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Lens proteome map and α-crystallin profile of the catfish *Rita rita*

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Crystallins are a diverse group of proteins that constitute nearly 90% of the total soluble proteins of the vertebrate eye lens and these tightly packed crystallins are responsible for transparency of the lens. These proteins have been studied in different model and non-model species for understanding the modifications they undergo with ageing that lead to cataract, a disease of protein aggregation. In the present investigation, we studied the lens crystallin profile of the tropical freshwater catfish *Rita rita*. Profiles of lens crystallins were analyzed and crystallin proteome maps of *Rita rita* were generated for the first time. αA-crystallins, member of the α-crystallin family, which are molecular chaperons and play crucial role in maintaining lens transparency were identified by 1-and 2-D immunoblot analysis with anti-αA-crystallin antibody. Two protein bands of 19-20 kDa were identified as αA-crystallins on 1-D immunoblots and these bands separated into 10 discrete spots on 2-D immunoblot. However, anti-αB-crystallin and antiphospho-αB-crystallin antibodies were not able to detect any immunoreactive bands on 1- and 2-D immunoblots, indicating αB-crystallin was either absent or present in extremely low concentration in *Rita rita* lens. Thus, *Rita rita* α-crystallins are more like that of the catfish *Clarias batrachus* and the mammal kangaroo in its αA- and αB-crystallin content (contain low amount from 5-9% of αB-crystallin) and unlike the dogfish, zebrafish, human, bovine and mouse α-crystallins (contain higher amount of αB-crystallin from 25% in mouse and bovine to 85% in dogfish). Results of the present study can be the baseline information for stimulating further investigation on *Rita rita* lens crystallins for comparative lens proteomics. Comparing and contrasting the α-crystallins of the dogfish and *Rita rita* may provide valuable information on the functional attributes of αA- and αB-isoforms, as they are at the two extremes in terms of αA-and αB-crystallin content.

Keywords: α-Crystallin, Biomarker, Cataract, Crystallin proteome map, Lens proteins, *Rita rita*

Vertebrate eye lens is a fascinating example of biological adaptation. It is a highly specialized tissue characterized by the presence of large amounts of water soluble proteins called crystallins. The crystallins are a diverse group of proteins that make up over 90% of the total soluble proteins of the vertebrate eye lens and function to maintain the transparency of the lens. They are divided into two superfamilies: α and βγ-crystallins. α-crystallin is found to be a member of the heat shock proteins (HSPs), while βγ-crystallins are considered relatives of microbial stress proteins[1]. Studies have shown that evolutionary divergence early on might have split the βγ-crystallins into two distinct families, β- and γ-crystallins[2]. The crystallin families are further divided into different subunits: αA and αB of the α-family, βA1-A4 and βB1-B3 of the β-family, and γA-F and S of the γ-family. Some taxon-specific crystallins like δ, ρ, ε, τ etc are also reported[3,4], which are generally the enzymes recruited as structural proteins in the lens to perform specialized functions[5].

Aging of normal lens leads to accumulation of post-translationally modified proteins that cause lens opacity called cataract. Cataract is a leading cause of human blindness accounting for about 17 million cases worldwide per year[6]. Presently, surgical intervention is the only approach to manage cataract. Understanding the molecular mechanism for cataract formation could be useful for designing alternative treatment or for designing drugs that may delay the onset of the process. Many model organisms like mouse[7], rat[8], zebrafish[9-12] and non-model organisms like crocodiles[3,4,13], anurans[14], chicken[15] are being used to study protein modifications occurring in lens and corneal crystallins during aging and cataractogenesis by proteomic analysis and for the comparative study of vertebrate eye development, function and ophthalmic diseases.

In aquatic animals like fishes lens damage has been reported to be significantly related to exposure to polycyclic aromatic hydrocarbons (PAHs)[16,17],

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organophosphate pesticides like monocrotophos[18,19], heavy metals such as lead (Pb^{2+})[20]. The toxic environmental contaminant arsenic (As^{3+}) has also been reported to alter lens αA-crystallin profile *in vivo* and induce cataract in the Indian major carp *Labeo rohita*[21]. Lens has been, therefore, a target tissue for searching biomarker suitable for aquatic environmental monitoring.

Rita rita, a freshwater catfish is predominantly found in the tropical rivers and occurs in the Indus and its affluent rivers, the Ganga and the Yamuna and also the Irrawaddy, Brahmaputra[22]. This fish is reported to be suitable for biomonitoring studies[23,24]. In the present study, lens proteome map of *Rita rita* has been generated for the first time and crystallin profiles as well as αA- and αB-crystllins expression have been investigated.

Materials and Methods

Preparation of lens protein extracts

Freshwater catfish *Rita rita* were collected from the river Ganga at Samudragarh, Kolkata and brought to the lab live. The fishes were sacrificed by over-anaesthetization by tricane methane sulphonate (MS 222, Sigma-Aldrich), lenses were carefully dissected, and stored frozen at - 40°C. The frozen lenses were thawed, cleaned to remove any contaminating tissues and then homogenized in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4), pH 7.3 containing protease inhibitor cocktail (Sigma P8340). The buffer was added at 200 µl per lens. The homogenates were centrifuged at 11,500 × *g* (13,000 rpm) at 4°C for 30 min in a high-speed refrigerated centrifuge (Biofuge *FRESCO*, Heraeus)[12] for removing the unsolubilized material. The extracts were aliquoted and stored at – 40°C till further use.

Protein estimation

Protein concentration of lens protein extracts was estimated by Lowry method[25] using BSA as the standard.

SDS-PAGE

The lens proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (1-D GE) in a Mini-Protean 3 (MP3) electrophoresis cell (Bio-Rad) using 5% (w/v) stacking gel and 10%, 12% and 15% separating gels[26]. The gels were either stained with Coomassie brilliant blue R250 (CBB) (or Coomassie-silver double-stained) for visualization of the protein bands.

2-D Gel Electrophoresis

For 2-dimensional polyacrylamide gel electrophoresis (2-D GE)[27], the lens proteins were resolved by isoelectric focusing in the first dimension, followed by SDS-PAGE in the second dimension. Isoelectrofocusing (IEF) was performed on immobilized pH gradient (IPG) strips (7 cm and 11 cm; pH 3-10, Bio-Rad and 11 cm; pH 5-8, Sigma) in a Protean IEF cell (Bio-Rad). The lens extract (about 150-200 µg protein) was premixed with 'rehydration buffer' (8 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10 ampholyte, and 0.001% bromophenol blue) and rehydration of the IPG strips was carried out for 12-16 h. The rehydrated strips were isoelectrofocussed at a current of 50 µA/strip at the stated voltage gradient: 250 V for 20 min, 8000 V for 2 h 30 min and 8000 V for 20000 V-h with an end voltage of ~30,000 V-h. After the IEF run, the IPG strips were equilibrated with the 'equilibration buffers' I and II (equilibration buffer I: 6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol, 130 mM DTT and equilibration buffer II: 6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol, 135 mM iodoacetamide) and then placed on 12% SDS-polyacrylamide slab gels for the second dimension run. The gels were either CBB stained or CBB-silver double-stained. Those IPG strips which were not run for second dimension immediately were stored at - 40°C.

1- and 2-D Immunoblot analysis

Immunoblot analysis was carried out to identify the αA- and αB-crystallins in the lens protein extract[28]. Transfer of proteins which were separated on 12% or 15% gels from polyacrylamide gels to nitrocellulose (NC) membranes (0.2 µm, N8017, Sigma) was carried out in a Mini TransBlot electrophoretic transfer cell (Bio-Rad) at 200 mA/2 h. Transfer was confirmed by staining the NC membrane with Ponceau S (P7170, Sigma). The primary antibodies used were anti-αA-crystallin affinity isolated antibody (C5990, Sigma), anti-αB-crystallin polyclonal antibody (AB1546, Millipore) and anti-phospho-αB-crystallin affinity isolated antibody (C7740, Sigma). Anti-rabbit IgG-peroxidase conjugate (A0545, Sigma) was the secondary antibody used. Diaminobenzidine (DAB) (D7304, Sigma) and H_2O_2 were used as substrates.

Results and Discussion

Protein estimation

Protein concentration of the lens extracts of *Rita rita* varied from 3.6-4.0 g/dL. Owing to such high protein

concentration, the lens extract often got crystallized, if not optimally diluted, when the 2-D sample buffer/IPG rehydration buffer was added to the sample for rehydrating the IPG strips. The ocular lens in vertebrates is composed of highly concentrated soluble proteins, the crystallins[9,13]. It contains more protein than common cells,~30-35% of the entire mass of the lens; correspondingly, the water content, which is usually ~95% in a cell is reduced in the lens to 65-70%. Analysis of these proteins and their characterization has been a challenge[29].

SDS-PAGE

A series of gels with different acrylamide concentration viz. 10%, 12%, 15% were run to obtain a finer resolution of the high and low abundant proteins (Fig. 1A-D). Coomassie and Coomassie-silver double-stained 10% SDS-polyacrylamide gels separated the lens extracts into 33 bands in the molecular mass range of 14 to >205 kDa (Fig. 1A-B). The high abundant crystallins resolved in the 20-29 kDa range. A 12% gel (Fig. 1C) was suitable for studying the high abundant proteins, whereas a 15% gel with silver staining was ideal for studying the low molecular mass low abundant proteins (Fig. 1D). In fact, when the lens proteins were extracted using PBS or any such buffer and centrifuged with an ordinary laboratory table top centrifuge at about 4500 × *g* and the supernatant was resolved on a 10% acrylamide gel, the high abundant proteins were visible with Coomassie-blue staining, although the other high and low molecular mass low abundant proteins were not resolved properly. That may be the reason for some earlier reports[30] showing the distribution of lens proteins in the pI range 4.3-9.0 and molecular mass range of 17.7-31 kDa, whereas actually the lens protein extracts contain proteins in a broad molecular mass range from 6 to > 206 kDa.

2-D Gel Electrophoresis

Silver-stained 2-D polyacrylamide gels (12%) separated the soluble lens protein extracts into 75-80 spots (Fig. 2A and C). Figure 2 shows representative 2-D gel profiles of the lens crystallins, where the lens extracts were focused on IPG strips of different pH range, pH 3-10 (Fig. 2A, C) and pH 5-8 (Fig. 2B, D) for the first dimension run. 2-D GE has been successfully used to describe the crystallin profiles and modifications that occur in the mammalian[7,8], chicken[15], crocodile[3,4,13], anurans[14], and fish[10-12] eye lens and cornea.

Fishes have adapted well to diverse habitats and survive in highly contrasting environmental conditions. They live in habitats as diverse as the warm waters of the tropics to the freezing waters of the Antarctics, from fresh waters to the extremely saline seawaters and from smallest brooks to large oceans, and therefore, employ different adaptation

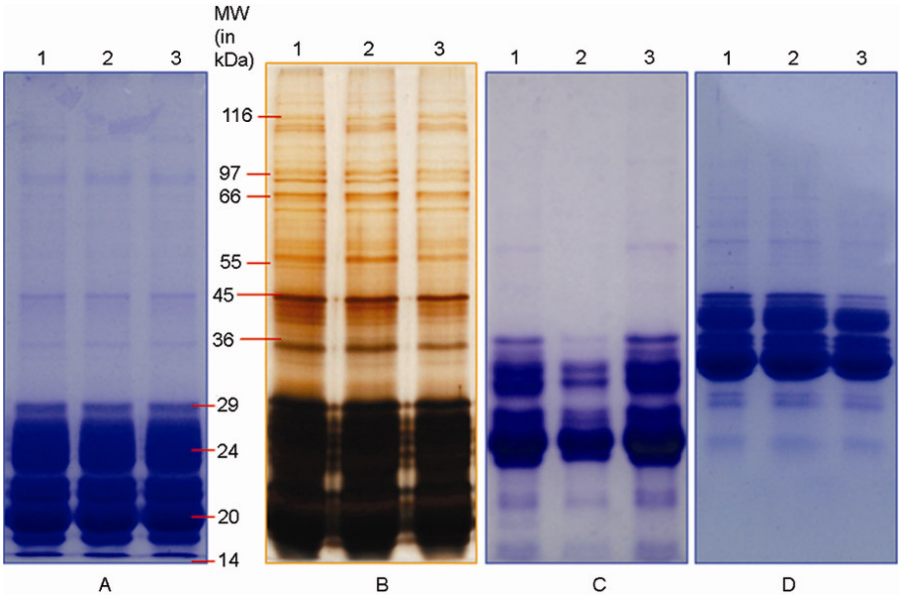

Fig. 1—SDS-PAGE profile of lens crystallins of *Rita rita* [The proteins were run at different acrylamide concentrations viz. 10% (A, B), 12% (C) and 15% (D) and the gels were Coomassie-stained (A, C, D) or Coomassie-silver double-stained (B) for visualization of the proteins. Lanes: 1-3 indicate soluble lens protein extract from different specimens. 30 µg of protein was loaded in each lane. Molecular weights indicated were determined by co-running the SigmaMarker (wide molecular weight range: 6,500-205,000 Da, M-4038)]

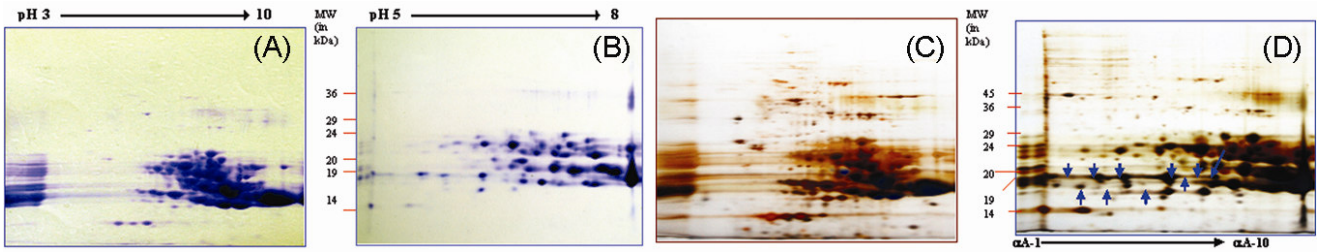

Fig. 2—Two-dimensional gel electrophoresis (2D-GE) profiles of adult *Rita rita* soluble lens proteins [Separation was performed on 11 cm IPG strips with pH gradients of 3-10 (A, C) and 5-8 (B, D). 150 µg protein was loaded for 2D-GE. Gels were either Coomassie-stained or Coomassie-silver double-stained. Molecular weights of proteins are indicated with reference to markers (M4038, Sigma). In Fig. 2D, the protein spots corresponding to αA-1 to αA-10 (with reference to 2-D immunoblot, Fig. 4) are shown with arrow heads]

mechanisms for survival. For example, the eye lenses of the Antarctic toothfish *Dissostichus mawsoni* are transparent at -2°C, whereas the cold-sensitive mammalian and tropical fish lenses display cold-induced cataract at 20°C and 7°C, respectively[31,32]. The complete lens transparency of *D. mawsoni* in the perennially freezing subzero temperature (-2°C) suggests a high degree of cold stability of their constituent lens proteins. Cold-cataract formation does not occur even at temperature as low as -12°C. It is also reported that a large amount of γ-crystallins are found in the fish species from evolutionarily diverse groups and this appears to be a general feature of the fish lens[31]. The abundance of small, polydiverse γ-crystallins (> 40%) allow tighter protein packing to produce the dense lenses needed for underwater vision[31]. Therefore, fish lenses are peculiar in many ways and studying the lens proteins of a varieties of fishes are likely to throw light on unknown facts that may add to the current understanding of the lens development and eye diseases. However, other than few species like zebrafish *Danio rerio*[9-12] and Antarctic toothfish *Dissostichus mawsoni*[31-33], information is lacking on other fish lens proteins.

In zebrafish, 2-D GE has resolved over 80 protein spots in soluble lens protein extracts[12]. Sixty of the spots have been identified as one of 28 different crystallins and another four as non-crystallins. Similarly, 2-D gel analysis of chicken lens crystallins has been resolved into ~70 protein spots, of which 40 spots have been identified as β-crystallins[15]. In the present study, soluble lens protein extracts of *Rita rita* were resolved to 75 protein spots, as determined by the 2-D image analysis software PD Quest (Bio-Rad).

Crystallins are the major structural proteins of the eye lens fiber cell cytoplasm. The crystallin polypeptides are localized and synthesized differently during the course of lens development and they differ significantly between species belonging to different classes of vertebrates[34,35]. Evolutionary analysis has demonstrated the relationship of crystallins to proteins involved in protection against stress. Among the different crystallins, α-crystallins play an important role in the formation of the unique physical properties of the eye lens, which provides a clear medium with a high refractive index. About 50% of the human lens proteins are composed of α-crystallins. The abundance of this protein signifies its importance in the lens not only as a structural protein, but also a molecular chaperone for β and γ-crystallins[10,36].

α-Crystallin has also been found to interact with other types of non-crystallin proteins and enzymes such as insulin, α-lactalbumin, alcohol dehydrogenase, and citrate synthase. Its dual function, as both a structural protein and a chaperone to prevent aggregation of other protein species, makes it a popular target for research[1]. Due to its long life in the lens, α-crystallin is also one of the best studied proteins with respect to post-translational modifications, including age-induced alterations[37]. In fish lens, γ-crystallins are the most abundant crystallin family and among its members, αA-crystallin is the major constituent in catfish[12,38]. The over-expression of αA-crystallin prevents γ-crystallin insolubility and cataract formation in the zebrafish cloche mutant lens[39]. Due to its role in normal lens development, we further worked on identification of αA-crystallin proteins by 1-D and 2-D immunoblot.

1- and 2-D Immunoblot analysis

To identify αA- and αB-crystallins, immunoblot analyses were carried out by running 1-D and 2-D gels of eye lens proteins. The bands were closely examined on the blots by analyzing the proteins at different acrylamide concentrations (12% and 15%). Two protein bands in the molecular mass range of 19-20 kDa were identified as αA-crystallins by 1-D immunoblot analysis on basis of their reactivity with

the anti-αA-crystallin antibody (Fig. 3A-B). However, we could not detect αB-crystallin, as immunoblot analysis with anti-αB-antibody failed to show any immunoreactive bands. Commercially available purified bovine α-crystallins were used as positive control and a 20 kDa immunoreactive band lighted up on immunoblot, when the NC membrane was probed with anti-αB-antibody (Fig. 3C). The blot was also probed with anti-phospho-αB-crystallin antibody to look for phosphorylated form of the αB-crystallin, if any. No immunoreactive band with *Rita rita* crystallin was visible, whereas with purified bovine α-crystallin four bands of 17, 18, 20 and 29 kDa were observed, of which the 20 kDa band was the major one (Fig. 3D). On 2-D immunoblot, two αA-crystallin bands got separated into ten discrete spots and these spots had different pI and molecular masses; these α-crystallin spots have been indicated as αA-1 to αA-10 (Fig. 4).

The commercially available anti-αA-crystallin antibody was raised in rabbit using purified αA-crystallin from bovine lens as the immunogen. This antibody detects αA-crystallin from bovine and rat samples and specifically recognizes a ~20 kDa protein, representing αA-crystallin from bovine lens samples. We observed strong immunoreactivity of this antibody with *Rita rita* lens proteins. On 1-D and 2-D immunoblots bands appeared on the NC membrane almost instantly on addition of the substrate. The strong immunoreactivity indicated that the αA-crystallin proteins of bovine and this catfish shared strong homology, although fish belongs to a clade phylogenetically distant from the bovines. However, in this study, anti-αB-crystallin and anti-phospho-αB-crystallin antibodies were not able to detect any immunoreactive bands or spots on 1- and 2-D immunoblots (Fig. 3C-D) and 2-D immunoblots (data not shown), implying that in the catfish *Rita rita,* αB-crystallin was either absent or present in extremely low concentration. Protein spot analysis from 2-D gels by more advanced techniques like matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) should be able to throw more light on distribution of αB-crystallin in *Rita rita.*

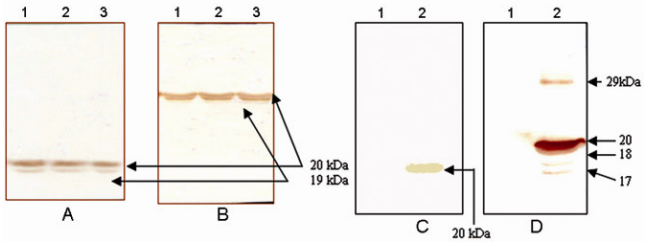

Fig. 3—(A and B): 1-D Immunoblots showing αA-crystallin bands on 12% (A) and 15% gels (B) [Anti-αA crystallin was used as the primary antibody (dilution 0.1 µg/ml) and anti-rabbit IgG-peroxidase conjugate (A0545, Sigma) was used as the secondary antibody (dilution 1/12000)]; (C): 1-D Immunoblots of lens crystallins with anti-αB-crystallin affinity isolated antibody [No band appeared in *Rita rita* eye lens extract (lane 1) whereas a strong band was seen in purified bovine α-crystallin (lane 2); and (D): 1-D Immunoblot of lens crystallins with anti-phospho-αB-crystallin affinity isolated antibody [No band appeared in lens crystallin extract of *Rita rita* eye lens (lane 1), whereas four immunoreactive bands were seen in purified bovine α-crystallin (lane 2)]

In the zebrafish lens, the relative amounts of the three α-crystallins viz. αA-, αBa- and αBb-crystallins have been determined[12]. α-Crystallins were observed in 13 different 2D-GE gel spots, suggesting several post-translational modifications (PTMs)[12]. By MALDI-TOF-MS on 2-D gels, Posner *et al.*[12] identified eight numbers of spots as αA-crystallins. α-Crystallin is the major protein of the mammalian lenses (up to 50%)[40], whereas in zebrafish it constitutes less than 10% of the lens proteins[12]; however, in some other fish species, α-crystallin proportions in lens have been reported to be 12% and 41.6%[41,42]. α-Crystallins under stress conditions can act as molecular chaperones by preventing the precipitation of partially unfolded target proteins[43,44] via the formation of a stable, soluble, high molecular-mass small HSP (sHSP)-target protein complex; however, unlike other molecular chaperones that are involved in protein folding, α-crystallins have no ability to refold target proteins[45]. It has been proposed that the chaperone activity of α-crystallin, i.e. its ability to prevent crystallin aggregation may be important in maintaining an optically transparent lens[44].

α-Crystallins consist of two polypeptides, αA- and αB-crystallin. The molar ratios of distribution of these

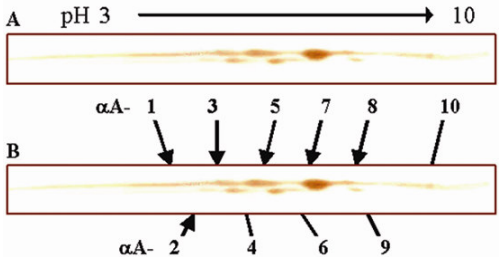

Fig. 4—2-D Western-blots showing αA-crystallin spots (12% gel) [(A): Original unlabelled blot showing protein spots which were recognized by anti-αA-crystallin affinity isolated antibody, and (B): Same blot-labeled to show the individual αA-crystallin spots, numbered from left to right. First dimension run was carried out on IPG strip; 7 cm, pH 3-10 (Bio-Rad) and second dimension run was carried out on 12% SDS-polyacrylamide gels. Antibodies used were same as mentioned in Fig. 2 A, B]

two isoforms is quite interesting (Table 1); it varies from 19:1 in catfish[46] to 1:5 in dogfish[47], suggesting that in catfishes 95% or more of the α-crystallins are αA-crystallin, whereas in the dogfish 85% of the α-crystallins are αB-crystallin. This is not true for all the lower vertebrates; in the zebrafish lens, the ratio (αA: αB) is 3:2, which is similar to humans (3:2) and other higher vertebrates, bovines and mouse (3:1). Again, contrastingly in another higher vertebrate kangaroo, the molar distribution (10:1) is similar to the lower vertebrates (catfish); more than 90% of the α-crystallins are of the αA-type. The structure and function of the αA-crystallin appear to have been conserved, but a αB-crystallin gene duplication in the zebrafish has led to a possible divergence of the structural and functional properties in the two polypeptides[10,11,31,46]. Comparing and contrasting the α-crystallins of the dogfish and *Rita rita* should be able to provide valuable information on the evolutionary basis and functional attributes of αA- and αB-isoforms, as they are at the two extremes in terms of αA- and αB-crystallin content.

The lead (Pb^{2+}) has been demonstrated to be toxic to lens because it alters lens biochemical homeostasis and also alters the post-translational protein expression profile of αA-crystallin and βA4-crystallin[20]. Calcium-binding to βB2- and βA3-crystallins has been recently reported and it has been suggested that all the β-crystallins are calcium-binding proteins. These findings have important implications for understanding the calcium-related cataractogenesis and maintenance of ionic homeostasis in the lens[48]. Recently, patterns of crystallin distribution in porcine eye lens have been reported[49]. There is close sequence homology between porcine and human αB-crystallins. Another interesting feature is that in porcine lens, β-crystallins are the major soluble proteins which comprise 45% of the total lens proteins, whereas the percentage of α-crystallins varies from 35% to 22% from outer to inner lens[49].

To conclude, in the present study, lens proteome map of the tropical catfish *Rita rita* has been generated for the first time. αA-crystallins have been identified and distribution of αA- and αB-crystallins has been studied. It would be interesting to identify the other members of crystallin family in *Rita rita*, including the β-, γ-crystallins and other taxon-specific crystallins, if any and compare the crystallin profiles with that of zebrafish and other species. The information generated in the present study may serve as the baseline data for further investigation on comparative lens proteomics which may help in advancing the current understanding on crystallin modifications leading to cataract. Further, as there are reports suggesting the lens to be a target organ for aquatic pollution monitoring[14,15], proteomic studies of *Rita* lens crystallins may also help in pollution biomarker discovery.

Table 1—Relative distribution of αA- and αB-crystallins in the higher vertebrates (mammalians) and lower vertebrates (piscines)

Species	αA:αB Crystallin	αA-Crystallin (%)	αB-Crystallin (%)	Ref
Higher vertebrates				
Human	3:2	60	40	50
Bovine	3:1	75	25	47
Mouse	3:1	75	25	7
Kangaroo	10:1	91	9	49
Lower vertebrates				
Dogfish	1:5	15	85	40
Zebrafish (*Danio rerio)*	3:2	60	40	12
Asian catfish (*Clarias batrachus)*	19:1	95	5	46
Fresh water catfish (*Rita rita)*	19:1	> 95	< 5	Present study

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