

# Triose phosphate isomerase, a novel enzyme-crystallin, and $\tau$ -crystallin in crocodile cornea

## High accumulation of both proteins during late embryonic development

Thandavarayan Kathiresan\*, Kannan Krishnan\*, Vaithilingam Krishnakumar, Raman Agrawal, Amit Anand, Dasari Muralidhar, Anurag K. Mishra, Vishnu M. Dhople, Ramesh K. Aggrawal and Yogendra Sharma

Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad-500 007, India

### Keywords

cornea; crocodile development;  $\tau$ -crystallin;  $\alpha$ -enolase; triose phosphate isomerase

### Correspondence

Y. Sharma, Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad-500 007, India

E-mail: yogendra@ccmb.res.in

R. K. Aggrawal, Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad-500 007, India

Fax: +91 40 2716 0591

Tel: +91 40 2716 0222

E-mail: rameshka@ccmb.res.in

\*These authors contributed equally to this work

(Received 24 March 2006, revised 11 May 2006, accepted 26 May 2006)

doi:10.1111/j.1742-4658.2006.05344.x

Several enzymes are known to accumulate in the cornea in unusually high concentrations. Based on the analogy with lens crystallins, these enzymes are called corneal crystallins, which are diverse and species-specific. Examining crystallins in lens and cornea in multiple species provides great insight into their evolution. We report data on major proteins present in the crocodile cornea, an evolutionarily distant taxon. We demonstrate that  $\tau$ -crystallin/ $\alpha$ -enolase and triose phosphate isomerase (TIM) are among the major proteins expressed in the crocodile cornea as resolved by 2D gel electrophoresis and identified by MALDI-TOF. These proteins might be classified as putative corneal crystallins.  $\tau$ -Crystallin, known to be present in turtle and crocodile lens, has earlier been identified in chicken and bovine cornea, whereas TIM has not been identified in the cornea of any species. Immunostaining showed that  $\tau$ -crystallin and TIM are concentrated largely in the corneal epithelium. Using western blot, immunofluorescence and enzymatic activity, we demonstrate that high accumulation of  $\tau$ -crystallin and TIM starts in the late embryonic development (after the 24th stage of embryonic development) with maximum expression in a two-week post-hatched animal. The crocodile corneal extract exhibits significant  $\alpha$ -enolase and TIM activities, which increases in the corneal extract with development. Our results establishing the presence of  $\tau$ -crystallin in crocodile, in conjunction with similar reports for other species, suggest that it is a widely prevalent corneal crystallin. Identification of TIM in the crocodile cornea reported here adds to the growing list of corneal crystallins.

Crystallins are defined as lens structural proteins, which are classified as ubiquitous  $\alpha$ -,  $\beta$ - and  $\gamma$ -crystallins, and taxon-specific crystallins [1]. The transparency and refractive properties of lens depends on crystallins. Taxon-specific crystallins are generally enzymes recruited as structural proteins in the lens to perform specialized functions, i.e., maintaining lens transparency [2]. Some examples of the species-specific crystallins are

$\delta$ - and  $\epsilon$ -crystallins present in birds and reptiles,  $\tau$ -crystallins in turtle and reptiles,  $\rho$ -crystallins in frog and camel lenses [3]. Several of these crystallins are metabolic enzymes:  $\delta$ -crystallin is argininosuccinate lyase [4];  $\tau$ -crystallin is  $\alpha$ -enolase [5]. They are believed to be recruited by gene sharing or by gene duplication [2,6].

Some enzymes such as aldehyde dehydrogenase class 3 (ALDH3), aldehyde dehydrogenase class 1

### Abbreviations

ALDH, aldehyde dehydrogenase; IEF, isoelectric focusing; TIM, triose phosphate isomerase.

(ALDH1) [7–10], transketolase [11] and isocitrate dehydrogenase [12] in bovine and human cornea, peptidylprolyl *cis-trans*-isomerase (cyclophilin) and  $\alpha$ -enolase/ $\tau$ -crystallin in chicken cornea [13,14] and gelsolin in zebrafish cornea [15] are found in unusually high concentrations. Because their expression in the cornea, specifically in the corneal epithelium, is unusually high, they are thought to be functioning as structural proteins rather than enzymes. Based on the analogy with lens crystallins, these enzymes are called corneal crystallins [16]. The criteria for a protein to qualify as a corneal crystallin are: (i) it must be expressed at a high concentration, (ii) it should generally be an enzyme, though it might not perform an enzymatic function, and (iii) it should probably express in corneal epithelium [9,16]. It is presumed that like lens crystallins, the abundant corneal proteins might contribute structurally to its transparency and optical properties [9,17]. Because cornea is the outermost window or gate of the eye, some of the abundant corneal proteins such as aldehyde dehydrogenase, also act as antioxidant filters and protect the cornea against oxidative insults [7,18,19]. These tissue- and species-specific crystallins represent recruitment of enzymes to serve a structural role in the tissue, and this phenomenon has been termed as gene sharing and gene duplication [2,4,6,17].

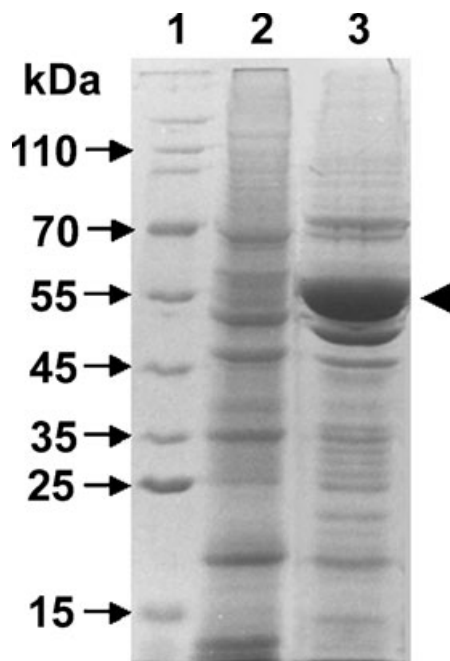
Some of the corneal crystallins are also lens crystallins.  $\delta$ -Crystallin, a major crystallin in birds and some reptile lenses is present in bovine cornea [14]. ALDH and glutathione *S*-transferase are both lens and corneal crystallins in some species [20,21]. However, not all corneal crystallins are found as lens proteins.  $\tau$ -Crystallin is present in the mammalian cornea but absent in mammalian lens. Just as there are taxon-specific lens crystallins, there are taxon-specific corneal crystallins e.g., gelsolin presents in zebrafish cornea, but not in bovine cornea [15]. Similarly, aldehyde dehydrogenase, which is present in bovine and human corneas, might not accumulate in high concentrations in the chicken cornea. It appears that corneas from various organisms express different enzyme-crystallins, which could be due to various divergent routes during evolution. It is to be mentioned that corneas of only a few species have been analyzed to date. Examining major proteins in the cornea of multiple species provides great insights into their evolution, as well as how crystallins adapt to varying physiological demands. It is therefore interesting to analyze corneas from diverse species and identify their corneal crystallins. In this regard, we have selected crocodile cornea for the presence of corneal crystallins. Crocodylians are evolutionarily interesting vertebrates, supposed to be of the Triassic era. Cornea

of this taxon or its closely related species such as alligator has not been analyzed previously. Any information about such a species would add significantly to our understanding of the origin and evolution of genes and their functions.

In this work, we have performed the proteomics analysis of the major proteins expressed in crocodile cornea. We have found that a taxon-specific lens crystallin,  $\tau$ -crystallin/ $\alpha$ -enolase, is expressed in the crocodile corneal epithelium as a corneal crystallin.  $\tau$ -Crystallin was first identified in turtle lens [22] and is also expressed in the lenses of duck [23] and lamprey [5]. We have reported earlier that  $\tau$ -crystallin is present in crocodile lens, though as a minor fraction [24].  $\alpha$ -Enolase/ $\tau$ -crystallin has been identified previously in the corneas of bovine and chicken [7,13,14]. In this study, we show that  $\tau$ -crystallin is present in crocodile cornea and have collected data on its expression and abundance in corneal epithelium. We demonstrate that the accumulation of  $\tau$ -crystallin in the corneal epithelium increases with embryonic development, which might coincide with the attainment of many optical properties of the cornea, such as transparency and curvature. In addition to  $\tau$ -crystallin, we have found triose phosphate isomerase (TIM), a dimeric enzyme that catalyzes the interconversion of D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate during glycolysis [25], as another major enzyme-crystallin in the soluble corneal fraction. Using immunofluorescence, we demonstrate that TIM accumulation in the cornea increases during late embryonic development. Our results suggest that  $\tau$ -crystallin is a widely prevalent corneal crystallin, and TIM is a novel enzyme-crystallin expressed in crocodile cornea.

## Results and Discussion

Though very diverse corneal crystallins have been identified in the corneas of bovine and chicken, no reptile cornea has been studied to date [11,12,14,15]. Crocodiles represent an evolutionarily important taxon among various vertebrate species because it is believed that they have survived and changed very little in hundreds of millions of years, in spite of the global catastrophe that caused the mass extinction of the dinosaurs and many other animal species 65 million years ago. Unlike the lens of many vertebrate species, the crocodile lens has several different crystallins ( $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -,  $\epsilon$ -,  $\tau$ -crystallins and at least one crystallin yet to be identified) [24], which makes this species very interesting for studying various crystallins. We were therefore interested in identifying and comparing the major crystallins expressed in the crocodile cornea,



**Fig. 1.** SDS/PAGE [12% (w/v) gel] stained with Coomassie brilliant blue. Lane 1, molecular mass markers; lane 2, crocodile total corneal extract; lane 3, bovine corneal epithelium extract. Arrowhead points to the prominent ALDH band.

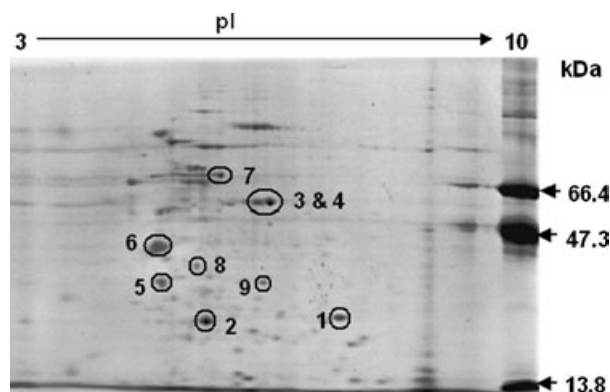
which would provide great insight into their evolution. Some species of crocodiles, being endangered, are not easily accessible for studies, thus further increasing the significance of any experimental data, more so if these are from the embryonic developmental stages.

SDS/PAGE yielded prominent protein bands in the water-soluble fraction of crocodile cornea with molecular masses of approximately 20, 35, 47 and 50 kDa (Fig. 1, lane 2). However, corresponding protein bands of the same intensity are absent in the SDS/PAGE of bovine corneal extract used for comparison (Fig. 1, lane 3). The bovine corneal sample shows a prominent 54 kDa band corresponding to ALDH3 (earlier known as BCP54) (Fig. 1, lane 3). Interestingly, a corresponding band is not seen in the SDS/PAGE of crocodile cornea. We further resolved the proteins expressed in crocodile cornea by 2D gel electrophoresis and identified them by MALDI-TOF.

#### Identification of proteins expressed in crocodile cornea by proteomics/MS methods

##### $\tau$ -Crystallin/ $\alpha$ -enolase is expressed as a major protein in crocodile cornea

Two-dimensional electrophoresis of the soluble fraction of corneal proteins revealed several spots scattered



**Fig. 2.** Protein profile of the total corneal extract of crocodile by 2D gel electrophoresis on a 12% (w/v) gel. First dimension separation by isoelectric focusing with pI range between 3 and 10; second dimension separation by SDS/PAGE and stained with sensitive Coomassie. Protein spots were excised and identified by MALDI-TOF. Spot 1, triose phosphate isomerase; spots 3 and 4,  $\tau$ -crystallin/ $\alpha$ -enolase; spot 5, tropomyosin 2; spot 6,  $\beta$ -actin. Spots 2, 7, 8 and 9 could not be identified because of low confidence score.

all over the gel. Two of the major spots (spots 3 and 4) at pI  $\approx$  6.5, adjacent to each other in the  $\approx$  50 kDa range (Fig. 2) were excised separately for MALDI-TOF analysis. Both the spots matched to  $\tau$ -crystallin with a score of 143 and 69 on MASCOT (Matrix Science, www.matrixscience.com) (more than the accepted score of 63) search for spot 3 and 4, respectively. The molecular mass (48 kDa) of these proteins (spots 3 and 4) also matched with  $\alpha$ -enolase/ $\tau$ -crystallin. In total, 13 peptides generated after tryptic digestion matched with  $\tau$ -crystallin using MASCOT search (Table 1). These MALDI-TOF results were further confirmed by ESI-MS-QTOF MS/MS analysis (data not shown). The difference in pI of two  $\tau$ -crystallin spots could be because of partial post-translational modification of the protein. Mobility difference of vertebrate  $\alpha$ -enolase isozyme has been observed earlier on a native 2D SDS/PAGE due to phosphorylation [26,27]. We suspect that there might be partial phosphorylation or some post-translational modification of  $\tau$ -crystallin resulting in differential mobility on 2D gel electrophoresis.

##### Triose phosphate isomerase (TIM) is a novel protein in crocodile cornea

Another prominent spot (spot 1, Fig. 2) was analyzed by MALDI-TOF. The scores on MASCOT (score 77) and PROFOUND (score 2.43) programs (Rockefeller University, New York, NY, USA) used for the identification were sufficiently high to establish the identity of

**Table 1.** Protein identification for spots 1, 3 and 4 from 2D gel shown in Fig. 2 using MALDI-TOF peptide mass fingerprinting. Spots from acrylamide gel were excised, protein was in-gel digested with trypsin, eluted and analyzed.

Spot on 2D gel	Measured mass (p.p.m.) (error in p.p.m.)	MASCOT score	PROFOUND score	Residues	Matched peptide sequences from database
Spots 3 and 4: $\tau$ -crystallin/ $\alpha$ -enolase	1455.721(-32)	143 and 181	2.43 and 2.27	81-92	NINEVEQEKIDR
	1464.706 (-12)			270-281	YNQILRIEEELGSK
	1690.906 (11)			407-420	YNQILRIEEELGSKAR
	1804.041 (58)			33-50	AAVPSGASTGIYEALELR
	1853.876 (-86)			184-199	IGAEVYHNLKNVIEK
	1853.876 (18)			254-269	DGKYDLDFKSPDDPSK
	1918.038 (6)			407-422	YNQILRIEEELGSKAR
	1922.058 (36)			16-32	GNPTVEVDLYTNKGLFR
	2003.205 (50)			404-420	LAKYNQILRIEEELGSK
	2195.989 (-36)			10-28	EIFDSRGNPTVEVDLYTNK
	2347.233 (21)			307-327	FTACVDIQVGGDDLTVTNPKR
	2533.182 (-37)			33-56	AAVPSGASTGIYEALELRDNDKTR
	2669.344 (9)			10-32	EIFDSRGNPTVEVDLYTNKGLFR
	Spot 1: Triose phosphate isomerase			1081.582 (11)	77
1538.881(64)		86-99	DLGATWVVLGHSER		
1695.044(94)		86-100	DLGATWVVLGHSEERR		
1457.804 (62)		101-113	HVFGEDELIGQK		
1735.019 (69)		176-190	TATPQQAQEVHEKLR		

this spot as TIM, an enzyme of the glycolytic cycle that catalyzes the interconversion of D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Out of the 17 peptides obtained from tryptic digestion of this spot, 14 matched to TIM (molecular size 27 kDa) with a score of 77 on MASCOT (Table 1). The accumulation of TIM has not been identified earlier in the cornea of any other species, and our data suggest that TIM qualifies as a new enzyme-crystallin present in the crocodile cornea.

### Other proteins in the crocodile cornea

We have attempted to identify the other major spots obtained on 2D gel. We have identified tropomyosin 2 (spot 5), actin (spot 6), and myosin (spot 7) by MALDI-TOF analysis (Fig. 2). Actin was reported as a major intracellular water-soluble protein in zebrafish along with gelsolin [15]. We could not establish the identity of other spots (spots 2, 8 and 9 in Fig. 2) because their scores on MASCOT and PROFOUND were below the confidence level. We have not analyzed the spots whose molecular masses were more than 70–80 kDa.

### Localization and embryonic development studies

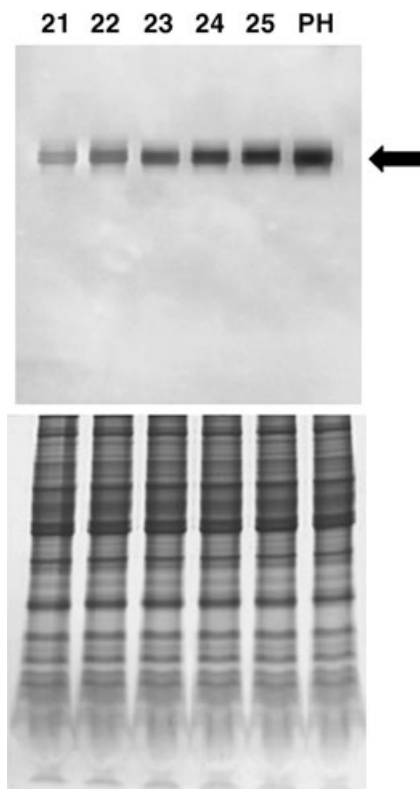
#### Different stages of embryonic development of crocodile

We followed the various stages of embryonic development of crocodile as described by Ferguson [28],

however, with minor modifications based on our experience with *Crocodylus palustris* development. The embryonic development of crocodile is divided into 28 stages, spanning over 65 days. In the embryo of stage 9 (9 days after egg laying), the optic cup is large and round but unpigmented. Up to stage 20 (28 days) the embryo size is very small and fragile. In the embryo of the 21st stage (31 days), a white ring in the iris surrounds the outline of the lens of the eye and is overlapped by both upper and lower eyelids. Scales are present on the dorsal and ventral aspects of the body of the embryo of the 21st stage. In the embryo of the 22nd stage (35 days), the eye is almost fully developed. Stages 23 and 24 represent 40 and 45 days after egg laying. The embryo of the 25th stage (51 days) is like a miniature version of the hatching. Hatching takes place after 65 days (after the 27th stage of development) of egg laying.

#### Expression of $\tau$ -crystallin in crocodile cornea during embryonic development

Although  $\tau$ -crystallin has been identified in the adult bovine cornea, its expression during embryonic development has not been studied. We have followed its expression in crocodile cornea at various stages of embryonic development by western blot analysis and immunofluorescence using a polyclonal rat antiserum raised against recombinant  $\tau$ -crystallin cloned from a crocodile lens [29]. The western blot data showed that



**Fig. 3.** Detection of  $\tau$ -crystallin by western blot using anti- $\tau$ -crystallin polyclonal IgG in the cornea collected from different stages of crocodile embryos and from a two-week posthatched animal. Numbers above the lanes indicate the stages of embryonic development (from 21st to 25th stages). PH indicates cornea from a two-week posthatched crocodile. Solid arrowhead points to the prominent band of  $\tau$ -crystallin. Lower panel: the blot after Ponceau staining, demonstrating that equal amount of proteins were transferred from gel to membrane.

$\tau$ -crystallin is present, albeit at a lower concentration, in the corneal extract of the cornea from the stage 21 embryo and its expression increased during further stages of development as seen from the intensity of the band (Fig. 3). These data suggest a gradual increase in  $\tau$ -crystallin expression, assuming that the antibody specificity to  $\tau$ -crystallin does not change with development.

We followed the expression of  $\tau$ -crystallin in crocodile cornea during embryonic development by immunofluorescence using the above antibody. Corneas obtained from different embryonic developmental stages (stages 21–25) and from a two-week old crocodile were sectioned and stained for the presence of  $\tau$ -crystallin. The cornea obtained from embryos of stages earlier than 21st were fragile and were not studied. Low or basal level of expression of  $\tau$ -crystallin was observed in the corneas obtained from embryos in the

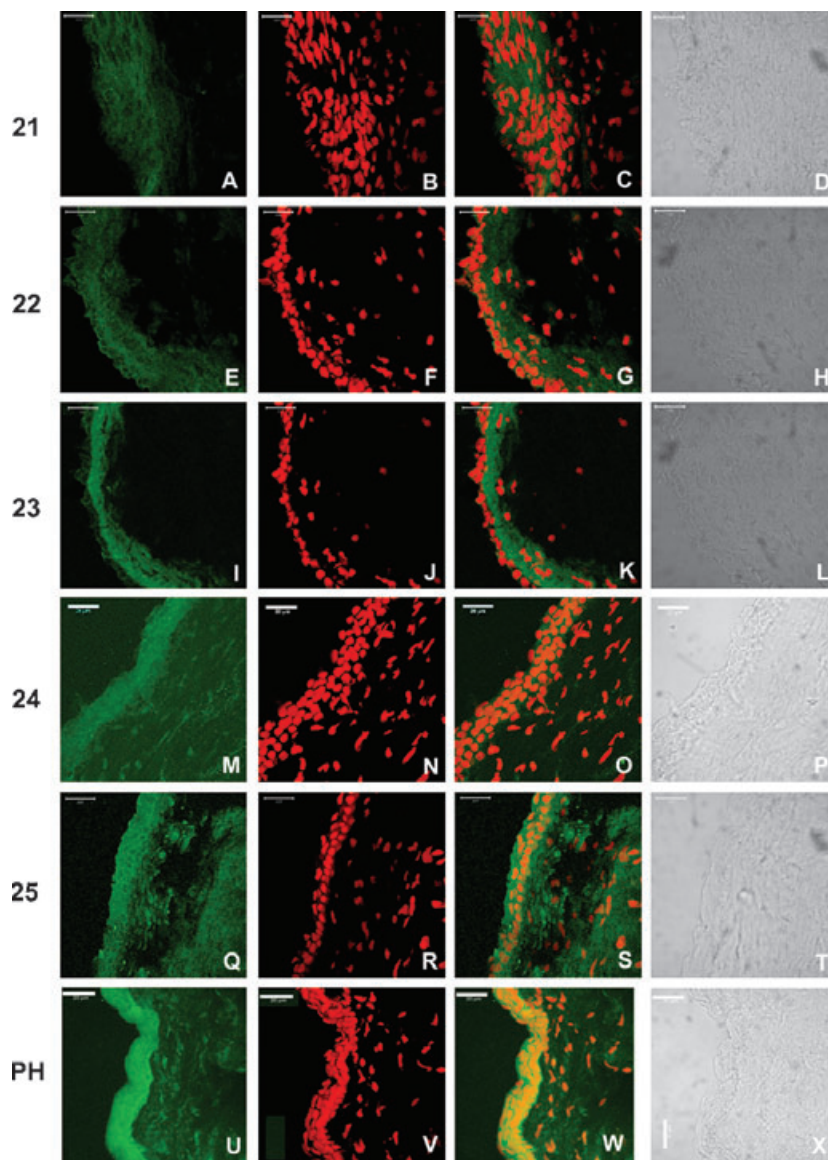
21st to 23rd stages of development (Fig. 4A,E,I). Weak but detectable intensity of the antibody staining was seen in the corneas, specifically in the corneal epithelium from the 24th stage onwards (Fig. 4M,Q). The intensity of  $\tau$ -crystallin staining in the cornea of a two-week posthatched animal was the highest (Fig. 4U). These results demonstrate that the accumulation of  $\tau$ -crystallin begins towards the later stages of embryonic development and reaches its maximum as the embryo attains maturity and hatches.

We also used  $\alpha$ -enolase activity as a measure of its level of expression in the cornea. The  $\alpha$ -enolase assay was performed with the water-soluble extract of crocodile cornea collected from various stages of embryonic development and from a two-week old animal. As seen from our analysis, the enzymatic activity increases in the corneal extract with development. The corneal soluble fraction from a two-week old animal exhibits higher expression of  $\tau$ -crystallin (Fig. 5) provided there is no change in its activity during development. These results further support the above data suggesting that the expression of  $\tau$ -crystallin in the cornea increases during development.

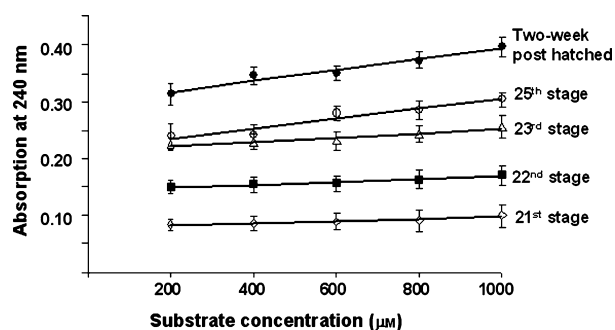
#### Accumulation of TIM in crocodile cornea during embryonic development

We also monitored the expression of TIM in the cornea during embryonic development by western blotting, immunofluorescence and enzymatic activity. The antibody used was raised against recombinant TIM cloned from *Saccharomyces cerevisiae*. As monitored by western blot, the cornea obtained from embryos in the 21st stage exhibited a comparatively low expression of TIM, which increased in the cornea during further development (Fig. 6).

Immunofluorescence data demonstrate a low level of expression of TIM in the cornea obtained from the 21st and 22nd stages of embryos (Fig. 7A,E), which increased with further development and reached its maximum in the cornea obtained from a two-week posthatched animal (Fig. 7U), confirming that TIM expression increases with development. We also observed that TIM is largely expressed in the corneal epithelium with low expression in the stromal region (Fig. 7). We also assayed the TIM activity in the total corneal extract and found that it possesses significant enzymatic activity even as early as the 21st stage of embryonic development. TIM activity increased in the corneal extract with the advancement of embryonic development (Fig. 8). Maximum activity was seen in the extract collected from a two-week-old crocodile cornea (Fig. 8). These results suggest that similar to



**Fig. 4.** Immunolocalization of  $\tau$ -crystallin in crocodile cornea during embryonic developmental stages and in a two-week post-hatched animal. (A–D) Stage 21 (31 days after egg laying); (E–H) stage 22 (35 days after egg laying); (I–L) stage 23 (40 days after egg laying); (M–P) stage 24 (45 days after egg laying); (Q–T) stage 25 (51 days after egg laying); (U–X) cornea from a two-week post-hatched crocodile. Column 1 (A, E, I, M, Q and U) shows anti- $\tau$ -crystallin IgG staining (green); column 2 (B, F, J, N, R and V), propidium iodide staining showing nuclei in red colour; column 3 (C, G, K, O, S and W) shows  $\tau$ -crystallin and propidium iodide staining overlapped; column 4 (D, H, L, P, T and X) represents transmission phase contrast. Scale bar 20  $\mu$ m.

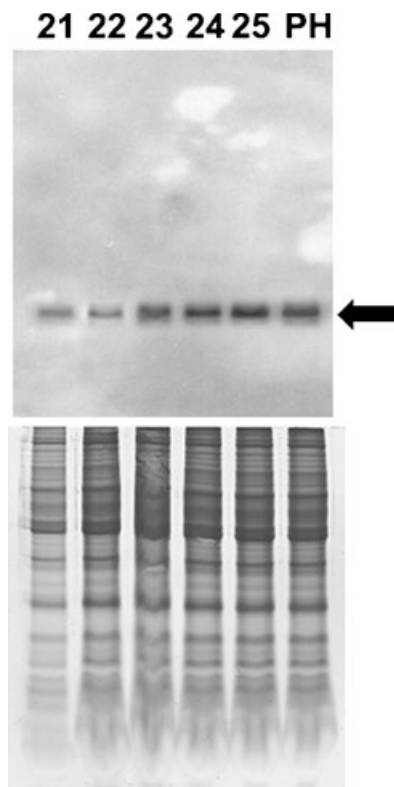


**Fig. 5.**  $\alpha$ -Enolase activity in corneal extract collected from the embryos of different developmental stages and from a two-week posthatched crocodile.  $\square$  21<sup>st</sup> stage;  $\square$  22<sup>nd</sup> stage;  $\triangle$  23<sup>rd</sup> stage;  $\circ$  25<sup>th</sup> stage;  $\bullet$  two-week posthatched crocodile.

$\tau$ -crystallin, accumulation of TIM also increases in the cornea with embryonic development.

#### $\tau$ -Crystallin and TIM are putative corneal crystallins in crocodile cornea

Our results demonstrate that (i)  $\tau$ -crystallin and TIM are among the major proteins expressed in crocodile cornea, (ii) they are expressed mainly in the corneal epithelium, and (iii) they accumulate during late stages of development. These are some of the criteria by which a crystallin is defined [9,16]. These proteins might be performing a structural role and are crystallins in crocodile cornea. We have observed that the cornea from an embryo of the 21<sup>st</sup> and 22<sup>nd</sup> stages is



**Fig. 6.** Detection of TIM by western blot using anti-TIM polyclonal IgG in different stages of embryos and from a two-week post-hatched crocodile. Numbers above the lanes indicate the stages of embryonic development (from 21st to 25th stage). PH indicates cornea from a two-week posthatched crocodile. Solid arrowhead points to the prominent band of TIM. Lower panel: the blot after Ponceau staining, demonstrating that equal amount of proteins were transferred from gel to membrane.

opaque. Therefore, our data on the accumulation of  $\tau$ -crystallin and TIM during the late stages of development suggest that they might have a role in providing the necessary optical properties, such as transparency and curvature to cornea during its development.

In conclusion, by proteomics and mass spectroscopic analysis, we have identified  $\tau$ -crystallin, a prevalent corneal crystallin, and TIM, a novel corneal crystallin, as two crystallins in the cornea of crocodile. Our data support the earlier observation that  $\tau$ -crystallin is a widely prevalent corneal crystallin, present in the cornea of bovine, chicken and crocodile. The expression of TIM in the corneas of other species is yet to be identified. Both crystallins accumulate during later stages of embryonic development. Our work further suggests the diversity of corneal crystallins. Crystallins in the corneas of diverse species need to be investigated for comparative purpose, which would help in understanding the evolution of transparency of lens and cornea.

## Experimental procedures

### Crocodile cornea

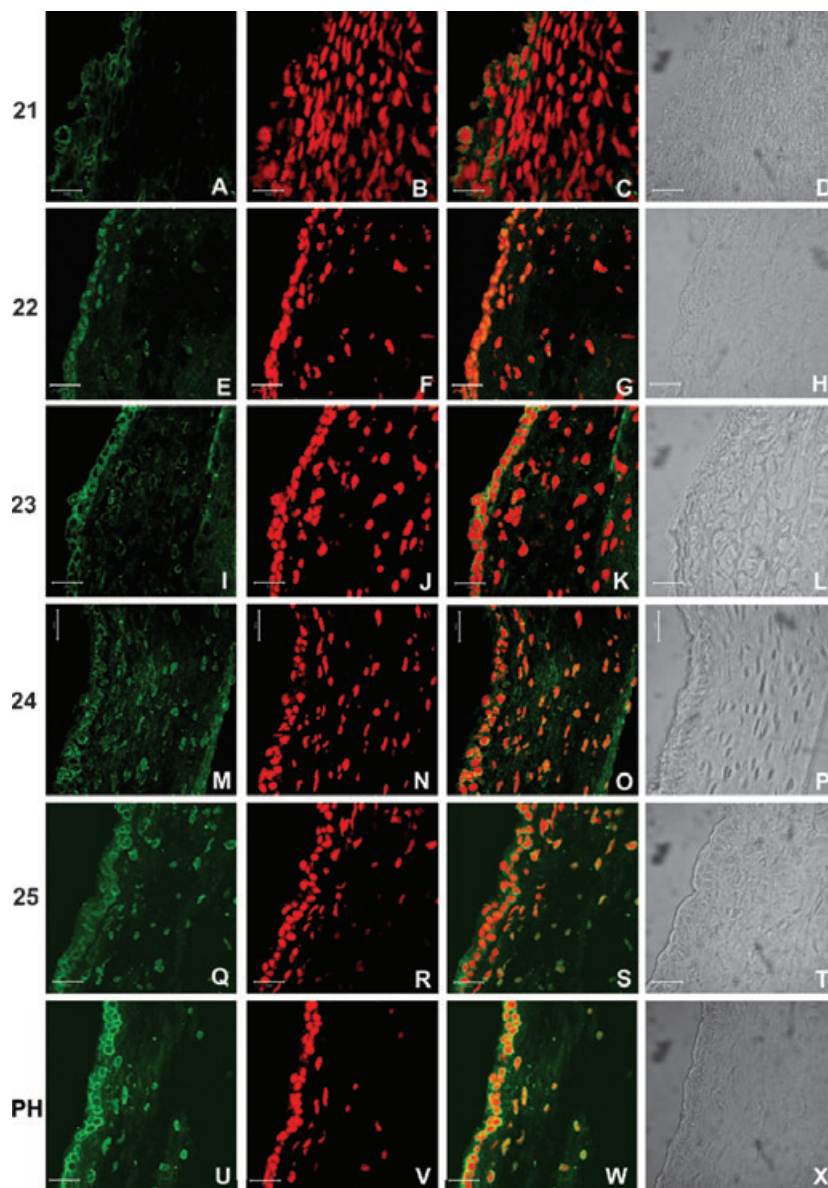
Fertilized eggs of Indian mugger (*Crocodylus palustris*) were collected from the Nehru Zoological Park, Hyderabad, India on day zero of egg laying and were incubated in the laboratory at 30–32 °C. Proper guidelines of ARVO were strictly followed in the handling of animals and were approved by the Institutional Ethics Committee. Embryonic developmental stages of crocodile have been classified previously [28]. Accordingly, there are 27 stages of embryonic development, after which hatching takes place. Corneas were dissected from the embryos between the developmental stages 20–25, and also from two-week old animals. Corneas collected from crocodile embryos were homogenized in 50 mM Tris, pH 7.5, 100 mM NaCl, 0.02% (v/v) sodium azide, 1 mM EDTA, centrifuged at 10 000 *g* for 15 min at 4 °C and the supernatant collected.

### 2D gel electrophoresis

2D electrophoresis was performed using immobilized pH gradient strips of 7 cm and pH range 3–10 (Bio-Rad, Hercules, CA, USA). The samples were resolved by isoelectric focusing (IEF) in the first direction and by SDS/PAGE [12% (w/v) acrylamide] in the second direction. In brief, 40–50  $\mu$ L aliquot of the sample containing about 60  $\mu$ g of the protein was premixed with rehydration buffer [8 M urea, 2% (v/v) CHAPS, 50 mM dithiothreitol and 0.2% (v/v) of 3–10 ampholytes] and the rehydration of the immobilized pH gradient strips was carried out for about 12–15 h. The rehydrated strip was subjected to IEF at a current of 50 mA·strip<sup>-1</sup> with an end voltage of 8000 V. After the IEF run, the gel strips were equilibrated with equilibration buffer and placed on a precast SDS-polyacrylamide gel. The gel was stained with Coomassie blue R-250 and destained. The image was acquired using Fluor-S Multi-imager (Bio-Rad) and the protein spots were identified based on their discrete presence on the gel and processed further for analysis.

### In-gel tryptic digestion

The selected spots were manually excised from the gel and were subjected to in-gel digestion using sequence grade bovine trypsin (Sigma, St Louis, MO, USA) reconstituted in 25 mM ammonium bicarbonate at pH 8.0. The molar ratio of protein to trypsin was maintained between 10 : 1 and 30 : 1, and the mixture was incubated at 37 °C for 16–18 h. After the incubation, peptides from the gel were extracted twice using 100  $\mu$ L of 50% (v/v) acetonitrile containing 5% (v/v) trifluoroacetic acid for about 30 mins by vortex. The extracts were pooled, lyophilized and used for MALDI-TOF analysis.



**Fig. 7.** Immunolocalization of TIM in crocodile cornea from early embryonic stages and from a two-week posthatched crocodile. (A–D) Cornea from embryo of 21st stage; (E–H) cornea from 22nd stage; (I–L) cornea from 23rd stage; (M–P) cornea from 24th stage; (Q–T) cornea from 25th stage; (U–X) cornea from a two-week posthatched crocodile. A, E, I, M, Q and U show anti-TIM IgG staining (green). B, F, J, N, R and V propidium iodide staining showing nuclei (red). C, G, K, O, S and W show TIM and propidium iodide staining overlapped. D, H, L, P, T and X represent transmission phase contrast. Scale bar 20  $\mu$ m.

### Mass spectrometry

The peptides were analyzed for peptide mass fingerprinting using the Voyager-DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) using  $\alpha$ -cyano-4-hydroxy cinnamic acid as matrix. The reconstituted peptides in 50% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid were spotted on a 96-well plate. The MALDI-TOF spectra were recorded in the Reflectron mode using the following parameters: accelerating voltage 20 kV, grid voltage 72%, delay time 200 ns, low mass gate 750, scan range 800–4000 and accumulation from 100 laser shots.

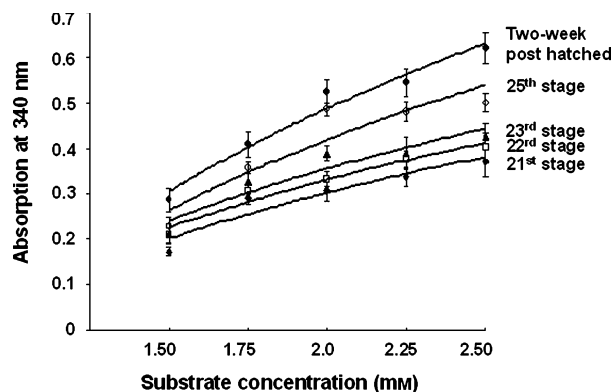
Determination of the internal sequence was done by MS/MS fragmentation of the peptides using QSTAR Pulsar ESI-QTOF (PE SCIEX, Applied Biosystems, Foster

City, CA, USA) and nano spray source. TOF-MS spectra were obtained in the range  $m/z$ , 500–1700 at 1000 V spraying voltage. The multiply charged species were subjected to fragmentation by CID using collision energy ranging from 30 to 50 eV.

### Protein identifications

A peak list was generated from the MALDI-TOF spectrum using DATA-EXPLORER ([www.ibm.com/dx](http://www.ibm.com/dx)) and finally, screened for the presence of nonpeptide peaks. The proteins were identified with peptide mass fingerprinting using NCBI protein database and by MASCOT (Matrix Sciences) and PROFOUND (Rockefeller University) search programmes. The search criteria fixed were partial methionine oxidation,





**Fig. 8.** TIM activity in the soluble corneal extract collected from the embryos of different developmental stages and from a two-week posthatched crocodile. ◆ 21st stage; □ 22nd stage; ▲ 23rd stage; ○ 25th stage; ● two-week posthatched crocodile.

carbamidomethylation of cysteines and up to maximum of two miscleavages with a mass error of 100 p.p.m. For the searches, molecular mass within  $\pm 5$  kDa and pI within  $\pm 0.5$  units of the molecular mass and pI observed in 2D gels were used.

Protein ID was accepted if the scores on MASCOT and PROFOUND were at least 63 and 1.5, respectively. Protein identification was further validated by examining the number of peptides matched (at least 5) and the sequence coverage (15–20%). The ID of some proteins was further confirmed by MS/MS fragmentation and searches were carried out using sequences that were analyzed manually by BIO-ANALYST (PE Sciex, Applied Biosystems).

### Purification of recombinant $\tau$ -crystallin and generation of polyclonal $\tau$ -crystallin/ $\alpha$ -enolase and TIM antibodies

Recombinant  $\tau$ -crystallin was cloned and overexpressed from crocodilian lens as described earlier [29]. Antibody against recombinant  $\alpha$ -enolase/ $\tau$ -crystallin was raised in a six-month-old mouse using the standard procedure. Animals were bled three days after the final booster. Serum was separated and antibody titer and specificity were assessed by western blot using recombinant  $\alpha$ -enolase. For the preparation of polyclonal antibody against TIM, we used recombinant TIM cloned from *Saccharomyces cerevisiae*, a kind gift from P Guptasarma and S Ahmed (IMTECH, Chandigarh, India).

### SDS/PAGE and western blot

Corneas of *Crocodylus palustris* were removed surgically and the epithelial cells were scraped and homogenized in 50 mM Tris, pH 7.5, 100 mM NaCl, 0.02% (v/v) sodium azide, 1 mM EDTA. After sedimenting the insoluble debris by spinning at 5000 g for 2 min total protein was estimated

using the Bio-Rad protein estimation kit. About 100  $\mu$ g protein was resolved using 12% (w/v) SDS-polyacrylamide gel and transferred on a nitrocellulose membrane (Hybond-C, Amersham Biosciences, Inc., Pittsburg, PA, USA). Examination of the blot after Ponceau staining demonstrated that equal amount of protein from the gel to membrane was transferred. These membranes were probed either with polyclonal mouse anti- $\alpha$ -enolase IgG (1 : 500) or with polyclonal anti-TIM IgG (1 : 300) followed by secondary antibody, anti-mouse-HRP conjugate (1 : 5000) and visualized using an ECL kit (Amersham Biosciences, Inc.).

### Immunofluorescence staining and confocal imaging

Cornea was frozen-sectioned (10  $\mu$ m) by a cryomicrotome (Leica Microsystems, Bensheim, Germany) and the sections were fixed in 100% methanol for one minute and washed with 1 $\times$  NaCl/P<sub>i</sub> pH 7.2. The slides were air-dried and stored till further use. The sections were rehydrated using 1 $\times$  NaCl/P<sub>i</sub> and the cells were permeabilized with 0.1% (v/v) Triton X-100 in NaCl/P<sub>i</sub> for 4 min. The cells were reacted separately with anti-enolase IgG (mouse polyclonal antibody) at 1 : 100 dilutions or with anti-TIM IgG (mouse polyclonal antibody) at 1 : 50 dilutions. After three washes in NaCl/P<sub>i</sub>, anti-enolase or anti-TIM was visualized with FITC-labeled sheep anti-mouse IgG (Jackson Laboratory, Bar Haine, Maine, USA) at a 1 : 200 dilution. The nucleus was stained with propidium iodide. Dual immunofluorescence confocal microscopy was carried out on a laser scanning confocal microscope (model LSM 510 META) (Carl Zeiss, Gottingen, Germany) using a 488 nm excitation and 540 nm long-pass emission filter.

### $\alpha$ -Enolase and TIM activities in corneal homogenate

The  $\alpha$ -enolase activity in the corneal homogenate of *Crocodylus palustris* was measured as described earlier [30]. Total protein (40  $\mu$ g) was taken with different concentrations of glyceraldehydes-2-phosphate (0.1–1 M) prepared in imidazole buffer pH 6.7. The substrate and enzyme were incubated at 37 °C for 30 min and the conversion of glyceraldehydes-2-phosphate to the phosphoenolpyruvate was measured by taking  $A_{240}$ , which was then plotted as a function of substrate concentration. TIM activity was measured using the method described by Plaut and Knowles [31]. Glyceraldehyde phosphate was used as a substrate. Data were plotted as change in absorption at 340 nm vs. substrate concentration.

### Acknowledgements

We thank Dr Lalji Singh for his interest in the work and initiating the program on crocodile development

and sex determination. We acknowledge Dr T. Ramakrishna Murthy for his critical comments, G. Srinivas for helping with frozen-sectioning, R. Nandini for confocal microscopy imaging and Dr Purnanad Guptasarma and Shabbir Ahmed for providing yeast TIM. We thank Hyderabad Zoological Park and Zoo Authority of India for allowing us to collect crocodile eggs. Raman Agrawal and Amit Anand are the recipients of a senior research fellowship from the Council of Scientific and Industrial Research (CSIR), Govt. of India.

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