Pakistan J. Zool., vol. 48(4), pp. 1147-1153, 2016.

Structure Function Relationship of Lactate Dehydrogenase/ ε–Crystallin from Lenses of Indian Spiny-Tailed Lizard (*Uromastyx hardwickii)*

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ABSTRACT

Eye lens is a fascinating object to study protein evolution. Lenses contain highly stable long-lived proteins with the least turnover. Lens transparency is maintained by water soluble proteins classified into ubiquitous (α, β and γ crystallin) and taxon-specific lens crystallins. Ubiquitous crystallins show great diversity among vertebrates and invertebrates due to selective pressure leading to adaptive conflict. Taxon-specific lens crystallins, on the other hand, are products of gene sharing or gene duplication phenomenon where metabolic enzymes are recruited as crystallins in addition to their structural role. Current investigation was designed to study the evolutionary changes and discriminating adaptations in lens proteins. For this purpose, Indian spiny-tailed lizard *Uromastyx hardwickii* was used as an evolutionary model. We report partial nucleotide sequence of LDH/ε-crystallin comprising of 628 bp corresponding to 209 amino acids from lenses of *Uromastyx hardwickii*. To get more insight about the structure-function relationship, bioinformatics tools were used. Homology between sequences of ε-crystallin and LDH and conservation of catalytic residues indicated that ε-crystallin is a result of recruitment through gene sharing phenomenon.

Article Information
Received 18 March 2015
Revised 8 December 2015
Accepted 4 1 January 2016
Available online 1 June 2016

Authors' Contribution
SZ conceived and designed the study. AA prepared lens samples, performed RT-PCR, *in silico* studies and wrote the article. AI executed the DNA sequencing and ZH gene expression studies.

Key words
Biochemical adaptation
Lactate dehydrogenase B
ε-crystallin, gene sharing
Uromastyx hardwickii

INTRODUCTION

Ocular lens is an interesting object to study evolution, cell differentiation, aging and selective adaptation. In lens, transparency and light refractive properties are maintained by water soluble proteins known as crystallins (Bloemendal, 1981). Crystallins are long-lived, stable proteins with the least turnover (de Jong *et al*., 1989) and can resist various toxic environmental pressures. Crystallins consist of two groups known as ubiquitous (α, β, γ crystallin) and taxon-specific lens crystallins. The members of former group are abundantly found in most of the living organisms. The latter group, however, has limited distribution and has been evolved by a gene recruitment phenomenon (Wistow, 1995).

Taxon-specific crystallins were first discovered in lenses from birds followed by many studies in other species (Williams and Piatigorsky, 1979). Birds and reptiles have diverged approximately 200 million years ago and still share several morphological characters (Keeton, 1972). Major taxon-specific lens crystallins in birds and reptiles are ε-crystallin and δ-crystallin (de Jong *et al*., 1981). ε-crystallin is closely related to LDH-B enzyme, is catalytically active and contains a dinucleotide fold for NADH binding (Wistow *et al*., 1987). Duck lens ε-crystallin and LDH-B are suggested to be the products of same gene, evolved by gene sharing phenomenon (Hendricks *et al*., 1988). ε-crystallin has evolved at a slower rate (2 to 4 substitutions per 100 residues per 100 million years) as compared to other crystallins. ε-crystallin was first observed in avian lenses (Stapel *et al*., 1985). Later, ε-crystallin was discovered in reptilian lens but was not found in other taxa (Stapel *et al*., 1985). The level of LDH/ε-crystallin varies from species to species. Wistow *et al*. (1990) reported up to 40% of total soluble proteins from lens of humming bird. In birds and reptiles, it was found up to 23% of total water-soluble proteins (Wistow *et al*., 1987, Stapel *et al*., 1985). The only complete sequence of ε-crystallin has been reported from duck lens (Hendricks *et al*., 1988) while among reptiles, members of order Crocodilia (caiman, crocodiles and alligator) (Stapel *et al*., 1985) and Squamata (*Gecko phelsuma* and *Uromastyx hardwickii*) showed existence of ε-crystallin (Roll *et al*., 1996; Atta *et al*., 2014). These investigations, however, mostly dealt with kinetic, immunostaining detection and partial sequence studies. Chiou and colleagues (Chiou *et al*., 1991) reported the presence of ε-crystallin in caiman lenses. They have conducted kinetic studies of caiman ε-crystallin and compared their results with duck ε-crystallin. Another study (Stapel *et al*., 1985) highlighted presence of ε-crystallin in alligator, Heron and tufted duck using two-dimensional electrophoresis. The presence of ε-crystallin in gecko lens was confirmed by using an immunostaining method (Roll *et al*., 1996). A recent study has reported partial characterization of LDH like crystallin from

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0030-9923/2016/0004-1147 $ 8.00/0

Uromastyx hardwickii at mRNA level (Atta *et al*., 2014). In this communication, we report the partial sequence of LDH/ε-crystallin like protein from the water-soluble lens fraction of *Uromastyx hardwickii*. Furthermore, the observed sequence has been explored to examine structure-function relationships.

MATERIALS AND METHODS

Sample collection

Fresh lenses from *Uromastyx hardwickii* were obtained after the approval of Institutional Review Board to conduct this study. All procedures were in accordance with the standards set forth in the Guide for the Care and Use of Laboratory Animals (National Academy of Science, National Academy Press, Washington, D.C.).

Gene expression analysis using RT-PCR

Total RNA was extracted from *U. hardwickii* lenses by using SV total RNA isolation system kit (Promega, USA) according to manufacturer's protocol. mRNA was reverse transcribed using SuperScript® III First-Strand Synthesis System (Invitrogen, UK). PCR of *ε-crystallin/LDH* gene was performed using total lens cDNA as template. Each 50 µl reaction volume contained cDNA (5µg), 2X Master Mix (GoTaq® Green Master Mix (2X) Promega, U.S.A) with 0.4µM sense and 0.4µM antisense primers respective to *LDH* gene. Primers were designed from *LDH* fingerprinting and phosphate-specific binding region (NAD(H) pyrophosphate binding site) (Birktoft *et al*., 1982). PCR blank and RT blank were used as negative controls. Primer sequences and annealing temperatures (Tm) are given in Table I. Four sense and one common antisense primer was used. PCR conditions were: denaturation at 94°C for 2 min, annealing at 53- 55°C for 1.5 min and extension at 74°C for 1 min. 35 cycles of PCR were performed for gene amplification in gradient thermocycler (Eppendorf).

PCR products were visualized by using agarose gel electrophoresis. 2% agarose gel was prepared in TBE buffer (45mM Tris, 45mM boric acid, 1mM EDTA) pH 8.5. Ethidium bromide (0.5µg/mL) was used to visualize PCR products under UV light using gel documentation system (Bio-Rad). 100 bp DNA ladder was used as marker.

DNA sequencing

Purified PCR products and 10 pmol forward and reverse primers were directly sent to First BASE Laboratories (Selangor, Malaysia) for DNA sequencing. Analysis of DNA sequences was done by using basic local alignment search tool (BLAST) (www.ncbi.nlm.nih.gov/blast). DNA sequence was submitted to GenBank data base.

Bioinformatics analysis

In silico translation was performed using translate tool of ExPASy server (http://web.expasy.org/translate/) and resulting amino acid sequence was submitted to online automated three-dimensional structure prediction server SWISS-MODEL (http://swissmodel.expasy.org/). The coordinates of human lactate dehydrogenase (PDB ID: 1I0Z) were used as a template which showed 90% sequence similarity with target sequence. The model was evaluated by PROCHECK (Laskowski *et al*., 1993) while domain identification was performed using InterPro (https://www.ebi.ac.uk/interpro). For multiple sequence alignment CLUSTALW online tool was employed (http://www.genome.jp/tools/clustalw/). BOXSHADE alignment tool (Version 3.21) was used for depicting multiple sequence alignment in color (http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic analysis was performed using protein maximum likelihood without molecular clock module of PHYLIP software (Retief, 1999). Tree was drawn using drawgram tool (Felsenstein, 1991). To assess the reliability of the dataset and validity of results, bootstrapping was performed (Hillis and Bull, 1993). Resulting trees with and without bootstrapping were compared and results were analyzed. Results with 70% or more score in bootstrapping were considered significant.

RESULTS AND DISCUSSION

U. hardwickii lives in desert and is highly exposed to UV radiations (Zain-Ul-Abedin and Barbara, 1977). Earlier studies have focused on biochemical changes of tissues/body fluids from *U. hardwickii* with reference to their variable environmental factors (Ahmed *et al*., 2006; Barka-Dahane *et al*., 2010). In the present study, we report partial nucleotide and protein sequence of LDH like crystallin from *U. hardwickii* lenses. Gene expression analysis using LDH gene primers (Table I) resulted in four PCR products (Fig.1) which were subjected to sequencing. Resulting sequences were overlapped and a final sequence of 628 bp was obtained (Fig. 2a). Nucleotide sequence was submitted to GenBank data base (KJ432281) and UniprotKB database (X2J6H4). The gene sequence was conceptually translated to 209 amino acids using ExPASy translate tool (Fig. 2b). The three dimensional structure of partial amino acid sequence (209 residues) was constructed using Human LDH (1I0Z) as a template (Fig. 3). Human LDH is a homotetrameric molecule comprising of 333 residues in each chain. Since we were unable to get a

Table I.- Sequences of forward (F), reverse (R) primers and predicted melting temperatures used for the study.

PCR primers	Primer sequence	Predicted Tm (°C)
Primer-1	F: 5` GGCCAAGTCGGGATGGC 3`	54
	R: 5` CCCTTGACCATGGTGGATAC 3`	62.4
Primer-2	F: 5`GAAATGATGGATCTACAGCATGG 3`	55
	R: 5` CCCTTGACCATGGTGGATAC 3`	62.4
Primer-3	F: 5` GTCCGCCAGCAAGAAGGG 3`	53
	R: 5` CCCTTGACCATGGTGGATAC 3`	62.4
Primer-4	F: 5` CGTCTTGATCTTGTGCAGAGG 3`	54
	R: 5` CCCTTGACCATGGTGGATAC 3`	62.4

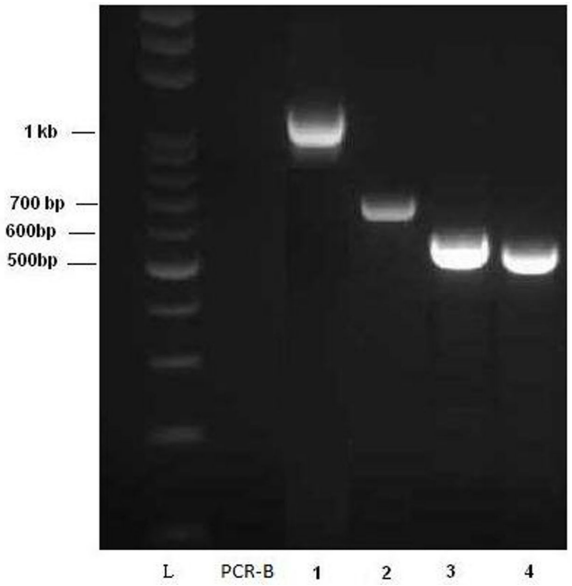

Fig. 1. Expression of LDH/ε-crystallin mRNA in eye lens of *Uromastyx hardwickii*. mRNA level of expression was analyzed by RT-PCR. cDNA was used as RT product with four different specific forward primers to amplify lens LDH. L, Ladder; PCR-B, PCR blank; lanes 1-4 represent gene expression using primers 1-4 as described in Table I. Lanes 1-2 show PCR products corresponding to 1000 bp and 700 bp, while Lanes 3 and 4 show PCR products corresponding to 500 bp.

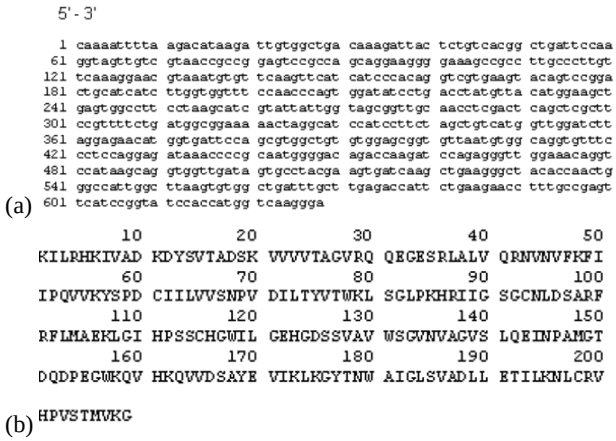

Fig. 2. 5`-3` nucleotide sequence of amplified PCR product of *LDH/ε-crystallin* like gene. It is an overlapping 628-bp fragment (a) along with the translated amino acid sequence (b). In the deduced sequence, first codon (AAA) and last codon (GGA) correspond to K72 and G280, respectively.

complete sequence, partial sequence was used for analysis. Structure analysis revealed retention of overall fold, catalytically crucial residues, cofactor binding site and antigenic loop (Fig. 4). Our modeled structure depicted tetrameric structure comprising of α-helices and β-sheets. The X-ray structure of the template indicated four monomeric units; each comprising of two domains namely Rossmann fold and substrate-binding domain (Read *et al.*, 2001). The predicted model had the same orientation for the active site region (100-111) and antigenic loop (221-227) as for the template (Fig. 3). Model accuracy was evaluated by PROCHECK which indicated that the predicted model contained more than 90% residues in core region and none in disallowed region suggesting a good model. InterPro analysis also revealed relationship with lactate/malate dehygrogenase (results not shown).

To examine the evolutionary relationship, sequences of LDH/ε-crystallin like protein (*U. hardwickii*, duck) and LDH (fish, amphibians, birds, mammals, reptiles) were used to construct a phylogenetic tree. Results of phylogenetic analysis (Fig. 5) revealed that the sequence of LDH/ε-crystallin like protein from *U. hardwickii* has evolved from lizards (*I. iguana* and *S. undulates*). Reliability of the tree is evident from high bootstrap values thus supporting observed topology.

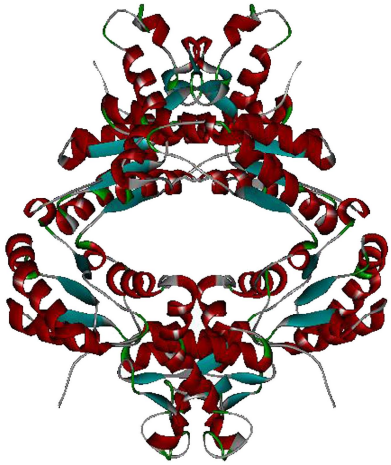

Fig. 3. Predicted model of tetrameric LDH/ε-crystallin like protein.

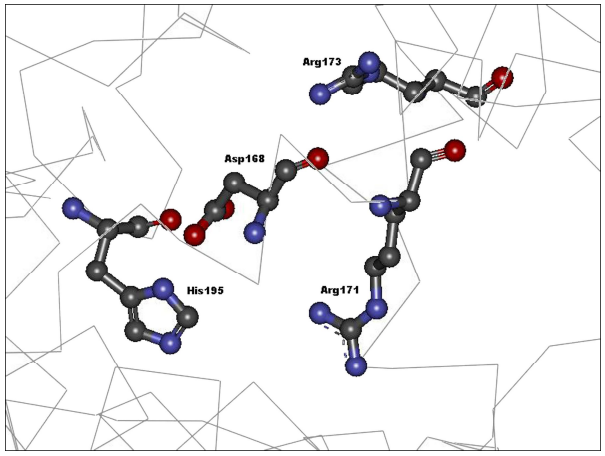

Fig. 4. Active site region of predicted model. Catalytically active site residues are shown in ball and stick representation. The remaining molecule is depicted as Cα backbone.

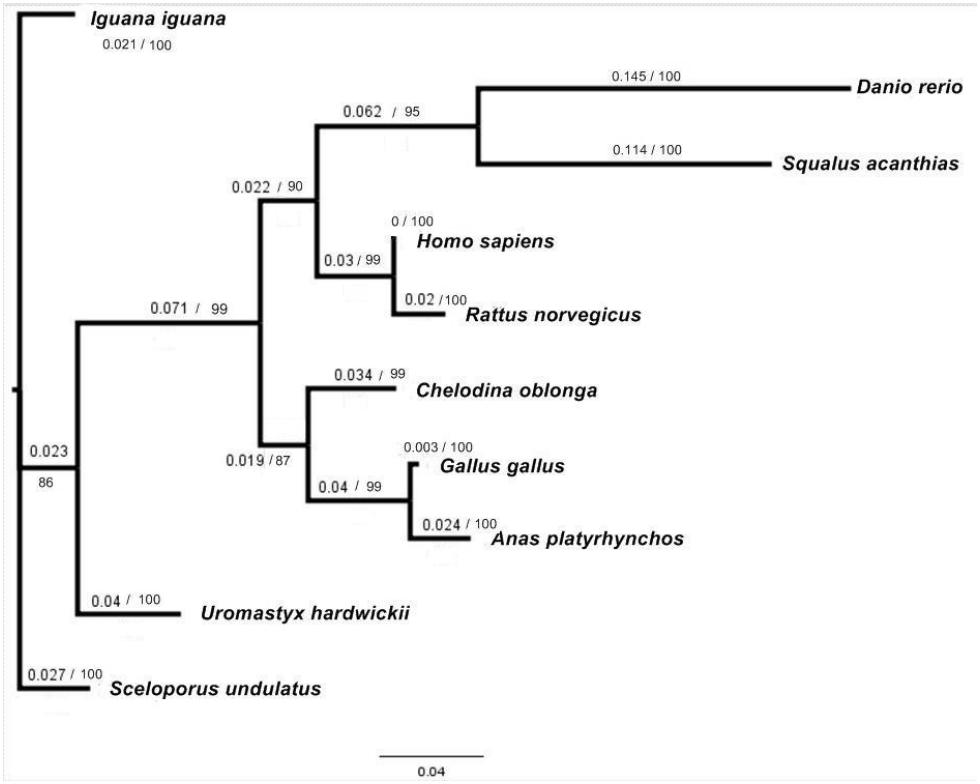

Fig. 5. Unrooted ML tree of LDH/ε-crystallin like protein using the PHYLIP program. The tree was constructed without assumption of molecular clock. Amino acid sequences and their respective accession numbers are as follows: *Squalus acanthias* (Q9YI05); *Danio rerio* (Q9PVK4); *Chelodina oblonga* Q6S5M5; *Rattus norvegicus* (P42123); *Anas platyrhynchos* (P13743); *Gallus gallus* (P00337); *Sceloporus undulatus* (Q9W7L4); *Iguana iguana* (Q6YL21); *Homo sapiens* (P07195); *Uromastyx hardwickii* (X2J6H4). Bootstrap (right) and branch length (left) values are indicated. Branch length represents number of amino acid substitutions per site while bootstrap value indicates the significance of the suggested tree.

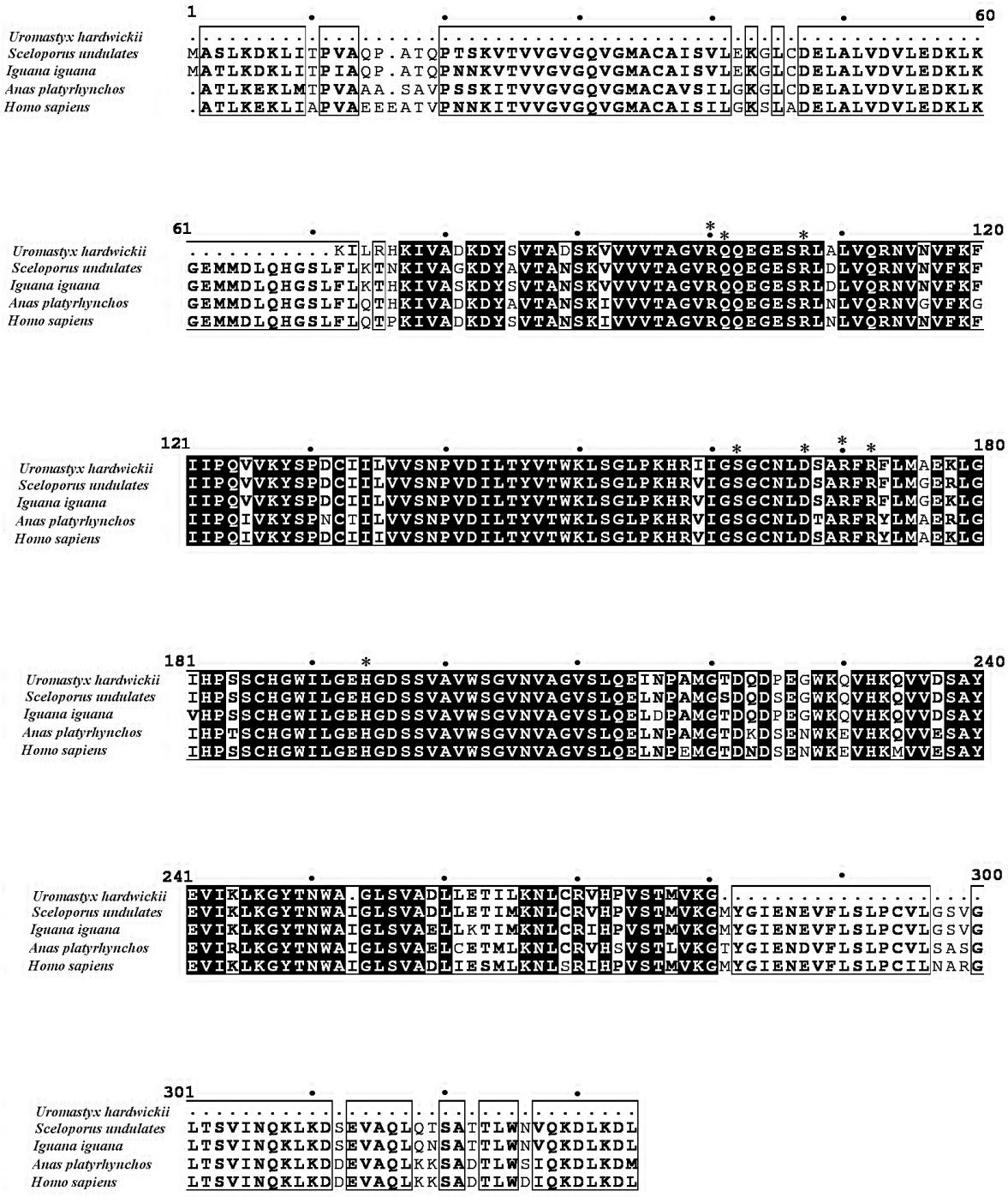

Fig. 6. Alignment of amino acid sequence of LDH/ε-crystallin like protein from *Uromastyx hardwickii* with ε-crystallin from *Anas platyrhynchos* duck lens (P13743), LDH from *Sceloporus undulates* (P79913), *Iguana iguana* (Q6YL21), and *Homo sapiens* (P07195). Highly conserved residues and semiconserved residues are boxed in black and white background, respectively. Residues involved in catalytic activity and substrate recognition include Arg100, Gln101, Arg107, Ser162, Asp167, Arg170, Arg172, and His194 which are represented by *. A dot is marked after an interval of ten residues.

Multiple sequence alignment of LDH/ε-crystallin like protein from *U. hardwickii* with ε-crystallin (duck) and LDH (*Iguana iguana, Sceloporus undulates* and *Homo sapiens*) is shown in Figure 6. Residues critical for catalytic activity and substrate recognition include Arg100, Gln101, Arg107, Ser162, Asp167, Arg170, Arg172, and His194 were found to be conserved in all sequences including LDH/ε-crystallin like protein from *U. hardwickii* (Fig. 6). His194 is suggested to act as catalytic acid which in turn is stabilized by Asp167 (Read *et al*., 2001) while Arg107 has shown to assist in transition state stabilization (Clarke *et al*., 1986). All of the critical residues are retained in LDH/ε-crystallin like protein from *U. hardwickii* suggesting the likelihood of the protein to be catalytically functional.

It has been observed that among taxon-specific crystallin, ε-crystallin distribution is restricted to avian and reptilian species. It is also established that ε-crystallin has sequence homology with LDH (Wistow *et al*., 1987). ε-crystallin was first reported in duck lenses (De Jong *et al*., 1981) followed by identification in various birds. In duck, epsilon crystallin was reported as an abundant lens crystallin and shows high sequence similarity with heart LDH-B (Hendriks *et al*., 1988). We reported earlier nucleotide sequence of ε-crystallin from *U. hardwickii* lenses comprising of 119 bp (Atta *et al*., 2014). In vertebrates, enzymes and enzyme-crystallins are encoded by identical genes (Piatigorsky and Wistow, 1991). It has also been documented that in non-lens tissues, expression of enzyme crystallins is low while within lens, higher concentrations are found to fulfill their requirement as a structural taxon-specific protein (Thomas and Dimonie, 1990). Although the presence of ε-crystallin could not be ascertained at protein level, mRNA and enzyme activity data suggests presence of LDH like protein in *U. hardwickii* lenses. Current data provides helpful information in the perspective of lens development mechanism and visual adaptive conflict in species under selective pressures during evolution.

ACKNOWLEDGMENTS

First BASE Laboratories (Selangor, Malaysia) is acknowledged for providing DNA sequencing facility.

Statement of conflict of interest

Authors have declared no conflict of interest.

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