

Crocodylian τ -Crystallin: Overexpression, Purification, and Characterization

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τ -Crystallin is a taxon-restricted crystallin found in eye lenses of reptiles and a few avian species but presumably absent in mammals. The level of τ -crystallin in the lens varies among different species. In the crocodile lens, it is the least abundant crystallin and is present in trace amounts. We present a method for cloning, overexpression, and purification of crocodylian τ -crystallin utilizing a combination of gel filtration and ion-exchange chromatography yielding an extremely purified protein. The protein gets profusely expressed resulting in a fairly high yield and exists as a monomeric entity of 47.5 kDa molecular mass. The recombinant τ -crystallin exists in a properly folded native state as probed by circular dichroism and fluorescence spectroscopy and exhibits enolase activity. © 2002 Elsevier

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τ -Crystallin is an important taxon-specific crystallin present in many reptilian and a few avian lenses (1). It is identical to α -enolase, a housekeeping enzyme of the glycolytic pathway indicating its recruitment for a dual role—as a structural protein in lens and as a metabolic enzyme in other tissues (2, 3). τ -Crystallin was first identified and characterized from lamprey and turtles (4, 5) and thus was named as τ (tau; derived from turtle) (5). Later, it was also found in duck lens and was shown to have sequence identity to α -enolase (3, 6).

Not much information is available on the structure and conformation of this crystallin except for a few reports from turtle and lamprey lenses. One of the reasons for this is the extreme difficulty in procuring the

source lenses from such rare animals owing to various national and international wildlife conservation acts. To overcome these constraints, it would be prudent if these crystallins were prepared by bacterial overexpression. We have been interested in τ -crystallin from crocodylian eye lens and have cloned and sequenced complete cDNA of this crystallin (7). It is the minor crystallin in the crocodile lens, comprising just about 3–4% of the total lens proteins, thus limiting the biochemical and biophysical studies on this crystallin. To overcome this problem, we have developed a method for overexpression and purification of crocodile τ -crystallin in bacterial expression host *Escherichia coli*. Moreover, we establish that the recombinant τ -crystallin possesses enolase activity in spite of being a monomer.

MATERIALS AND METHODS

RNA Isolation

Total RNA was extracted from the embryonic lens (developmental stages 21–25) of Indian mугger (*Crocodylus palustris*) using Trizol (Gibco BRL). cDNA was synthesized using Superscript II RNaseH reverse transcriptase kit (Life Technologies) using a mixture of random primers and oligo(dT) primer.

Primer Design and RT-PCR

Primers for τ -crystallin were designed from the sequence of τ -crystallin cDNA (7). The forward primer was selected so as to have an *Nde*I site at the initiating codon ATG. The reverse primer was selected from the 3'-UTR² region just downstream of the stop codon and

² Abbreviations used: CD, circular dichroism; DTT, dithiothreitol; IPTG, isopropyl β -D-thiogalactoside; PMSF, phenylmethylsulfonyl fluoride; FPLC, fast protein liquid chromatography; UTR, untranslated region; LB, Luria–Bertani; Trp, tryptophan.

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was engineered to introduce a *Xho*I site. The sequences of both primers were: forward, 5'-CAACATATGTCAGT-TCTCAAGG-3'; and reverse, 5'-GGCAGCTGCTGTTC-TCGAGATAA-3'.

PCR was performed on a MJ Research thermal cycler in a total volume of 50 μ l. The conditions for PCR were denaturation at 94°C for 1 min, annealing at 45°C for 1.5 min, and extension at 72°C for 2 min for 30 cycles. The PCR product (1320 bp) was checked on a 1.5% agarose gel, eluted using Qiagen gel extraction kit, and verified by DNA sequencing using the same set of primers. The sequence (1320 bp) was analyzed by BLAST software program of NCBI (8).

Subcloning in pET21a

The PCR product was blunt-end cloned in a plasmid bluescript pBSKSII and the insert was released by double digestion using *Nde*I and *Xho*I restriction enzymes (New England BioLabs). This insert with overhangs was subcloned in a pET21a expression vector (Novagen) in *Nde*I-*Xho*I site, which provided the first codon ATG at the *Nde*I site. This clone was transformed into BL21(DE3) expression host.

τ -Crystallin Expression in *E. coli*

The transformed BL21(DE3) *E. coli* cells were grown in LB medium at 37°C up to $A_{600} = 0.6$ before being induced with IPTG at 1 mM final concentration. The growth was allowed for 3 h postinduction and A_{600} was recorded before harvesting the cells. Equal amount of proteins before and after induction were checked on 10% SDS-PAGE followed by Coomassie brilliant blue or silver staining as the case may be.

Gel Filtration Chromatography

Cells (0.25 g wet wt) from 100 ml of induced culture were harvested by centrifugation and resuspended in 3 ml of lysis buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF). One-hundred microliters of 10 mg/ml lysozyme was added and the mixture was incubated on ice for 30 min followed by sonication to complete the cell breakage. The sample was spun at 15,000 rpm for 30 min at 4°C, the pellet was removed, and the supernatant was loaded onto a Bio-Gel A-1.5m column (2 \times 80 cm). Fractions (3 ml) from the Bio-Gel A-1.5m column were checked on 10% SDS-PAGE and those having τ -crystallin were pooled together.

Mono Q FPLC

Pooled fractions from Bio-Gel A-1.5m having overexpressed τ -crystallin were loaded on to a Mono Q HR 5/5 FPLC column. The buffer employed was 50 mM

Tris, pH 8.0, 1 mM EDTA, 1 mM DTT, and the gradient was developed from 0 to 1 M NaCl.

Superose 12 FPLC

Superose 12 column (1 \times 30 cm) was equilibrated with 50 mM Tris, pH 8, containing 1 mM DTT, 1 mM EDTA. The purified protein (80 μ g) was loaded on to the column at a flow rate of 0.25 ml/min. For calculating the molecular weight, the column was calibrated with gel filtration standards (low molecular mass, Pharmacia Biotech) and the size of the protein was estimated based on the elution volume.

SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis of proteins was performed on a discontinuous buffer system on 10% polyacrylamide. Protein bands were visualized either by Coomassie brilliant blue or by silver staining and photographed.

Enolase Assay

Enolase activity was assayed spectrophotometrically by measuring the change in phosphoenolpyruvate concentration at 240 nm in 50 mM imidazole-HCl, pH 6.7, 75 mM KCl, 5 mM MgSO₄, 1 mM EDTA as described earlier (9, 10). Activity was expressed as micromoles of the substrate converted into product in 1 min at 30°C.

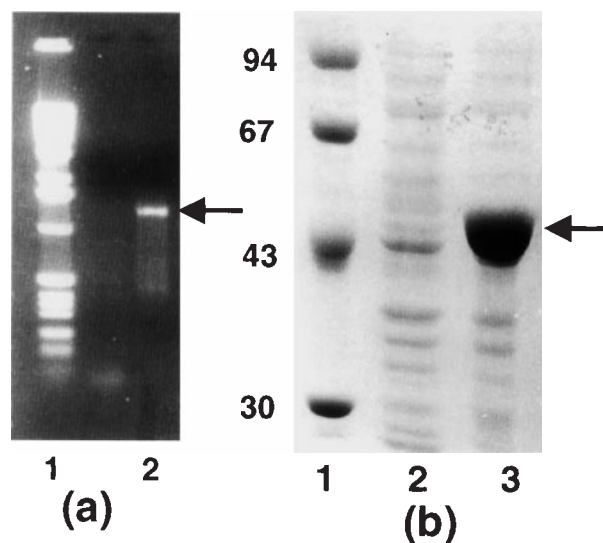


FIG. 1. (a) PCR product with the complete coding sequence of 1320 bp. Lane 1, 1-kb DNA ladder; lane 2, PCR product for τ -crystallin cDNA. (b) 10% SDS-PAGE showing overexpression of τ -crystallin. Lane 1, standard molecular weight markers; lane 2, control, uninduced cell lysate; lane 3, IPTG-induced cell lysate overexpressing τ -crystallin.

Circular Dichroism

Near- and far-UV CD spectra were recorded in a Jasco J-715 spectropolarimeter in a 1.0- and 0.05-cm path-length cell in 50 mM Tris, pH 8.0, containing 100 mM NaCl, 1 mM DTT, and 1 mM EDTA. Ellipticity values were expressed in millidegrees.

Steady-State Fluorescence

Emission spectra were recorded using an excitation wavelength of 280 nm in a correct spectrum mode on a Hitachi F-4010 spectrofluorometer. The buffer used was 50 mM Tris, pH 8, 100 mM NaCl, 1 mM DTT, and 1 mM EDTA. The excitation and emission bandpasses were set at 5 nm.

RESULTS AND DISCUSSION

cDNA Subcloning and Protein Expression

For cloning and overexpressing the τ -crystallin gene, we performed PCR over the cDNA synthesized from the total RNA from crocodile embryonic lens. Figure 1a shows the PCR product of the complete τ -crystallin coding region (1320 bp). The product obtained pertains to the complete gene from the start to the stop codon with *NdeI* and *XhoI* sites at the 5'- and 3'-ends, respectively, as confirmed by DNA sequencing. This blunt end product was cloned into the plasmid bluescript pBSKSII and the resulting recombinant plasmid was subjected to *NdeI*-*XhoI* double digestion to release the insert with the corresponding restriction overhangs. This insert

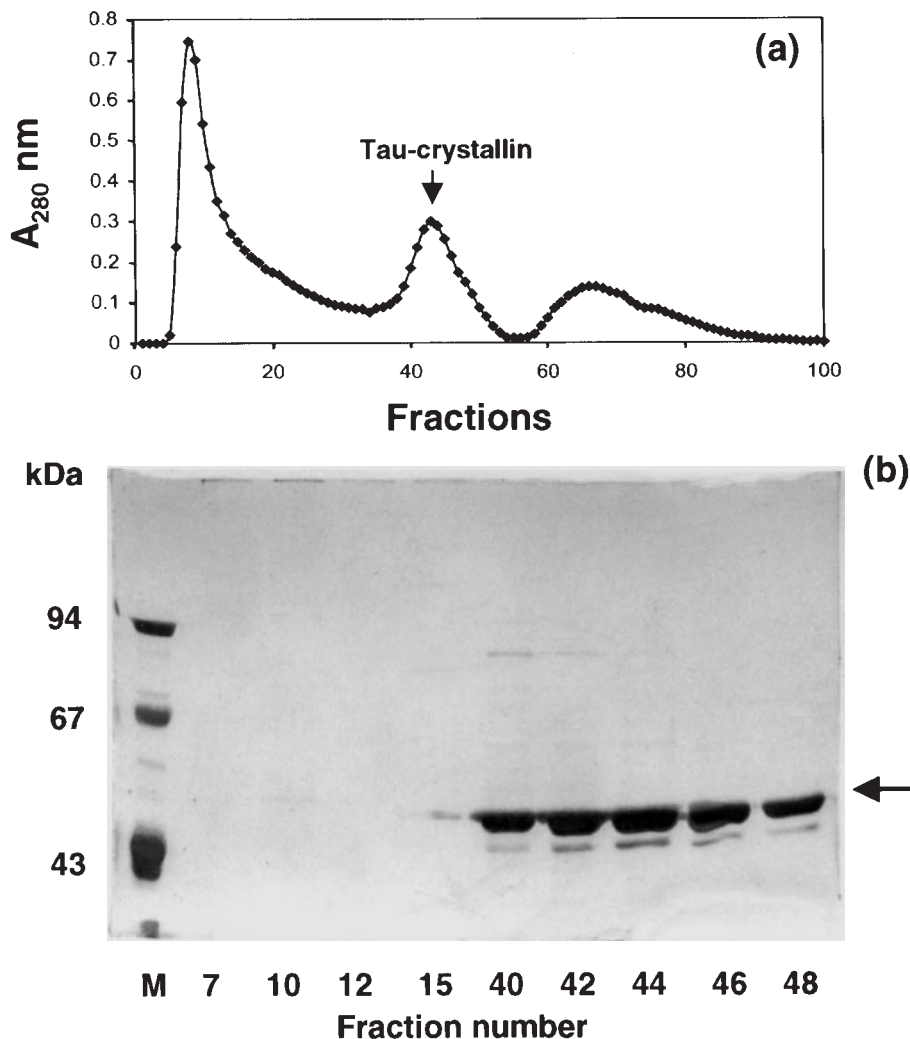


FIG. 2. (a) Bio-Gel A-1.5m chromatography of the whole cell lysate in 50 mM Tris, 100 mM NaCl, 1 mM DTT, 1 mM EDTA. The whole cell lysate was loaded and fractions were monitored at 280 nm. (b) SDS-PAGE analysis of peak fractions from Bio-Gel A-1.5m column. Arrow indicates τ -crystallin band.

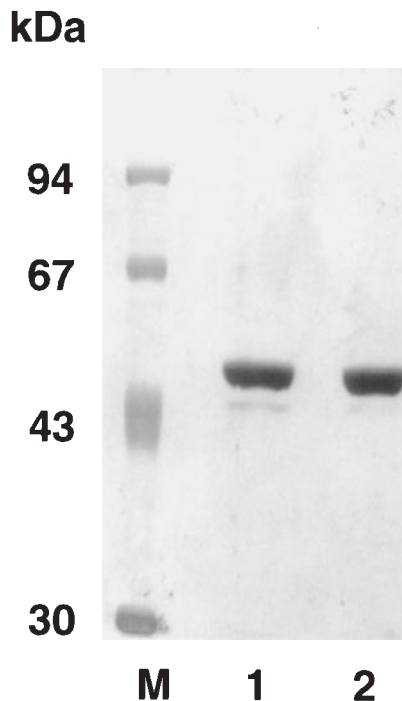


FIG. 3. Silver-stained SDS-PAGE of the Mono Q flowthrough. M, molecular weight markers; lane 1, peak fraction (peak 2, Fig. 2a) from Bio-Gel A-1.5m column; lane 2, Mono Q flowthrough having purified τ -crystallin.

with overhangs was subcloned into the *NdeI*-*XhoI* linearized pET21a expression vector to generate the τ -crystallin expression clone (pET21a- τ).

The pET21a- τ clone was transformed into BL21(DE3) host and the protein expression was induced by IPTG as described under Materials and Methods. Figure 1b shows the expression of this protein in transformed BL21(DE3) host *E. coli* cells both under the control uninduced condition and after IPTG induction that resulted in profuse expression of τ -crystallin to the extent of almost $\sim 80\%$ of the total cellular protein. The overexpressed protein confirms the expected molecular mass of 47.5 kDa as calculated based on the sequence analysis. Following expression, the protein was found to remain in the soluble fraction and did not form inclusion bodies.

Chromatographic Purification

We then performed the purification of the overexpressed protein. After cell lysis, the sample was spun to get rid of pellet and the supernatant was subjected to gel filtration on Bio-Gel A-1.5m column. Figure 2a shows the Bio-Gel A-1.5m chromatogram of the total cell lysate, which fractionated as three well-resolved peaks. SDS-PAGE analysis (Fig. 2b) of the peak fractions shows that τ -crystallin elutes in the middle peak. The protein had an apparent purity of $\sim 90\%$ on SDS-

PAGE after silver staining as well as Coomassie blue staining (Fig. 2b).

Though the τ -crystallin could be purified almost completely on Bio-Gel A-1.5m chromatography, the complete purification of the protein was achieved by ion-exchange FPLC using Mono Q column (data not shown). In this case, the sole contaminating band (Fig. 2b) was retained on the column, whereas the τ -crystallin eluted unbound as an uncontaminated, singular species as determined by SDS-PAGE (Fig. 3). Beginning with 100 ml of bacterial culture, the yield of the purified τ -crystallin was about 4 mg (Table 1).

Quaternary Structure

The molecular weight of the protein under denatured conditions on SDS-PAGE shows the expected molecular mass of around 48 kDa. We also determined the molecular weight and quaternary structure by gel filtration on Superose 12 column. τ -Crystallin is known to be identical to α -enolase in amino acid sequence (3). α -Enolase is known to exist as a dimer whereas τ -crystallin from eye lens is a monomer. In order to see if the bacterially expressed protein is a monomer or a dimer, we performed gel filtration under native conditions. The protein, under the conditions employed, eluted as a monomer (Fig. 4). We further attempted to induce dimerization in the protein using magnesium ions, as they are implicated in α -enolase activity and possibly in dimerization. Recombinant τ -crystallin was found to remain monomer in the presence of 8 mM Mg^{2+} (data not shown), suggesting that the parameters

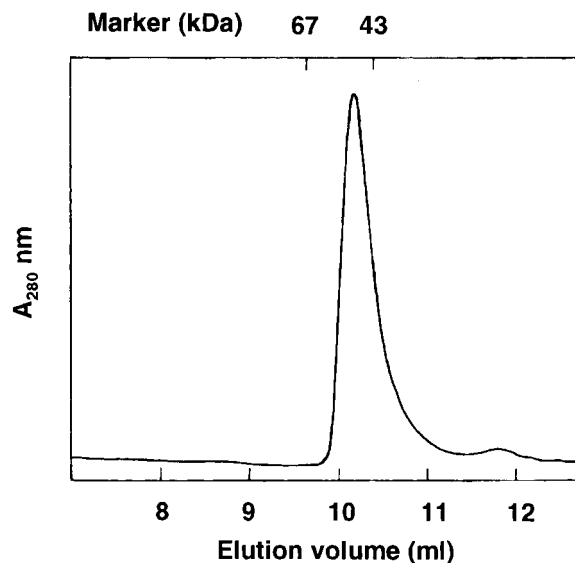


FIG. 4. Superose 12 (1 \times 30-cm column) FPLC profile showing the elution of recombinant τ -crystallin. The chromatography was performed in 50 mM Tris, pH 8, containing 100 mM NaCl, 1 mM DTT, 1 mM EDTA. The flow rate was 0.25 ml/min. The column was calibrated with molecular weight standards.

TABLE 1
Purification and Activity Profile of τ -Crystallin at Different Steps

	Total protein (mg)	Yield (%)	Total activity (μ mol/min)	Sp act (μ mol/min/mg)	Fold purification
Cell lysate	27.05	100	1000	37.0	—
Bio-Gel A-1.5m pooled peak	7.68	28.4	1836	239	6.47
Mono Q flowthrough	4	14.8	1000	250	6.76

deciding the monomeric/dimeric fate could be complicated.

Enolase Activity

Recombinant τ -crystallin was assayed for enolase activity and was found to have a fairly good enzymatic activity as shown in Table 1. It is noteworthy to mention that nearly all enolases are reported to be dimeric (11) and in view of this, the recombinant τ -crystallin provides an interesting example of a monomeric enolase.

Secondary and Tertiary Structure

We determined the secondary structure of the recombinant τ -crystallin by far-UV CD. The two minima at 222 and 208 nm indicate that the structure of the protein is dominated largely by α -helix (Fig. 5a). The CD profile is in good agreement with the only published report of the secondary structure of τ -crystallin from turtle lens and matches closely in appearance (5). This

suggests that the bacterially expressed τ -crystallin is folded to its native conformation, making it suitable for structural and functional studies.

Near-UV CD of τ -crystallin was performed to probe its tertiary structure, which is shown in Fig. 5b. There is a minimum at about 296 nm for Trp, followed by a very broad and dominant positive peak between 260 and 280 nm for aromatic amino acids. This composite peak is suggestive of the presence of a good tertiary structure. There are no data available on the tertiary structure of τ -crystallin from crocodile lenses or even closely related species such as alligator. The tertiary structure of the overexpressed protein does not match with the τ -crystallin of turtle. This, however, is expected since there are species-specific variations in the protein sequences (7). Far- and near-UV CD spectra in combination present τ -crystallin with its own characteristic signature profile.

Trp Microenvironment

Steady-state protein fluorescence is a powerful tool to probe the folding state of the molecule by assessing the microenvironment of Trp, which also provides an insight into its tertiary structure. We recorded emission spectra of τ -crystallin with the excitation wavelength set at 280 nm. The emission maximum is seen at around 330 nm, which indicates that Trp is buried in the hydrophobic environment. The emission maximum of crocodilian τ -crystallin is close to that of turtle τ -crystallin, which is 328 nm (5). Taken together, the far- and near-UV CD spectra and the fluorescence spectra strongly suggest that the recombinant protein is properly folded and is in a native conformation (Fig. 6).

In summary, we present a novel and simple method for the over-expression and purification of a properly folded τ -crystallin. Earlier purification of τ -crystallin from turtle lenses involved a multistep procedure consisting of isoelectric focusing, gel filtration, and ion-exchange chromatography (5). The advantages of this method are the use of a simplified chromatographic procedure involving only two steps, which results in a high yield of the protein with high purity. This purification procedure can be applied even on large scales making it a method of choice. The availability of purified τ -crystallin will facilitate the detailed biochemical and

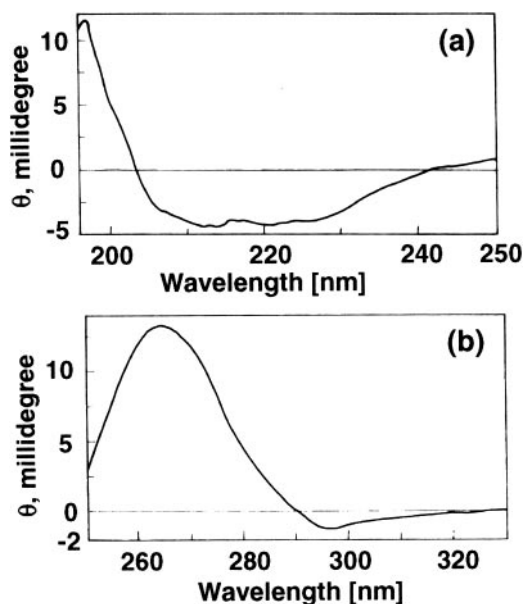


FIG. 5. (a) Far-UV and (b) near-UV CD of τ -crystallin in 50 mM Tris, pH 8, 100 mM NaCl buffer containing 1 mM DTT and 1 mM EDTA. Protein concentration was 0.44 mg/ml. Cells of 0.05- and 1-cm pathlengths were used for far- and near-UV CD, respectively. Ellipticity values are represented in millidegrees.

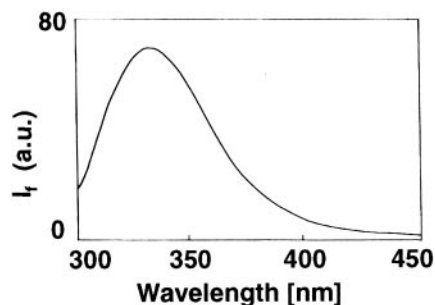


FIG. 6. Steady-state fluorescence of τ -crystallin. Protein solution (0.44 mg/ml) in 50 mM Tris, pH 8, containing 100 mM NaCl, 1 mM DTT, and 1 mM EDTA was subjected to excitation at 280 nm and the emission spectrum was recorded in the correct spectrum mode. Excitation and emission bandpasses were set at 5 nm.

structural studies of this important lens constituent, which would otherwise need to be isolated from the rare and endangered species such as crocodiles, where it is the least abundant crystallin. We further suggest that the bacterially expressed τ -crystallin is a monomer with enolase activity. The structural studies are required to elucidate the differences in τ -crystallin and dimeric enolases.

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