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LENS PROTEOMICS: EXPLORING ADAPTIVE CONFLICT IN *UROMASTYX HARDWICKII* LENS PROTEINS

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ABSTRACT

During last decade, proteomics has emerged as a powerful tool for high throughput screening, identification and quantification of proteins from biological samples. In current study, we have employed proteomics technique to explore proteins from eye lenses of *Uromastyx hardwickii*, an Indian spiny-tailed lizard. Water soluble and insoluble proteins from *Uromastyx hardwickii* lens were separated and characterized by 2-DE (two dimensional electrophoresis). After in-gel digestion, well resolved spots were identified by nano- liquid chromatography-electrospray tandem mass spectrometry (nLC-MS/MS). We have identified αA-, βA2-, βA4-, βB2- and βB3-crystallins as ubiquitous crystallins and δ-, τ-crystallins as taxon-specific lens crystallins in water soluble fraction. αA- and βB3-crystallins were found to be most abundant water soluble proteins in *Uromastyx hardwickii* lens. Among non-crystallins (cellular proteins), β-actin was identified. From water insoluble fraction, vimentin, gelsolin, gamma enolase like protein, filensin like protein and ATP synthase subunit B were identified. Our results illustrate advantage of proteomics technology for identification of lens proteins. Furthermore, the study provides distribution patterns of ubiquitous and taxon specific crystallins from *Uromastyx hardwickii* that is likely to be useful in understanding evolutionary lineage of this organism.

Key words: Lens proteomics, ubiquitous lens crystallin, taxon specific lens crystallin, *Uromastyx hardwickii*, water insoluble lens protein.

INTRODUCTION

Ocular lens of vertebrates is highly specialized tissue present from jelly fish to human with stable proteins having no turn over (Harding and Crabbe, 1984). Lens transparency, refractive index (Bloemendal, 1981) and lens fiber remodeling (Piatigorsky, 1981) are maintained by lens proteins. Lens comprises of long lived water soluble and insoluble proteins. Water soluble proteins are mainly classified into ubiquitous crystallins, taxon specific crystallins and non-crystallins. Ubiquitous crystallins comprise of α, β and γ crystallin which are most prevalent in vertebrates and invertebrates (Bloemendal, 1981). Taxon specific lens crystallins, however, were evolved after ubiquitous group and are recruited from metabolic enzymes in response of environmental stress (Wistow, 1993). This process of recruitment has successfully continued in selective set of species until now showing their constant evolutionary advantage (de Jong *et al*. 1989). In most of the species of reptiles and birds, however, γ-crystallins is gradually reduced and diminished due to the over expression of recruited enzyme crystallins as structural protein (Wistow and Piatigorsky, 1988). Besides water soluble crystallins, eye lens also contains non crystallin proteins including various metabolic enzymes which are involved in protein, DNA, RNA biosynthesis and degradation (Hockwin and Ohrloff, 1981). Lens fiber cells also express water insoluble proteins along with water soluble lens protein during lens differentiation process (Bloemendal *et al.* 1972). Lens cytoskeletal proteins, lens specific beaded filament proteins and fiber cell membrane proteins are major water insoluble proteins. Major cytoskeletal proteins are spectrin, beta-actin, vimentin and tubulin. These proteins play very important role in maintaining membrane architecture and remodeling during cell differentiation (Piatigorsky, 1981). Beaded filament proteins are known as filensin and phakinin (Graw and Löster, 2004). Lens fiber cell membrane plays crucial role in maintaining transparency as it manages transport activity including movement of water, ions and small molecules (Piatigorsky, 1981).

Proteomics is fast-evolving high throughput technique used for the screening, identification and quantification of complex protein mixture in biological samples. Proteomic approach using two dimensional electrophoresis (2-DE) followed by mass spectrometric analysis facilitates the detailed study of protein expression. Investigations of protein expression from different species provide significant comparative data to study their evolution (de Jong and Hendriks, 1986; Wistow and Piatigorsky, 1988). Proteomic analysis data has been reported from some mammalian (Hoehenwarter *et al.* 2008), amphibians (Keenan *et al*. 2012), fishes (Posner *et al.* 2008) and bird lenses (Wilmarth *et al.* 2004). Differential proteomics analysis was reported in human diabetes and cataract lenses (Zhu *et al.* 2013). Detailed comparative proteomic map from zebra fish and mouse lens has also been presented to study the

relationship between insolubility and truncation of water soluble lens proteins (Ueda *et al.* 2002; Posner *et al.* 2008). Till date, proteomics analysis of reptilian lens protein has not been reported.

In present study, water soluble and insoluble lens proteins from a reptile, *Uromastyx hardwickii* were characterized and identified. We have used proteomic tools including two dimensional gel electrophoresis (2-DE) and nLC-MS/MS analysis to study the water soluble and insoluble proteins from *Uromastyx hardwickii* lens.

MATERIALS AND METHODS

Sample collection: The study was conducted after approval from Institutional Review Board. Instructions given in the *Guide for the Care and Use of Laboratory Animals* (National Academy of Science, National Academy Press,Washington, D.C.) were followed throughout the study. *Uromastyx hardwickii* eyes were dissected and lenses were removed and stored at -80°C till further utilized.

Extraction of Total Lens Protein: Fresh lenses were homogenized in 50mM Sodium phosphate buffer, pH 7.0 on ice using glass homogenizer and centrifuged at 15,000 x g for 20 min at 4°C. The supernatant and pellet were collected and labeled as water soluble fraction (WSF) and water insoluble fraction (WIF), respectively. Total protein was estimated by Bradford kit method (Bio-Rad).

SDS-PAGE: Molecular weights of WSF and WIF fractions were determined by sodium dodecyl sulpate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) using 12% (w/v) polyacrylamide gel. Samples were dissolved in 3 X sample dissociation buffer and heated at 95º C for 3 minutes. After samples loading, electrophoresis was run at constant voltage of 65 volts till the dye front reached the bottom of the gel. Gel was fixed over night in gel fixing solution (50% ethanol, 2% phosphoric acid, Milli Q water) followed by washing thrice with Milli Q water. Staining of gel was performed with Coomassie brilliant blue R-250 for visualization of protein bands. Gel images were acquired by Gel Doc (Quantity One software, Bio-Rad).

Two dimensional electrophoresis (2-DE): Four lenses were homogenized in lysis buffer (0.5 M Tris-HCl pH 6.8, 0.25 M EDTA, Urea, 0.5 M dithiothreitol (DTT), Glycerol, NP40, ampholyte buffer pH 3-10) and centrifuged at 15,000 x g for 20 min at 4°C. The pellet (WIF) and supernatant (WSF) were collected separately. To remove salts and impurities from protein samples, 2-D cleanup kit (Bio-Rad) was used. Protein pallet was dissolved in rehydration buffer (8 M urea, 2% 3-[(3-cholamidopropyl) dimethylammonio-2-hydroxy-1-propanesulfonate (CHAPS), 2% 3-10 IPG buffer, 50 mM DTT, and a trace amount of bromophenol blue). Protein (75-80 mg) was loaded on immobilized pH gradient (IPG) strips (7cm, pH: 3-10 NL, Bio-Rad) by using passive rehydration method. Mineral oil was layered on IPG strip to prevent evaporation. First dimension or IEF was performed to a total of 10000 V/h at 20 ºC using Multiphor II Amersham Biosciences apparatus. After IEF run, IPG strip was equilibrated in equilibration buffer I (0.5 M Tris-HCl pH 6.8, 12 M Urea, 10 % SDS, 60% glycerol, 10 mg/ml DTT) and then in equilibration buffer II (0.5 M Tris-HCl pH 6.8, 12 M Urea, 10 % SDS, 60 % glycerol, 25 mg/ml Iodoacetamide) for 30 minutes each. Second dimension was performed on 12% polyacrylamide gel at 65 volts for 2 hrs. To visualize protein spots, gel was stained in coomassie brilliant blue R-250. Gel images were acquired using PD Quest software (Bio-Rad).

Identification of proteins using LC-MS/MS: To identify water soluble and insoluble proteins, well resolved spots were excised from the gels using Ex-Quest spot cutter (Bio-Rad). Protein spots were destained with 100 mM Ammonium Bicarbonate and 1:1 Acetonitrile:water. In-gel tryptic digestion was performed with 2ng/µL trypsin in 50 mM ammonium bicarbonate overnight at 37^0C and resulting peptides were extracted with 25 mM Ammonium Bicarbonate, Acetonitrile and 10% Formic acid. Extracted peptides were vacuum dried using Vacufuge® vacuum concentrator (Eppendorf®). The dried peptides were reconstituted with 0.1% Formic acid and subjected to linear ion trap MS (LTQ XL Thermo Fisher Scientific) interfaced with nano LC system for nLC-MS/MS analysis. A 1 µL sample was injected through an auto-sampler into the nLC system at the flow rate of 300 nL/min. The peptides were eluted using 75 µm I.D x15 cm Pep-Map 100 C-18 nano column. The column was equilibrated with 96.8 % A (0.1% Formic acid) and 3.2% B (98% Acetonitrile, 2% water, 0.1% Formic acid). Peptide separation was achieved with multi-step gradient from 3.2% to 80% solution B over 70 min. The obtained peptide spectra were searched against SWISS-PROT or NCBI nr databases using Mascot algorithm through Proteome discoverer 1.2. Parameters used for Mascot search were: Peptide mass tolerance ± 1.5 Da, MS/MS tolerance ± 0.5 Da, carbamidomethylation at Cys and Oxidation at Met as fixed and variable modifications respectively. Protein identification was carried out with Mascot scores above the threshold level ($p< 0.05$).

RESULTS

SDS-PAGE analysis of lens water soluble and water insoluble proteins: Water soluble and water insoluble protein fraction from *Uromastyx hardwickii* lens were

applied on SDS-PAGE (Fig. 1). In both fractions, many protein bands in a range of 14-200 kDa were observed.

Identification of water soluble lens proteins*:* Water soluble fraction of *Uromastyx hardwickii* lens was resolved on 2 DE using IPG strip pH: 3-10NL. Gel images were acquired and analysed by PDQuest software (Bio-Rad). Well resolved spots were excised from 2 DE gel by using Ex-Quest spot cutter (Bio-Rad).

Identification of ten spots by mass spectrometry revealed members of ubiquitous crystallin group (αA, βA4, βA2, βB2 and βB3), taxon specific crystallins (δ and τ) and cellular proteins (Table1).

We found two spots of αA-crystallin with same molecular weight of 17 kDa but different pI (Fig. 2). pI of spot 9 is 5.6 and spot 10 is approximately 4.7. In lenses of *Uromastyx hardwickii*, both acidic and basic group of β-crystallin were identified by mass spectrometry analysis (Table 1). Among acidic β-crystallins group, identified proteins were βA2, βA4 while basic group included βB2 and βB3. Predicted molecular weight of βA2 and βA4 is 22.4 and 25.6 kDa respectively. pI value of βA2 and βA4 were 6.42 and 6.39 respectively. In basic β-crystallins, βB3 was found abundantly in *Uromastyx hardwickii* lens. MS analysis of spot 4 showed that βB3-crystallin having predicted molecular weight 22 kDa but in our 2-DE pattern, molecular mass of spot 4 was approximately 26 kDa and its pI is 5.39. Spot 5 on 2-DE profile was identified as βB2-crystallin having 25 kDa molecular mass and its pI value is 8.35. Two members from taxon-specific crystallins group including δ2, and τ-crystallin were identified from lenses of *Uromastyx hardwickii*. δ2-crystallin was identified from 2-DE gel spot 2 (Table 1). Spot 1 on Fig. 2 was identified as τ-crystallin/α-enolase by nLC-MS/MS analysis. Fig. 3 depicts representative MS/MS scan of an in-gel tryptic digest of a spot from 2-DE. Although a recent report indicates presence of ε-crystallin in *Uromastyx hardwickii*, we were unable to identify the same using proteomics approach (Atta *et al.* 2014). Among non-crystallins, one cellular protein was identified as β-actin (Table. 1).

Identification of water insoluble lens proteins: Pellet of WIF was dissolved in rehydration buffer. For the characterization of lens water insoluble fraction (~80 µg), 2-DE (IPG strip pH: 3-10 NL) was performed (Fig. 4). We excised well resolved spots from coomassie blue stained 2 DE gel by using Ex-Quest spot cutter (Bio-Rad). Only five spots gave significant result (Table 2). The most abundant protein spot was identified as filensin like protein. Other identified spots included gelsolin, vimentin, gamma enolase, ATP synthase and cytoskeletal protein. Score, % coverage, calculated molecular weight and pI values of water insoluble proteins are shown in Table 2.

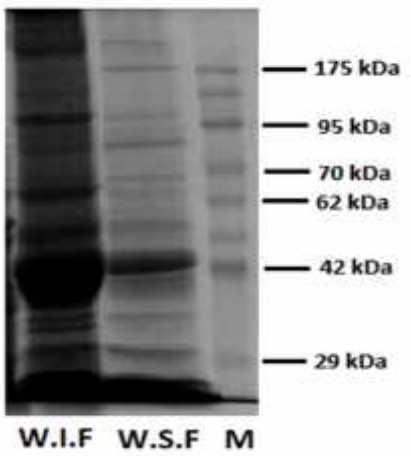

Fig. 1: SDS-PAGE profile of water soluble and insoluble lens proteins from *Uromastyx hardwickii*. W.S.F: Water soluble lens fraction, W.I.F: Water insoluble lens fraction, M: Protein molecular weight marker.

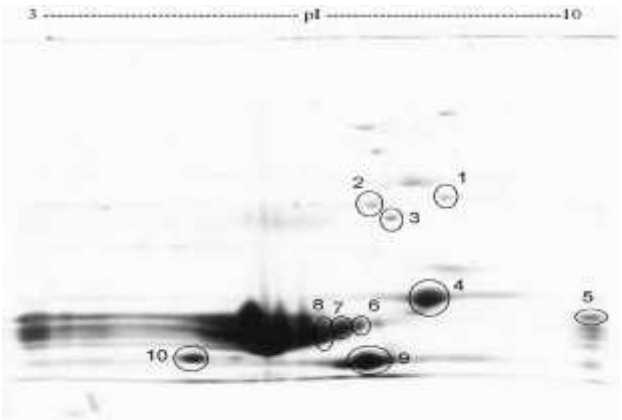

Fig. 2: Two dimensional gel electrophoresis profile of water soluble lens proteins from *Uromastyx hardwickii* using 7cm IPG strip pH: 3-10 NL. Labelled spots were further identified by nLC-MS/MS.

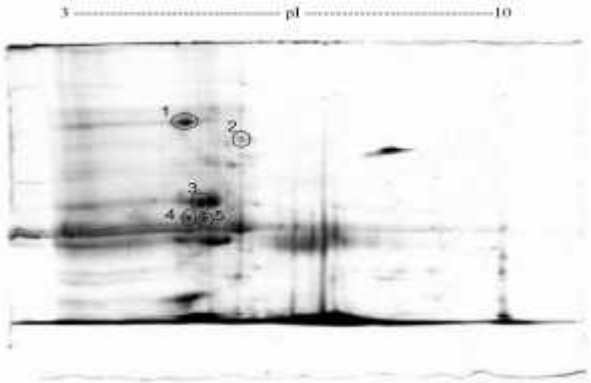

Fig. 3: Two dimensional gel electrophoresis profile of water insoluble lens proteins from *Uromastyx hardwickii* using 7cm IPG strip pH 3-10 NL. Labelled spots were further identified by nLC-MS/MS.

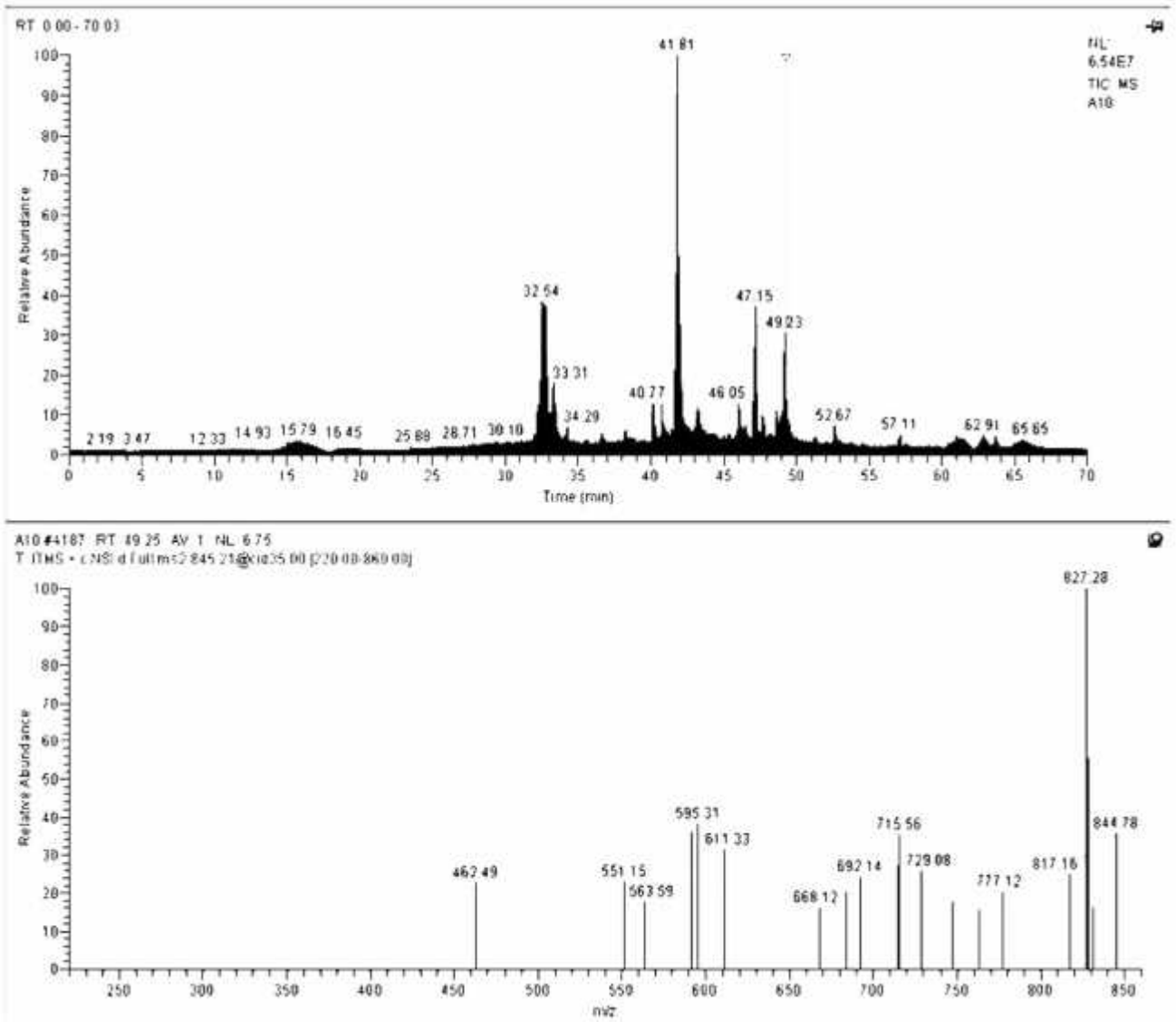

Fig. 4: Mass spectrum of tryptic peptides from a spot in 2-DE gel (top). Top five peptides with m/z values were selected for MS/MS analysis (bottom).

Table 1: Water soluble proteins from *Uromastyx hardwickii* lens as identified by nLC-MS/MS.

Spot No.	Protein	Accession No.	Mass (kDa)	pI	Coverage %	Score	Specie
1	τ-crystallin (α-enolase)	NP001020559	47	6.37	7	237	*Mus mscullus*
2	δ2-crystallin	AAA83149	51.7	6.15	2	104	*Anas platyrhynchos*
3	β-actin	AAC59891	42	5.3	46	786	*Takifugu rubripes*
4	βB3-crystallin	IPI005999618	22	5.39	7	37	*Chelonia mydas*
5	βB2-crystallin	Q05714	25	8.35	16	278	*Gallus gallus*
6	βA2-crystallin	AAN78174	22.4	6.42	20	137	*Iguana iguana*
7	βA2-crystallin	AAN78174	22.4	6.42	6	70	*Iguana Iguana*
8	βA4-crystallin	NP001080829	25.6	6.39	20	433	*Xenopus larvis*
9	αA-crystallin	P02506	19.8	5.78	26	358	*Tupinambis teguixin*
10	αA-crystallin	CAF02103	17.1	5.63	7	76	*Lygodactylus picturatus*

Table 2: Water insoluble proteins from *Uromastyx hardwickii* lens as identified by nLC-MS/MS.

Spot No.	Protein	Accession No.	Mass (kDa)	pI	Coverage %	Score	Specie
1	Filensin like	XP_003215278	75.8	4.87	3	136	*Anolis carolinensis*
2	Gelsolin	XP_002188662	85	6.01	5	256	*Taeniopygia guttata*
3	Vimentin	XP_004578639	53.6	5	25	660	*Ochotona princeps*
4	Gamma enolase like	XP_003202726	47.6	4.81	56	1225	*Meleagris gallopavo*
5	ATP synthase subunit β	Q5ZLC5	56.6	5.59	9	211	*Gallus gallus*

DISCUSSION

Current investigation deals with proteomics analysis of lens proteins from lizard *Uromastyx hardwickii.* We conducted this study to explore water soluble and insoluble lens proteins from *Uromastyx hardwickii*. Although genome sequence of *Uromastyx hardwickii* is not available, we found matches with other lizards/vertebrates and were able to assign many proteins with reasonable significance. This is the first report on proteomic analysis of water soluble and insoluble proteins from a member of reptilian family. We have found αA-, βA2-, βA4-, βB2- and βB3-crystallin as ubiquitous lens crystallins. In taxon-specific group, τ- and δ-crystallin were identified by nLC-MS/MS. β-Actin (non-crystallin) was also identified from *Uromastyx hardwickii* lens.

In current investigation, αA-crystallin was abundantly found from *Uromastyx hardwickii* lens. αA-crystallin also acts as chaperon which prevents insolubility (denaturation) of their surrounding proteins (Horwitz, 1992). In 2-DE gel (Fig 2), we observed two spots of αA-crystallin with different pI. It has been suggested that different forms of same protein along with variable mass might represent truncated proteins while same proteins with different isoelectric points are likely to result from modification (Posner *et al.* 2008). Our observation indicated same protein with different pI that might reflect a possible modification in αA-crystallin. However, N-terminal sequence analysis is still needed to examine the exact nature of αA-crystallin modifications. αA-crystallin was found to be an abundant protein in *Uromastyx hardwickii* lens that may be involved in maintaining transparency as well as acting as a chaperon. Among ubiquitous lens crystallins, we also identified βA2, βA4, βB2 and βB3. Two spots of βA2-crystallin were identified from 2-DE map having same mass with slight difference in their pI value. The βA4-crystallin and βA2-crystallin spots were very near to each other in 2-DE gel. Among acidic β-crystallins, βA4-crystallin was more abundant than βA2-crystallin. Furthermore, βB2- and βB3-crystallin were identified from basic β-crystallin group in which, the latter was present in larger concentration than the former.

Beside ubiquitous group, some vertebrates also contain bi-functional taxon specific crystallins that have evolved through gene recruitment phenomenon (Wistow *et al.* 1994). The rationale for recruitment of different enzymes as lens crystallins is still unclear. The requirement of catalytic activity is unlikely to be the reason for the presence of high concentrations of enzyme crystallin in lens as, in few cases, enzyme crystallins have lost their catalytic role either due to post-translational modification or due to gene duplication phenomenon (Piatigorsky and Wistow, 1991). The gene coding for recruited enzyme is likely to be exposed to different selective pressures during the process of evolution in order to retain catalytic activity along with high expression to maintain transparency and proper refractive properties of the lens (Horwitz, 1992). Such environmental pressures and adaptive conflict in animals is helpful in understanding the structure-function relationship of crystallins as species move from water to land, ground to air and from dark to light (Wistow *et al.* 2005). These proteins have been a subject of interest to study gene recruitment phenomenon in many organisms. In a previous study, ε-crystallin/LDH like protein was identified as taxon specific crystallin from *Uromastyx hardwickii* lens (Atta *et al.* 2014). In this investigation we have found τ- and δ-crystallin from *Uromastyx hardwickii* lens. τ-crystallin was found in reptiles (Williams *et al*. 1985), birds (Kim *et al*. 1991) and some fishes (Stapel and de Jong, 1983), suggesting an ancient origin in evolution of vertebrates. In case of reptiles, evidence for τ-crystallin was found only in turtle (Williams *et al.* 1985) and crocodile lenses (Wistow, 1995). δ-crystallin have been found to be recruited first as taxon specific lens crystallins during evolutionary process (Williams and Piatigorsky, 1979). In order squamata, Calotes versicolor (Pal *et al.* 1982) and Gecko (Roll *et al.* 1996) have been studied for water soluble lens proteins. Gecko adapts three taxon specific lens crystallins that are τ, δ, and ι-crystallin (Roll and de Jong, 1996). In *Uromastyx hardwickii* lens, the presence of metabolic enzymes as adaptive conflict with dual role along with ubiquitous crystallins might serve as protective mechanism for survival. Among non-crystallins, we have identified β-Actin. β-Actin plays an important role in the architecture of the cell, formation of cellular junctions and cell motility (Cavey and Lecuit, 2009).

Earlier studies have mostly focused on water soluble lens proteins while limited studies have been conducted on water insoluble lens proteins from mammalian eye lens (Ruttenberg, 1965; Lasser and Balazs, 1972). Water insoluble lens proteins became most suitable subject for researcher because plasma membrane and lenticular matrix proteins play vital role in maintaining lens transparency, membrane architecture and remodeling during cell differentiation process (Piatigorsky, 1981). In mammal, insoluble lens cytoskeleton proteins have been also reported (Fleschner, 2002). In this study, we identified Filensin like protein, Gelsolin, Vimentin, Gamma enolase like protein and ATP synthase subunit B from insoluble fraction of *Uromastyx hardwickii* lens.

Current study provides baseline data of lens crystallins from a member of reptilian family, *Uromastyx hardwickii* which has huge evolutionary distance from human but it has an important position in evolutionary lineage. This lizard is diurnal, hibernating, ground-dwelling animal and survives in all challenges of high temperature, arid area and limited diet resources (Ramesh

and Sankaran, 2013). For survival, *Uromastyx hardwickii* is well adapted animal in all situations to cope with their surrounding environment pressures. Due to their diversified environment, *Uromastyx hardwickii* is a fascinating animal model in different aspects of research (Ahmed *et al*. 2006; Barka-Dahane *et al.* 2010). We have identified αA-, βA2-, βA4-, βB2-, βB3-, δ-, τ-crystallin and β-actin as water soluble lens proteins. Among insoluble lens proteins, vimentin, gelsolin, gamma enolase like protein, filensin like protein and ATP synthase subunit B were found from lenses of *Uromastyx hardwickii.* Our data set provides helpful information in the perspective of tissue specific or specie specific protein expression, lens development mechanism and visual adaptive conflict in species under selective pressures during evolution. Proteomics analysis of lens proteins is likely to be helpful in exploring new avenues of visual system research.

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