζ -crystallin include protection against oxidative stress [10], binding of RNA [11] and single-stranded DNA [12,13], and as a pH response element-binding protein [14].

Our laboratory has found that chronic exposure of guinea pigs to UVA light produces detrimental effects in the central region of the lenses of the animals including increased light scattering, disulfide crosslinking of proteins and high molecular weight aggregate formation [15,16]. We hypothesize that these effects are caused by absorption of UVA light in the guinea pig lens by NADPH bound to ζ -crystallin, leading to the generation of reactive oxygen species (ROS) such as superoxide anion and H_2O_2 . By absorbing UVA light in the lens, ζ -crystallin may act to protect the guinea pig retina, as well as retinas of the camel and the Japanese tree frog [2,3], from UVA-induced damage. Indeed, retinas of guinea pigs that have been made aphakic have been reported to be damaged by physiological levels of UVA light [17]. Similar events may occur in the aging human lens where the binding of the UVA chromophore kynurenine to crystallins may lead to subsequent generation of damaging ROS [18].

The purpose of the present study was to employ recombinant methods to produce substantial amounts of ζ -crystallin which could later be used for *in vitro* investigation of mechanisms of UVA-induced damage in the lens. ζ -crystallin has been cloned previously from cDNA libraries of guinea pig lens epithelial cells [19], guinea pig lenses [20], guinea pig and mouse liver [21] and human liver [5]. Other investigators have found that attempts to express recombinant guinea pig ζ -crystallin in *E. coli* resulted in low yields since the expressed protein deposited mainly into inclusion bodies, an occurrence that was overcome by coexpression of the molecular chaperone GroEL/ES [20]. In the current study, his-tagged recombinant mouse ζ -crystallin was overexpressed in *E. coli* in substantial amounts without the need for coexpression of a chaperone, and the protein was purified to homogeneity. The purified his-tagged recombinant mouse ζ -crystallin was found to have the same quinone oxidoreductase activity as that for purified native guinea pig lens ζ -crystallin.

MATERIALS AND METHODS

Construction of a ζ -crystallin bacterial expression vector

A mouse ζ -crystallin bacterial expression vector was produced by Dr. Norman Curthoys, University of Colorado (unpublished methods of A. Tang and N.P. Curthoys), and provided as a gift. To produce the vector, a *BSSH* II fragment of plasmid pMLR107 containing a full-length cDNA of mouse ζ -crystallin [21] was used as the template for a polymerase chain reaction (PCR). The following two primers were synthesized to amplify the entire coding sequence and to introduce an *Nde* I site at the 5' end and a *Bgl* II site at the 3' end (underlined and in bold),

1. 5'-GCCATATGGCAACTGGGCAGAAAGTTGATGAGG-3' and
2. 5'-CCTCTTATGAGACCTGTGTCACTGGGCAGATCTCG-3'.

The *Nde* I *Bgl* II fragment of the PCR product was inserted into the *Nde* I and *Bam* HI sites of the pET-15b vector (containing a *his*₆ tag; henceforth referred to as "his-tag"). The resulting vector, pET-15b- ζ (Figure 1) was verified by restriction digestion and sequencing from both ends of the inserted segment. The vector was shipped to Oakland University in XL1-Blue *E. coli* cells.

Isolation of pET-15b- ζ vector from XL1-Blue *Escherichia coli* cells

XL1-Blue *E. coli* maintenance cells containing the pET-15b- ζ vector were inoculated using a wire loop into 2 x 1.5 ml Luria broth (LB) containing ampicillin (100 μ g/ml), and grown overnight at 37°C. The cells were pelleted at 13,000 rpm for 1 min. The supernatant was

decanted, taking care not to disturb the pellet. The cells were lysed and cleaned using a FastPlasmid™ Mini kit (Brinkmann Instruments, Inc., Westbury, NY) per the manufacturer's instructions.

Transformation of ζ -crystallin cDNA into BL21-DE3 *E.coli* cells

The vector pET-15b- ζ carrying cDNA encoding his-tagged mouse ζ -crystallin was transformed into *E. coli* competent cells (BL21-DE3) using a Stratagene transformation protocol (North Torrey Pines Road, La Jolla, CA) [22] per the manufacturer's instructions.

Production of his-tagged recombinant mouse ζ -crystallin

Each of four baffled culture flasks containing 400 ml of yeast extract tryptone (YT) broth were seeded with 8 ml of the transformed *E.coli* strain (BL21-DE3), and grown overnight in Luria broth (50 ml) containing ampicillin (100 μ g/ml). The bacteria were grown at 37°C in an incubator (100–115 rpm) to a logarithmic phase ($OD_{600} = 0.7$ – 1.0). ζ -crystallin expression was induced by adding isopropyl-1-thio- β -D-galactopyranoside (IPTG) (#420322, Calbiochem) to a final concentration of 1 mM, and the mixture left to grow overnight.

Harvesting and lysis of bacterial cells

E.coli cells were harvested and lysed according to procedures described previously [23,24]. After overnight incubation, cells containing his-tagged mouse ζ -crystallin were separated from the culture media by centrifugation at 4,000 rpm for 20 min at 4°C, and the supernatant discarded. Falcon tubes containing cell pellets were placed into a dry ice/ethanol bath for 2–5 min, transferred to cold water for 60 seconds to melt the ice along the walls of the tube, and a sterile plastic spatula used to break the pellet free. The pellets were transferred and suspended in 200 ml of neutral (N)-lysis buffer (50 mM Tris-HCl, pH 7.5, containing 0.3 M NaCl and 0.5 mM EDTA) in a 500 ml flask. Lysozyme (0.5 mg/ml) was added to the buffer and incubated for 2 hrs at room temperature (RT) to digest the cell wall. The *E. coli* cells were lysed using 20 ml of 10x Mg/Mn salt solution (100 mM $MgCl_2$ and 10 mM $MnCl_2$). DNA was removed by DNase (10 μ g/ml) with incubation for 30 min at RT. The resulting *E. coli* lysate (~220 ml) was placed into a dialysis bag (10 kD molecular weight cut-off) in 4 L of buffer (10 mM Tris-HCl, pH 7.4, containing 0.5 mM EDTA). The dialysis buffer was changed twice over a 16–24 hr period. At the end of the dialysis, cell debris was removed as a pellet by centrifugation at 12,000 rpm for 30 min at 10°C.

Purification of his-tagged recombinant mouse ζ -crystallin

Conditions for the purification of recombinant his-tagged mouse ζ -crystallin were optimized for maximal yield and purity by employing a DEAE Sepharose column followed by nickel affinity chromatography. DEAE Sepharose ion exchange (HiPrep 16/10 QFF) was conducted using Fast Performance Liquid Chromatography (FPLC) (BioLogic, Bio-Rad Laboratories, Hercules, CA). Dialyzed and centrifuged recombinant mouse ζ -protein supernatant (see above) was applied to a DEAE-Sepharose Fast Flow column (GE Healthcare, Piscataway, NJ) that had been equilibrated with dialysis buffer (10 mM Tris-HCl, pH 7.4, containing 0.5 mM EDTA). ζ -crystallin protein was eluted with an increasing salt gradient of up to 1 M NaCl in dialysis buffer. ζ -crystallin-containing fractions were pooled and dialyzed against 3 L of 20 mM Tris-HCl buffer (pH 7.5), containing 200 mM NaCl. The dialyzed solution was applied to a 1 ml Nickel Nitrilotriacetic acid (Ni-NTA) column and eluted by gravity flow, after the column had been equilibrated with dialysis buffer. The purification of the recombinant mouse ζ -crystallin was based on the his-tag protocol. After the protein was loaded onto the Ni-NTA column, it was washed with 10 bed volumes of 20 mM Tris-HCl buffer (pH 7.5), containing 200 mM NaCl and up to 20 mM imidazole. 2 ml fractions of eluate were collected throughout the entire purification protocol. Recombinant ζ -crystallin was eluted from the Ni-NTA column

with dialysis buffer containing 200 mM imidazole. The different stages of purification (i.e. ion exchange and affinity chromatography) were monitored by SDS-PAGE, and ζ -crystallin confirmed by Western blotting, as previously described [25]. Protein concentrations were determined by the Pierce BCA method, with BSA as the standard. Antibody produced in rabbit against guinea pig lens ζ -crystallin was a gift from J. Samuel Zigler, Jr. (Johns Hopkins University, Baltimore, MD). Protein bands were visualized by chemiluminescence (ECL Western Blot Detection System; GE Healthcare, Arlington Heights, IL).

Purification of native guinea pig lens ζ -crystallin

In order to compare the quinone oxidoreductase activity of recombinant mouse ζ -crystallin with that of native guinea pig lens ζ -crystallin, ζ -crystallin was purified from guinea pig lens cortex. Male Hartley guinea pigs, 18 months old, were obtained from Kuiper Rabbit Ranch (Indianapolis, IN). Isolated lenses were frozen and divided into cortex and nucleus with use of a cork borer. Purification of ζ -crystallin was performed with use of FPLC (Bio-Rad Laboratories, Hercules, CA) by the method of Rao et al. [6]. Briefly, cortex from four lenses was homogenized in 20x volume of buffer A (20 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and 10 mM β -mercaptoethanol). The homogenate was centrifuged at 14,000 rpm for 30 min at 4°C, and the supernatant dialyzed overnight with two changes of half strength buffer A. Soluble protein was applied to Blue Sepharose (HiTrap Blue HP) equilibrated with half strength buffer A. Elution of protein was performed sequentially with equilibration buffer containing 100 mM NaCl and 1 M NaCl, respectively. Pooled fractions containing ζ -crystallin were dialyzed in equilibration buffer and applied to a CM agarose Fast Flow column (HiTrap CM FF) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Fractions containing ζ -crystallin were eluted with equilibration buffer containing 0.5 M NaCl. Fractions were pooled, dialyzed overnight in phosphate buffered saline (PBS), pH 7.3, and then assayed immediately for quinone oxidoreductase activity. Protein concentration was determined by the Bio-Rad Coomassie Brilliant G-250 dye method with BSA as the standard.

Enzyme assay – NADPH:quinone oxidoreductase

The procedure for measurement of enzyme activity of ζ -crystallin was as described by Rao et al. [8]. Briefly, a total of 1 ml reaction mix contained 0.1 M Tris-HCl (pH 7.8), 0.2 mM EDTA, 0.1 mM NADPH and 0–7 μ g per assay of ζ -crystallin, incubated at 25°C. The reaction was initiated by adding a substrate, 5-hydroxy-1,4-naphthoquinone (juglone) (#H47003, Sigma), to a final concentration of 50 μ M, and the reaction measured as a decrease in NADPH at 340 nm for 2 min. Enzyme activity was calculated using a molar extinction coefficient of 6.22×10^6 for NADPH. One unit of enzyme activity was defined as 1 μ mole NADPH consumed per min, after subtraction of blanks.

RESULTS

Purification of his-tagged recombinant mouse ζ -crystallin

Purification of his-tagged recombinant mouse ζ -crystallin was conducted with use of cation exchange and affinity chromatography. Protein supernatant obtained from *E. coli* lysate (see Methods) was first applied to DEAE Sepharose in a low ionic strength buffer. ζ -crystallin was then eluted from the column with the same buffer containing 1 M NaCl, and the protein appeared at ~5–20% salt (Figure 2, cross-hatched area, 169–179 min elution time). Further purification of the protein was achieved by affinity chromatography using a Ni-NTA column (Figure 3), and elution with buffer containing 500 mM imidazole. This two step procedure resulted in purification of his-tagged recombinant mouse ζ -crystallin to homogeneity (Figure 4). The final yield was 3.0% from an initial 184.9 mg of crude protein, and purification of quinone oxidoreductase activity was 6.8-fold (Table I). The recovery of total enzyme activity was 20.1% (Table I).

Purification of native guinea pig lens ζ -crystallin

In order to compare the quinone oxidoreductase activity of purified his-tagged recombinant mouse ζ -crystallin with that of a native ζ -crystallin, ζ -crystallin was isolated and purified from guinea pig lens. Guinea pig lens ζ -crystallin was eluted from a Blue Sepharose column using a salt gradient of 25 to 62% (elution profile not shown). Further purification was accomplished by anion exchange using a CM Fast Flow column (Figure 5, cross-hatched area, 128–133 min elution time). SDS-PAGE analysis showed that purification was achieved to homogeneity (Figure 6). The final yield was 2.1% from an initial 151.4 mg of crude protein, and purification of quinone oxidoreductase activity was 11.5-fold (Table II). The recovery of total enzyme activity was 24.3% (Table II).

Comparison of the two purified ζ -crystallins

Specific activities of purified his-tagged recombinant mouse ζ -crystallin and native guinea pig lens ζ -crystallin compared favorably at 2.44 and 2.18 U/mg protein, respectively (Tables I and II). The molecular masses of each purified ζ -crystallin, as determined by SDS-PAGE (Figure 7), were as expected based on the theoretical values of ^{*}37,432 Da for his-tagged recombinant mouse ζ -crystallin and 35,202 Da for native guinea pig ζ -crystallin.

DISCUSSION

In this study, his-tagged recombinant mouse ζ -crystallin was successfully expressed in *E. coli* and purified to homogeneity, yielding a single protein band of approximately 37 kD on SDS-PAGE (Figure 4). To our knowledge, this is only the second time that recombinant techniques have been used to produce ζ -crystallin. Other researchers have coexpressed recombinant guinea pig ζ -crystallin in *E. coli*, along with the molecular chaperone GroEL/ES, in order to prevent formation of ζ -crystallin inclusion bodies [20]. In the current study, we observed no inclusion body formation during generation of his-tagged recombinant mouse ζ -crystallin. The yield of pure recombinant protein, 24 mg per liter of crude *E. coli* supernatant (Table 1), was 5 times that obtained previously for purification of recombinant guinea pig ζ -crystallin [20]. There are a number of possible reasons for these differences. They may be related in some way to the 9% difference existing between the sequences of the mouse and guinea pig ζ -crystallins [4,26]. Also, while Luria broth (LB) was employed by previous researchers [20] to express recombinant ζ -crystallin, yeast extract tryptone broth was used in the present study, after LB failed to produce a high protein yield. In addition, while the previous researchers expressed ζ -crystallin protein in the presence of antibiotics, the current study employed no antibiotics at the same stage of expression. It is possible that the use of antibiotics may have decreased the basal expression level below that required for plasmid stability [27]. In addition, the previous study used a pET21a vector which contains additional features, not present in the pET15b vector used in the current study, and which may have interfered with optimum expression [20]. For example, while the pET15b vector contains only a his-tag and a thrombin cleavage site, the pET21a vector contains, in addition to an optional his-tag, an N-terminus T7.Tag and f1 origin [28]. Finally, while the current study used affinity chromatography for the end state purification of recombinant ζ -crystallin, the previous study used gel filtration [20]. Affinity chromatography is known to typically produce a higher protein yield and a higher protein purity, compared to gel filtration [29].

^{*}The theoretical molecular mass of mouse ζ -crystallin is 35,268 Da. His-tagged recombinant mouse ζ -crystallin has twenty additional amino acids, MGSSHHHHHSSGLVPRGSH, at the N-terminus of the sequence, including the his₆-tag, residues 5-10, and a thrombin cleavage site, residues 14-19. Thus, the molecular mass of a his-tag protein is 2,181 Da. There is also a loss of a water molecule during the bonding of a his-tag protein with the mouse ζ -crystallin. Thus, the molecular mass of his-tagged mouse recombinant ζ -crystallin is 37,432 Da (35,268.5 + 2,181.3 - 17.9).

For the purpose of comparison with his-tagged recombinant mouse ζ -crystallin, native guinea pig lens ζ -crystallin was purified to homogeneity by the method of Rao and Zigler [6] to produce protein yielding a single band of approximately 35 kD on SDS-PAGE (Figure 6). Cortex from four guinea pig lenses yielded approximately 3 mg of pure ζ -crystallin (Table II), which was roughly half the ζ -crystallin expected to be present in this amount of lens cortical tissue.

The quinone oxidoreductase activity of purified his-tagged recombinant mouse ζ -crystallin, using 5-hydroxy-1,4-naphthoquinone (juglone) as a substrate, was 2.44 U/mg protein (Table 1). This activity compared well with that of native guinea pig lens ζ -crystallin, 2.18 U/mg protein (Table II), indicating that the active site and the NADPH binding site of the recombinant protein functioned normally, and that activity of the his-tagged enzyme was not affected by the presence of an extra 20 amino acid residues on the N-terminus. Residues important for quinone oxidoreductase activity of guinea pig ζ -crystallin have been reported to include Asn-48, Val-50, Thr-71, Ile-131, Cys-248, Gly-268, Ser-270 and Leu-271 [26], although quinone oxidoreductase active site residues are not entirely conserved in all species. The NADPH binding region of ζ -crystallin, a consensus sequence GXXGXXG, including residues 156–162, is known as the Rossmann fold [30].

This study's result of 2.44 U/mg protein for the activity of his-tagged recombinant mouse ζ -crystallin (Table 1) matched well with the 2.62 U/mg protein obtained by Goenka and Rao [20] for the activity of recombinant guinea pig ζ -crystallin; however, these authors used 9,10-phenanthrenequinone as a substrate, instead of 5-hydroxy-1,4-naphthoquinone used in the current study. Other investigators have observed substantially higher quinone oxidoreductase activities for ζ -crystallin using 9,10-phenanthrenequinone as a substrate, compared to 5-hydroxy-1,4-naphthoquinone, as well as other quinones [8,11].

This study's result of 2.18 U/mg protein for the activity of native guinea pig lens ζ -crystallin was nearly equal to the 2.10 U/mg protein obtained by Rao et al. [8] who also used 5-hydroxy-1,4-naphthoquinone as a substrate. The result was lower than the 2.87 U/mg protein obtained by Goenka and Rao [20] for the activity of native guinea pig lens ζ -crystallin; however, as mentioned above, these authors used 9,10-phenanthrenequinone as a substrate, which may yield higher activities compared to other quinone substrates.

In summary, his-tagged recombinant mouse ζ -crystallin has been purified to homogeneity, and found to possess quinone oxidoreductase activity comparable to purified native guinea pig lens ζ -crystallin. The method permits the production of substantial amounts of recombinant ζ -crystallin for conducting studies on the biological role of this interesting protein, which exists in such high concentrations in the lenses of certain species.

Acknowledgments

This work was supported in part by NIH grants EY02027 and EY 014803. We thank Dr. Norman Curthoys, University of Colorado, for the pET15b plasmid carrying cDNA for ζ -crystallin, and Dr. J.S. Zigler, Jr., Johns Hopkins University, for antibody to ζ -crystallin. We appreciate the assistance of the following undergraduate students with purification of recombinant ζ -crystallin: Robert Baker, Elizabeth Zbrozek, Zeeshan Ibadat, Renis Deda and Mark McCoy.

References

1. Huang QL, Russell P, Stone SH, Zigler JS Jr. Zeta-crystallin, a novel lens protein from the guinea pig. *Curr Eye Res* 1987;6:725–732. [PubMed: 3595182]
2. Garland D, Rao PV, Del Corso A, Mura U, Zigler JS Jr. Zeta-Crystallin is a major protein in the lens of *Camelus dromedarius*. *Arch Biochem Biophys* 1991;285:134–136. [PubMed: 1990971]

3. Fujii Y, Kimoto H, Ishikawa K, Watanabe K, Yokota Y, Nakai N, Taketo A. Taxon-specific zeta - crystallin in Japanese tree frog (*Hyla japonica*) lens. *J Biol Chem* 2001;276:28134–28139. [PubMed: 11371565]
4. Rao PV, Gonzalez P, Persson B, Jornvall H, Garland D, Zigler JS Jr. Guinea pig and bovine zeta-crystallins have distinct functional characteristics highlighting replacements in otherwise similar structures. *Biochemistry* 1997;36:5353–5362. [PubMed: 9154917]
5. Gonzalez P, Rao PV, Zigler JS Jr. Molecular cloning and sequencing of zeta-crystallin/quinone reductase cDNA from human liver. *Biochem Biophys Res Commun* 1993;191:902–907. [PubMed: 8466529]
6. Rao PV, Zigler JS Jr. Zeta-crystallin from guinea pig lens is capable of functioning catalytically as an oxidoreductase. *Arch Biochem Biophys* 1991;284:181–185. [PubMed: 1989495]
7. Lee DC, Gonzalez P, Wistow G. Zeta-crystallin: a lens-specific promoter and the gene recruitment of an enzyme as a crystallin. *J Mol Biol* 1994;236:669–678. [PubMed: 8114084]
8. Rao PV, Krishna CM, Zigler JS Jr. Identification and characterization of the enzymatic activity of zeta-crystallin from guinea pig lens. A novel NADPH:quinone oxidoreductase. *J Biol Chem* 1992;267:96–102. [PubMed: 1370456]
9. Rodriguez IR, Gonzalez P, Zigler JS Jr, Borrás TA. Guinea-pig hereditary cataract contains a splice-site deletion in a crystallin gene. *Biochim Biophys Acta* 1992;1180:44–52. [PubMed: 1390943]
10. Babiychuk E, Kushnir S, Belles-Boix E, Van Montagu M, Inze D. *Arabidopsis thaliana* NADPH oxidoreductase homologs confer tolerance of yeasts toward the thiol-oxidizing drug diamide. *J Biol Chem* 1995;270:26224–26231. [PubMed: 7592828]
11. Fernandez MR, Porte S, Crosas E, Barbera N, Farres J, Biosca JA, Pares X. Human and yeast zeta-crystallins bind AU-rich elements in RNA. *Cell Mol Life Sci* 2007;64:1419–1427. [PubMed: 17497241]
12. Kranthi BV, Balasubramanian N, Rangarajan PN. Isolation of a single-stranded DNA-binding protein from the methylotrophic yeast, *Pichia pastoris* and its identification as zeta crystallin. *Nucleic Acids Res* 2006;34:4060–4068. [PubMed: 16914438]
13. Gagna CE, Chen JH, Kuo HR, Lambert WC. Binding properties of bovine ocular lens zeta-crystallin to right-handed B-DNA, left-handed Z-DNA, and single-stranded DNA. *Cell Biol Int* 1998;22:217–225. [PubMed: 9974216]
14. Tang A, Curthoys NP. Identification of zeta-crystallin/NADPH:quinone reductase as a renal glutaminase mRNA pH response element-binding protein. *J Biol Chem* 2001;276:21375–21380. [PubMed: 11294877]
15. Simpanya MF, Ansari RR, Leverenz V, Giblin FJ. Measurement of lens protein aggregation in vivo using dynamic light scattering in a guinea pig/UVA model for nuclear cataract. *Photochem Photobiol* 2008;84:1589–1595. [PubMed: 18627516]
16. Giblin FJ, Leverenz VR, Padgaonkar VA, Unakar NJ, Dang L, Lin LR, Lou MF, Reddy VN, Borchman D, Dillon JP. UVA light in vivo reaches the nucleus of the guinea pig lens and produces deleterious, oxidative effects. *Exp Eye Res* 2002;75:445–458. [PubMed: 12387792]
17. Rudy MA, Zigman S, Girsch SJ, Schenk E. Sub-threshold near-UV radiation effects on aphakic guinea pig retinas. *Curr Eye Res* 1982;2:39–45. [PubMed: 7128182]
18. Korlimbinis A, Aquilina JA, Truscott RJ. Protein-bound UV filters in normal human lenses: the concentration of bound UV filters equals that of free UV filters in the center of older lenses. *Invest Ophthalmol Vis Sci* 2007;48:1718–1723. [PubMed: 17389504]
19. Rodokanaki A, Holmes RK, Borrás T. Zeta-crystallin, a novel protein from the guinea pig lens is related to alcohol dehydrogenases. *Gene* 1989;78:215–224. [PubMed: 2777081]
20. Goenka S, Rao CM. Expression of recombinant zeta-crystallin in *Escherichia coli* with the help of GroEL/ES and its purification. *Protein Expr Purif* 2001;21:260–267. [PubMed: 11237687]
21. Gonzalez P, Hernandez-Calzadilla C, Rao PV, Rodriguez IR, Zigler JS Jr, Borrás T. Comparative analysis of the zeta-crystallin/quinone reductase gene in guinea pig and mouse. *Mol Biol Evol* 1994;11:305–315. [PubMed: 8170370]
22. Stratagene. Transformation Protocol of BL21(DE3) competent cells - Instruction Manual. [Accessed in July, 2005]. <http://www.stratagene.com/products/displayProduct.aspx?pid=552>

23. Andley UP, Mathur S, Griest TA, Petrash JM. Cloning, expression, and chaperone-like activity of human alphaA-crystallin. *J Biol Chem* 1996;271:31973–31980. [PubMed: 8943244]
24. Cobb BA, Petrash JM. Characterization of alpha-crystallin-plasma membrane binding. *J Biol Chem* 2000;275:6664–6672. [PubMed: 10692476]
25. Simpanya MF, Ansari RR, Suh KI, Leverenz VR, Giblin FJ. Aggregation of lens crystallins in an in vivo hyperbaric oxygen guinea pig model of nuclear cataract: dynamic light-scattering and HPLC analysis. *Invest Ophthalmol Vis Sci* 2005;46:4641–4651. [PubMed: 16303961]
26. Edwards KJ, Barton JD, Rossjohn J, Thorn JM, Taylor GL, Ollis DL. Structural and sequence comparisons of quinone oxidoreductase, zeta-crystallin, and glucose and alcohol dehydrogenases. *Arch Biochem Biophys* 1996;328:173–183. [PubMed: 8638928]
27. Mierendorf R, Yeager K, Novy R. The pET system: your choice for expression. *Innovations (Novagen newsletter)* 1994;1:1–3.
28. Novagen.pET. *E. coli* T7 expression vectors. [Accessed on July 19, 2009].
<http://www.emdbiosciences.com/html/NVG/pettablemenued.html>
29. Jack, GW. Immunoaffinity chromatography. In: Kenney, A.; Fowell, S., editors. *Methods in Molecular Biology*. Humana Press Inc.; Totowa: 1992. p. 125-130.
30. Rossmann MG, Moras D, Olsen KW. Chemical and biological evolution of nucleotide-binding protein. *Nature* 1974;250:194–199. [PubMed: 4368490]

Figure 1. Construction of the pET-15b- ζ vector. The *Nde* I / *Bgl* II fragment of the PCR product (containing a his₆ tag and the coding region of ζ -crystallin) was inserted into the *Nde* I and *Bgl* II sites of the pET-15b vector.

Figure 2.

Purification of his-tagged recombinant mouse ζ -crystallin using DEAE Sepharose. ζ -crystallin was eluted with an increasing salt concentration to 1 M NaCl in 10 mM Tris/HCl (pH 7.4), containing 0.5 mM EDTA. The cross-hatched area represents pooled fractions of ζ -crystallin, the presence of which was determined by analysis of each fraction by SDS-PAGE. Pooled fractions were dialyzed and purified further with the use of Ni-NTA column (Figure 3).

Figure 3. Purification of his-tagged recombinant mouse ζ -crystallin on a Nickel-NTA column. Pooled fractions obtained from a DEAE Sepharose column (Figure 2) were applied to a Ni-NTA column, and ζ -crystallin was eluted with buffer containing increasing concentrations of imidazole up to 500 mM. ζ -crystallin eluted in fractions 33–37.

Figure 4. SDS-PAGE and Western blot analysis at various stages of purification of recombinant his-tagged mouse ζ -crystallin using DEAE Sepharose and Nickel-NTA chromatography. A, MW markers. B, Coomassie stain, DEAE Sepharose purification (Figure 2). C, Coomassie stain, Nickel-NTA purification (Figure 3). D, Western blot of lane C using guinea pig ζ -crystallin antibody.

Figure 5.

Guinea pig lens ζ -crystallin, purified previously with Blue Sepharose, was purified further on a CM Fast Flow (HiTrap CM FF agarose) column. ζ -crystallin was eluted from the column with an increasing salt concentration of up to 0.5 M NaCl in 10 mM Tris-HCl buffer (pH 7.4), containing 0.5 mM EDTA and 5 mM β -mercaptoethanol. ζ -crystallin eluted between 0 and 50% salt of 0.5 M NaCl (128–133 min elution time).

Figure 6. SDS-PAGE analysis at various stages of purification of native guinea pig lens ζ -crystallin using Blue Sepharose and a CM FF column (Figure 5). A, MW markers. B, Crude guinea pig lens water soluble cortical protein. C, ζ -crystallin after Blue Sepharose purification. D, ζ -crystallin after purification by CM FF column chromatography.

Figure 7. SDS-PAGE analysis of purified his-tagged recombinant mouse and native guinea pig lens ζ -crystallins. A, MW markers. B, Coomassie stain of his-tagged recombinant mouse ζ -crystallin after Ni-NTA purification. C, Coomassie stain of native guinea pig lens ζ -crystallin after CM FF purification.

Table 1

Purification of his-tagged recombinant mouse zeta crystallin from *Escherichia coli*

Purification Steps	Total Protein (mg)	Yield (%)	Total Activity ^b (U)	Specific Activity (U/mg protein)	Purification (fold)
Crude supernatant ^a	184.9	100	66.7	0.36	(1)
DEAE Sepharose	16.8	9.1	14.6	0.87	2.4
NI-NTA column	5.5	3.0	13.4	2.44	6.8

^aThe starting material was 230ml of crude *E. coli* supernatant.

^bOne unit is defined as the oxidation of 1 μ mole of NADPH/min at 25°C. Activity refers to that as a quinone oxidoreductase using 5-hydroxy-1,4-naphthoquinone (juglone) as a substrate. All results except enzyme activity are averages from 3 separate isolation procedures. The enzyme activity data are averages from 3 analyses for one of the isolations.

Table 2

Purification of native guinea pig lens zeta crystallin

Purification Steps	Total Protein (mg)	Yield (%)	Total Activity ^b (U)	Specific Activity (U/mg protein)	Purification (fold)
Crude supernatant ^a	151.4	100	28.8	0.19	(1)
Blue Sepharose	21.6	14.3	12.3	0.57	3.0
CM Fast Flow column	3.2	2.1	7.0	2.18	11.5

^aThe starting material was 5.3 ml of crude guinea pig lens cortical supernatant from four lenses.

^bOne unit is defined as the oxidation of 1 μmole of NADPH/min at 25°C. Activity refers to that as a quinine oxidoreductase using 5-hydroxy-1,4-naphthoquinone (juglone) as a substrate. All results except enzyme activity are averages from 3 separate isolation procedures. The enzyme activity data are averages from 3 analyses for one of the isolations.