

2014

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Atta, A., Ilyas, A., Hashim, Z., Ahmed, A., & Zarina, S. (2014). Lactate dehydrogenase like crystallin: A potentially protective shield for Indian spiny-tailed lizard (*Uromastyx hardwickii*) lens against environmental stress? *The Protein Journal*, 33(2), 128-34. <http://dx.doi.org/10.1007/s10930-014-9543-4>
Available at: <http://dx.doi.org/10.1007/s10930-014-9543-4>

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Lactate dehydrogenase like crystallin: a potentially protective shield for Indian spiny-tailed lizard (*Uromastix hardwickii*) lens against environmental stress?

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Abstract:

Taxon specific lens crystallins in vertebrates are either similar or identical with various metabolic enzymes. These bifunctional crystallins serve as structural protein in lens along with their catalytic role. In the present study, we have partially purified and characterized lens crystallin from Indian spiny-tailed lizard (*Uromastix hardwickii*). We have found lactate dehydrogenase (LDH) activity in lens indicating presence of an enzyme crystallin with dual functions. Taxon specific lens crystallins are product of gene sharing or gene duplication phenomenon where a pre-existing enzyme is recruited as lens crystallin in addition to structural role. In lens, same gene adopts refractive role in lens without modification or loss of pre-existing function during gene sharing phenomenon. Apart from conventional role of structural protein, LDH activity containing crystallin in *Uromastix hardwickii* lens is likely to have adaptive characteristics to offer protection against toxic effects of oxidative stress and ultraviolet light, hence justifying its recruitment. Taxon specific crystallins may serve as good models to understand structure-function relationship of these proteins.

Key words:

ϵ -crystallin, gene recruitment, gene sharing, lactate dehydrogenase, reptilian lens, taxon specific crystallin.

Abbreviations

LDH Lactatedehydrogenase

PVDF Polyvinylidene difluoride

RP-HPLC Reverse phase -High performance liquid chromatography

UV Ultra-violet.

1. Introduction:

Lenses of vertebrate eyes play vital part in maintaining transparency and refractive index [1]. Main component of lenses are structural proteins, called crystallins. Due to their unique properties, role in transparency, evolutionary history and distribution patterns, crystallins have been subject of interest since decades. They are mainly classified into two groups, namely ubiquitous crystallins and Enzyme or taxon specific crystallins. First group contains α , β and γ crystallins which are most prevalent in vertebrate and invertebrate lenses while latter includes taxon specific crystallins which are identical or closely related with metabolic enzymes prevalent in scattered group of species [2]. Taxon specific crystallins were first observed in birds followed by discovery in lenses of other species [3]. These crystallins are shown to be either the product of gene sharing [4] or gene duplication phenomenon [5]. During gene sharing phenomenon, same gene adopts refractive role in lens without modification/loss of pre-existing function [4]. The τ -crystallins of *Crocodylus palustris* is an example of gene sharing phenomenon containing sequence similarity with α -enolase from brain, heart, and gonad [6]. In gene duplication, however, original gene is duplicated to produce two copies of gene among which one copy retains its original catalytic activity while other become structural protein in lens [5]. In avian lens, *argininosuccinate lyase* gene is duplicated; one copy of gene maintained its role as enzymes (δ 2-crystallin), while

other gene has evolved as structural protein by losing little or all catalytic activity [3].

A major protein component in birds and reptiles is ϵ crystallin which is homologous to glycolytic enzyme lactate dehydrogenase (LDH) [7, 8]. Lactate dehydrogenase is responsible for converting glucose to lactate during anaerobic condition [9], subsequently, forming ATP. LDH has various isozymes which are tissues specific; such as LDH-A and LDH-B are found in muscle and heart, respectively. During evolution, the distribution of LDH isozymes has varied in different organs but function remained same [10]. Scientists suggested that distribution of LDH isozymes in different organs might be due to gene duplication or gene sharing phenomenon under selective pressures [1]. Birds and reptiles have diverged almost 200 million years ago and still share some morphological characters; however, difference in their proteins composition has been observed [11, 12]. Staple et al. [8] have reported presence of ϵ -crystallin in many avian lenses. In case of reptiles, evidence for ϵ -crystallin existence was found only in caiman, crocodiles and alligator which belong to order crocodylus. So far, there is only one report for presence of ϵ -crystallin in gecko *phelsuma*, a member of order squamata [13].

In this study, *Uromastix hardwickii* was used as an experimental model. *Uromastix hardwickii* belongs to reptilian family, order squamata, and has certain unique characteristics of amphibians, birds and mammals [14]. It is a terrestrial, hibernating, burrowing and diurnal animal commonly found in desert. Due to

diversified environment *Uromastix hardwickii* lives in, it is a model animal to understand the gene recruitment phenomenon and biochemical adaptations. The lens crystallins of *Uromastix hardwickii* has not been studied so far. In the present investigation, partial purification and characterization of ϵ -crystallin was performed using chromatographic techniques including gel filtration, RP-HPLC and affinity chromatography. Furthermore, ϵ -crystallin/LDH gene expression and DNA sequencing studies were also conducted.

2. Materials and Methods:

Sample collection and protein extraction:

The study was conducted after approval of Institutional Review Board, University of Karachi. All research procedures followed 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.). Fresh lenses from *Uromastix hardwickii* were removed and homogenized in 50 mM sodium phosphate buffer, pH 7.0 on ice in a ratio of 1/10 (w/v). Homogenate was centrifuged at $15,000 \times g$ for 20 min at 4°C. The supernatant was collected and labeled as water soluble fraction. The protein concentration was determined by Bradford protein assay kit (BioRad).

2.2 Protein purification:

Gel filtration chromatography was performed for the separation of lens proteins. 100 mg protein was loaded on Sephacryl S-300 gel filtration column (90x2.5cm) and eluted at room temperature with 0.01M phosphate buffer saline (pH 7.4). Fractions were collected at the flow rate of 12 – 15 ml/hr. Both crude water soluble lens proteins and peak-2 from gel filtration chromatography were fractionated by RP-HPLC (Perkin Elmer USA). 150 µl sample was injected to RP C18 (25x46mm) column equilibrated with 0.1 % Trifluoroacetic acid (solvent A). Proteins were eluted using a gradient of solvent A and solvent B (Acetonitrile containing 0.1% Trifluoroacetic acid) attaining 70 % B in 40 minutes. Elution was monitored at 280 nm. Affinity chromatography was also employed to separate proteins from gel filtration chromatography peak-2 and crude lens protein extract. Fresh lenses (5) were homogenized in 20 mM Tris-HCl buffer, pH 8.0 (equilibration buffer) and centrifuged at 15,000 x g for 20 min at 4 °C. Approximately 7.2 mg protein from water soluble fraction in equilibration buffer was applied on gel affinity column (10 x 1.5 cm) packed with Affi-gel Blue (BioRad). After elution of unbound fractions in equilibration buffer, bound proteins were eluted with same buffer containing 1M NaCl. Same procedure was used for affinity purification of catalytically active gel filtration fraction (peak-2) after an overnight dialysis in equilibration buffer at 4 °C.

2.3 Measurement of enzymatic activity:

Lactate dehydrogenase activity was determined in water soluble fraction, individual peaks of gel filtration chromatography and affinity chromatography purified fractions

using Randox Ld pyruvate lactate assay kit (Cat no. LD 401). The activity was determined by monitoring the decrease in absorbance at 340 nm for 3 min [15].

2.4 SDS-PAGE:

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the procedure of Laemmli [16] using 12% (w/v) resolving gel. Protein bands were stained using 0.2% coomassie brilliant blue (CBB) R-250 and were visualized using PD Quest software (BioRad).

2.5 N-terminal sequencing:

Partially purified protein fraction of affinity chromatography was subjected to SDS-PAGE and electroblotted onto a PVDF membrane. The bands were excised and analyzed for N-terminal amino acid sequencing using a Procise automated protein sequencer (Applied Biosystems, Inc).

2.6 nLC-MS/MS analysis:

Bands from SDS-PAGE gel of partially purified affinity chromatography fraction (Fig. 5) excised and digested with trypsin. The tryptic peptides were subjected to analysis using Thermo LTQ XL linear ion trap mass spectrometer interfaced with nano-LC system. Sample (1 μ L) was injected through an auto-sampler into the nLC system. The column (75 μ m I.D x15 cm Pep-Map 100 C-18 nano 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 at the flow rate of 300 nL/min over 70 min. Mass

spectra of nLC-MS/MS data were analyzed for protein identification using Mascot search engine against NCBI nr (www.ncbi.nlm.nih.gov/RefSeq/).

2.6 Gene expression analysis:

Total RNA was extracted from *Uromastix hardwickii* lenses by using SV total RNA isolation system kit (Promega, USA) according to manufacturer's protocol. Total RNA was reverse transcribed into cDNA by using reverse transcription kit (Invitrogen, UK). PCR amplification of total lens cDNA was performed by using GoTaq® Green Master Mix (2X) (Promega, U.S.A). Each 25 µl reaction volume contained template cDNA, Master Mix (2X) reaction Buffer (pH 8.5), 400µM dNTPs and 3mM MgCl₂ with sense (5'GGGACTGACCAAGATCCAGA3') and antisense (5' CCCTTGACCATGGTGGATAC 3') primers of *LDH* gene. PCR blank and RT- blank were used as negative control. Annealing temperatures (T_m) of both reverse and forward primers was 62.4°C and expected gene product size was 180 bp.

2.7 Agarose gel electrophoresis:

PCR product was visualized by using agarose gel electrophoresis. Agarose (2%) gel was prepared in TBE buffer (45mM Tris, 45mM Boric acid, 1mM EDTA) pH 8.5. Agarose was heated in TBE buffer until solution became clear. 0.5µg/ml ethidium bromide was added in warm agarose solution. After agarose gel solidification, PCR products (12µl) were loaded in wells. TBE buffer was used as running buffer at 80 mA for 40 min. DNA ladder (100 bp) was used as marker.

The DNA bands were visualized under UV light using gel documentation system (Bio-Rad).

2.8 DNA Sequencing:

PCR products were directly used for DNA sequencing analysis using DNA sequencing system 3130 (Applied Biosystem, U.S.A.). DNA sequencing reactions was carried out by Big-dye Terminator Cycle sequencing kit. Analysis of DNA sequences was done by using basic logical alignment search tool BLAST (www.ncbi.nlm.nih.gov/blast). Multiple sequence alignment was constructed using CLUSTALW (www.genome.jp/tools/clustalw/).

3. Results:

Total protein content determined by Bradford protein assay from water soluble fraction was found to be 1.5 mg/lens. Elution profile of crude lens homogenate from *Uromastix hardwickii* on Sephacryl S-300 column is presented in Fig. 1. Total lens homogenate was fractionated into five peaks which were all examined for LDH activity. Only peak-2 was found to be catalytically active (37.14 U/L). SDS-PAGE of peak-2 revealed more than one protein bands is shown in Fig. 2. Elution profile of total lens homogenate and rechromatography of peak-2 from Sephacryl S-300 by reverse phase HPLC is depicted in Fig.3. Peak-2 rechromatography yielded a peak which was subjected to nLC-MS/MS analysis which revealed a match with γ s crystallin from Iguana *iguana* (Ac. No: AAV54036) with 7% coverage. Affi-gel purified fraction had an activity of

284.76 U/L LDH (Fig. 4). SDS-PAGE analysis of affi-gel fraction revealed two bands of 22 kDa and 14 kDa which showed 22% and 20% band density, respectively (Fig. 5).

N-terminal sequencing analysis of 22 kDa protein band resulted in sequence of 9 residues. BLASTp analysis of observed sequence identified 22 kDa band to be truncated β A2-crystallin (Fig. 6). Second protein band of 14 kDa, however, gave no results. Both bands were also subjected to nLC-MS/MS analysis. Resulted peptides matched (26% coverage) with β A2-crystallin of Iguana *iguana* having a molecular weight of 22.4 kDa (Ac. No: AAN78174), thus confirming 22kDa band to be β A2-crystallin. The second band revealed a match with γ s-crystallin from Iguana *iguana* (Ac. No: AAV54036) with 6% coverage.

Gene expression from lens and liver using ϵ -crystallin/LDH primers is shown in Fig. 7. LDH expression was found to be higher in liver as compared to the lens. DNA sequencing of PCR product showed nucleotide sequence of 119 bp corresponding to 39 amino acids (Fig. 8). Alignment of resulting nucleotides with selected sequences is shown in Fig. 9.

4. Discussion:

Taxon specific crystallins have been a subject of interest to study gene recruitment phenomenon in many organisms. Apart from being abundant, the taxon specific lens crystallins have retained catalytic function along with structural role in lens [4]. 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 [5]. 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 [17]. 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 [18].

Among many enzyme crystallins identified so far, ϵ -crystallin has shown limited distribution restricted to avian and crocodylian species. It is already established that ϵ -crystallin have sequence similarity with LDH enzyme. ϵ -crystallin was first identified in duck lenses where it was one tenth of the total protein [8]. Later, it was explored in many members of avian family. In reptilian family, however, the presence was reported in members of order crocodylus including alligators, caiman and crocodiles while in case of order squamata, only one member (gecko *phelsuma*) [13], has shown presence of ϵ -crystallin. In present investigation, we have partially purified and identified ϵ -crystallin from lens of *Uromastix hardwickii* (order squamata). We have used gel filtration and RP-HPLC for the separation of ϵ -crystallin/LDH. Gel filtration separation resulted in five peaks of which, fraction 2 showed LDH activity. RP-HPLC fractionation of crude water soluble lens crystallins showed one fraction with enzymatic activity. Affinity chromatography

using Affi Gel Blue was then used for further purification of ϵ -crystallin/LDH. Affi-Gel blue gel has been used for the final step purification of dinucleotide fold containing enzymes such as lactate dehydrogenase enzyme [19] and phosphofructokinase enzymes [20]. SDS-PAGE analysis of affinity eluted fraction (fig 5), revealed two bands of 22 and 14 kDa. N-terminal analysis identified 22 kDa protein band as truncated β A2-crystallin. β -crystallin are oligomeric proteins with blocked N-terminal. However, presence of truncated β -crystallins has also been reported. β -crystallin has co-eluted with 14 kDa protein during affinity purification. This interaction is not surprising since similar interaction has been observed in rabbit lenses between β and λ -crystallin [21]. Another study reported interaction among α A-crystallin and δ -crystallin [22]. β A2-crystallin from *Uromastix hardwickii* showed maximum similarity (98 % homology) in nine residues with Iguana *iguana* β A2-crystallin, consisting of 189 residues long protein. Efforts to identify 14 kDa protein band by N-terminal sequence analysis were unsuccessful, most likely due to blocked N-terminal. MS analysis, however, identified this band to be γ s crystallin. Affi gel eluted fraction showed high LDH activity (284.76 U/L) but we could only identify β A2 and γ s crystallin from this fraction. We were able to confirm the presence of ϵ -crystallin/LDH expression at mRNA level in lens. Sequence alignment of ϵ -crystallin/LDH showed *I. iguana* and *S. undulates* (Fig. 9) demonstrate 40%, 51% alignment score respectively. The region containing 23 nucleotides is highly conserved in *Uromastix hardwickii*, *Iguana iguana* and *Sceloporus undulates*. Partial sequence of *Uromastix hardwickii* showed more sequence similarity with *Sceloporus undulates* than *Iguana iguana*.

It is interesting to note that recruited enzymes are mostly derived from oxidoreductases and dehydrogenases which require NADH or NADPH as cofactors. Lenses of certain species contain high content of these nucleotides suggesting active redox cycle *in vivo* [23]. Due to high reducing nucleotide (NADH/NADPH) binding capability, these enzyme crystallins not only help to keep up the redox balance against oxidative stress but are also beneficial as near UV filters [24]. Reduced form of NAD absorbs strongly in near ultra violet region, hence presence of ϵ -crystallin in birds and crocodiles is likely to provide protection against UV glare to facilitate prey hunting [25]. Since *Uromastix hardwickii* lives in desert and has maximum UV exposure [14], presence of LDH like crystallin may act as protective shield against oxidative stress and UV radiation. Thus, presence of enzyme crystallin may be regarded as defense mechanism against environmental stress.

Uromastix hardwickii is burrowing and hibernating animal and lives in desert. During day time, they accommodate in digger burrows in order to avoid ultra violet light exposure [14] while in hibernation period during winter; they hide underground for six months without eating and drinking. Metabolic activities are continued in all situations for their survival, and energy generation process entirely depends on anaerobic glycolysis [26]. During anaerobic condition, lactate dehydrogenase expression in different tissues is very high indicating the role in fulfilling energy requirement. High lactate dehydrogenase expression might be the biochemical adaptation to compensate partially low temperature and anaerobic

condition in nocturnal lizards [27]. In eye lens, however, presence of catalytic activity is still questionable.

The recruitment of enzyme crystallins demonstrates a model of molecular evolution in which changes occur before or instead of gene duplication. ϵ -crystallin is a product of recruitment of a pre-existing enzyme to structural role during which the protein retains its catalytic role outside lens. ϵ -crystallin represents an example of gene sharing phenomenon as two distinct phenotypes have been produced. The bi-functional enzyme crystallin has thus acquired a new role by modification of gene expression during the process of evolution. Further investigations are required to evaluate various characteristics of recruited metabolic enzyme as the process may have wide implication in protein evolution and differential gene expression mechanism.

Acknowledgements:

DNA sequencing analysis and N-terminal protein sequencing were provided by Centralized Science Laboratory, University of Karachi, Pakistan and RI-INBRE Centralized Core Facility, University of Rhode Island, USA, supported by grant # 5P20GM103430-13 from the National Institute of General Medical Sciences of the National Institute of Health.

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Legends:

Fig1: Elution profile of Uromastix *hardwickii* eye lens extract from Sephacryl S-300 column (90x2.5cm). Approximately 100 mg proteins were eluted using 0.01M phosphate buffer saline (pH 7.4) at room temperature and 3 ml fractions were collected. Solid line indicates protein concentration monitored at 280 nm while dotted line indicates LDH activity.

Fig 2: SDS-PAGE analysis of different fractions obtained from gel filtration chromatography. In each well, 30 μ g protein was loaded. C= crude water soluble proteins, Lane 1-5= gel filtration peaks, M= molecular weight marker.

Fig 3: RP-HPLC separation profile of crude lens homogenate (solid line) and peak-2 from Sephacryl S-300 fractionation (dashed line). Sample (~1 mg) was separated using a gradient of 0.1% Trifluoroacetic acid (solvent A) and solvent B (Acetonitrile containing 0.1% Trifluoroacetic acid) in 40 minutes (70% B).

Fig 4: LDH activity of Affi-Gel blue gel purified peak-2 from Sephacryl S-300 fractionation. Solid line indicates absorbance at 280 nm while dashed line indicates LDH activity. Water soluble fraction (~ 7.2 mg) was equilibrated in equilibration buffer (20 mM Tris-HCl buffer, pH 8.0) while bound protein was eluted with equilibration buffer containing 1M NaCl.

Fig 5: SDS-PAGE profile of affinity partially purified fraction (Affinity), sephacryl separated fraction (sephacryl) and (5 μ l) protein marker (M). Each well contained ~30 μ g sample.

Fig 6: Truncated N-terminal amino acid sequence alignment of lens β A2-crystallin of *Uromastix hardwickii* (*U. hardwickii*) with known β A2-crystallin of *Anolis carolinensis* (Ac), *Columba livia* (Cl), *Iguana iguana* (Ii), *D.mawsoni* (Dm) and *Oryzias latipes* (Ol).

Fig 7: Expression of LDH mRNA in lens and liver of *Uromastix hardwickii*. mRNA expression level was analyzed by RT-PCR. cDNA was used as RT product with specific primers to amplify lens *LDH* gene. L: Ladder; PCR-B: PCR blank; *LDH* Lens: lactate dehydrogenase gene expression in lens; *LDH* Liver: lactate dehydrogenase gene expression in liver.

Fig 8: 5`-3` nucleotide sequences and translated amino acids sequence of amplified PCR product of ϵ -*crystallin/LDH* gene.

Fig 9: Nucleotide sequence alignment of lens ϵ -*crystallin/LDH* of *Uromastix hardwickii* (U.h) with known *LDH* of *Iguana iguana* and *S. undulatus* by CLUSTALW. (*) shows matches and (.) show mismatches of nucleotides.

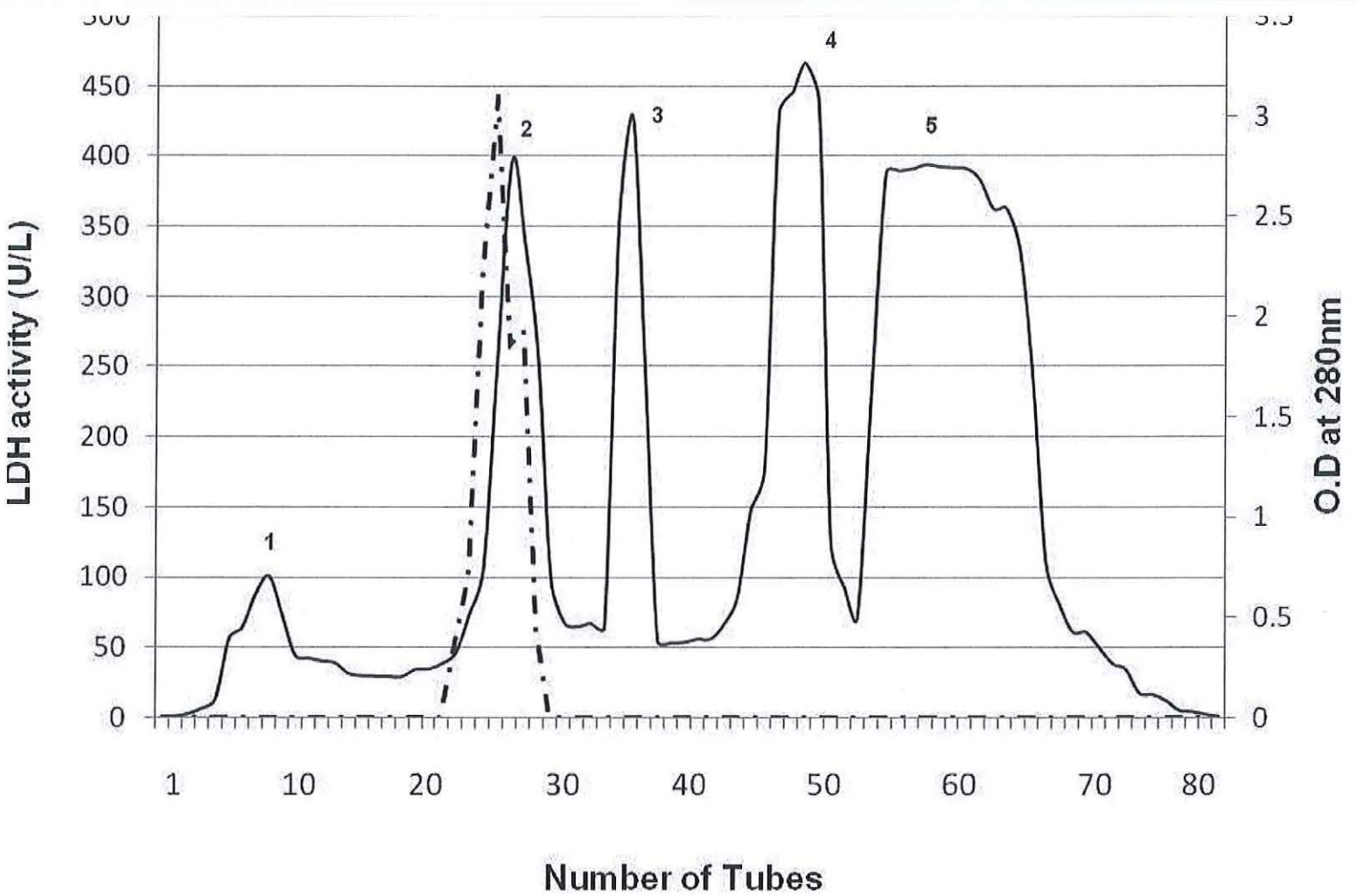


Fig-1

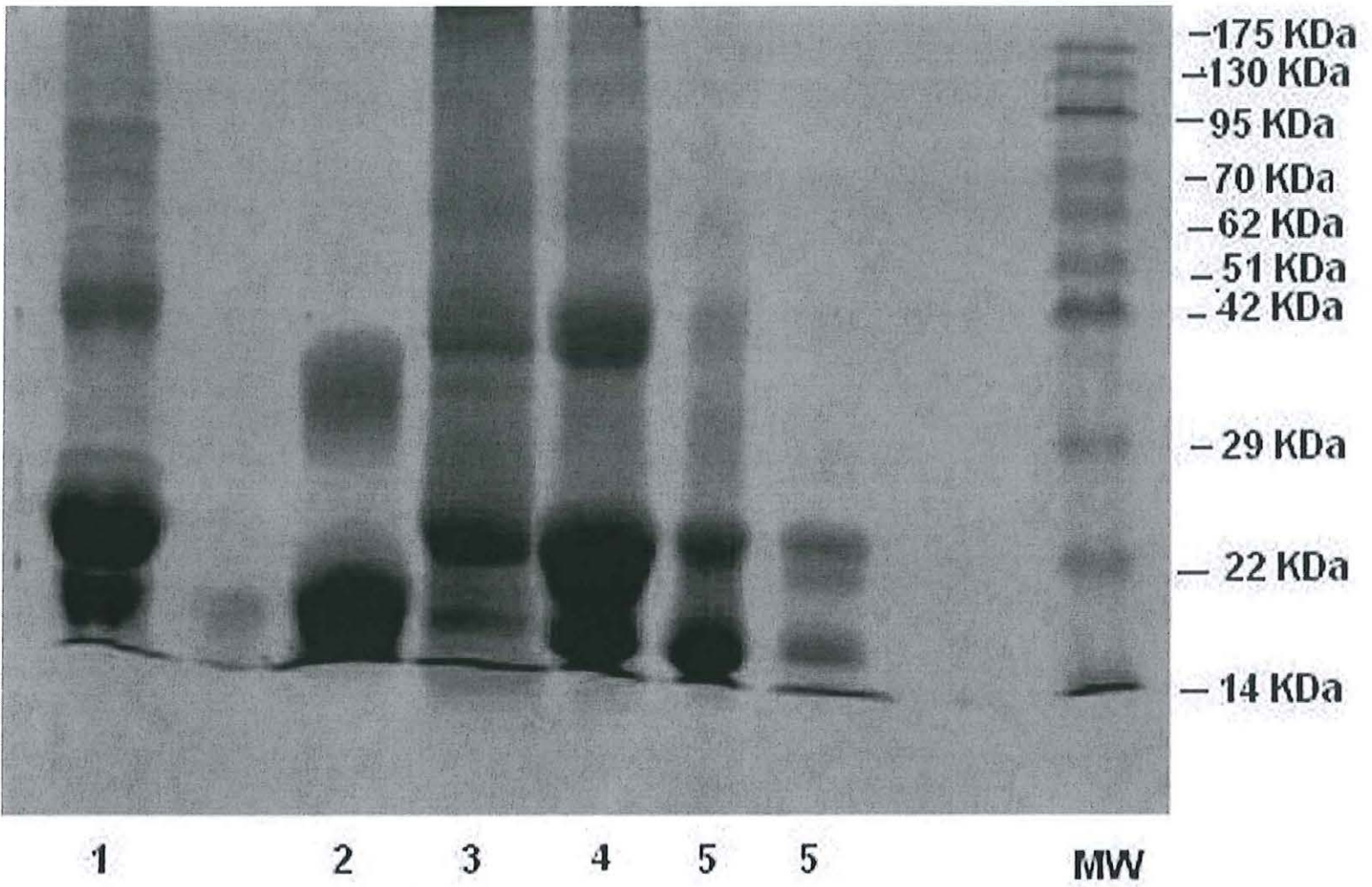


Fig 2

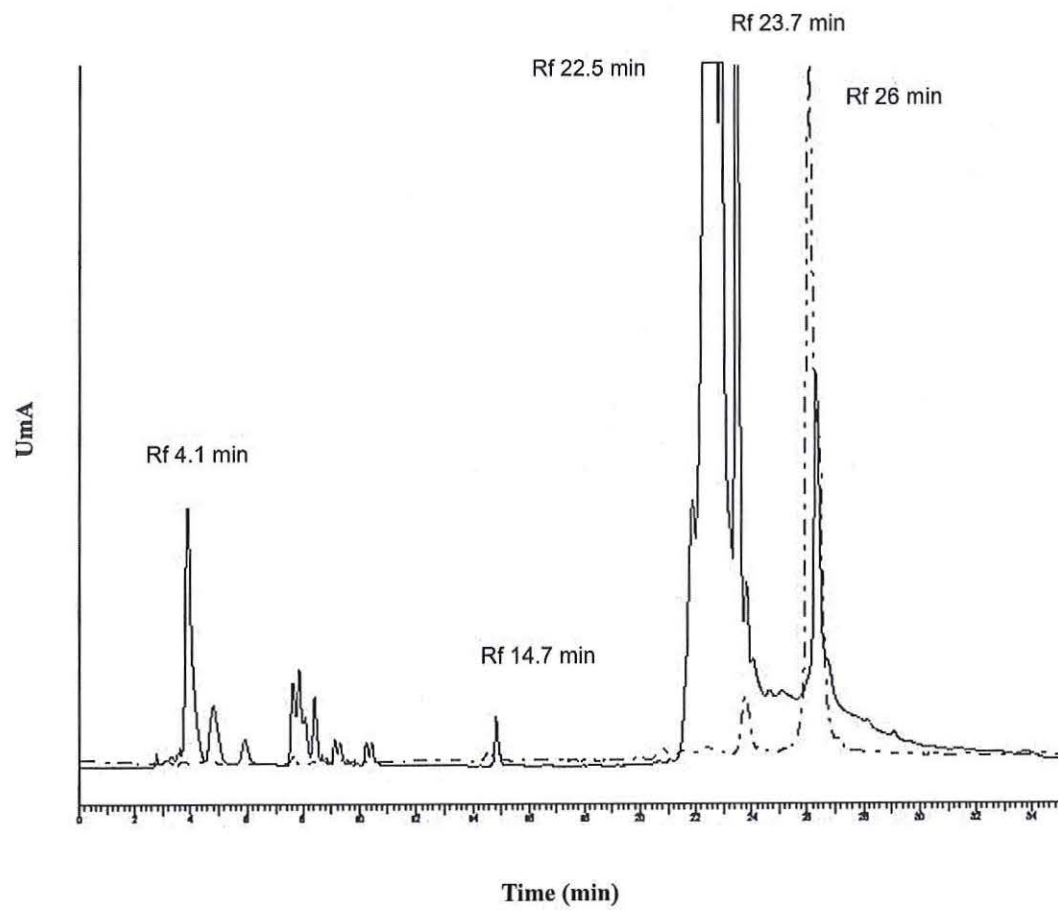


Fig 3

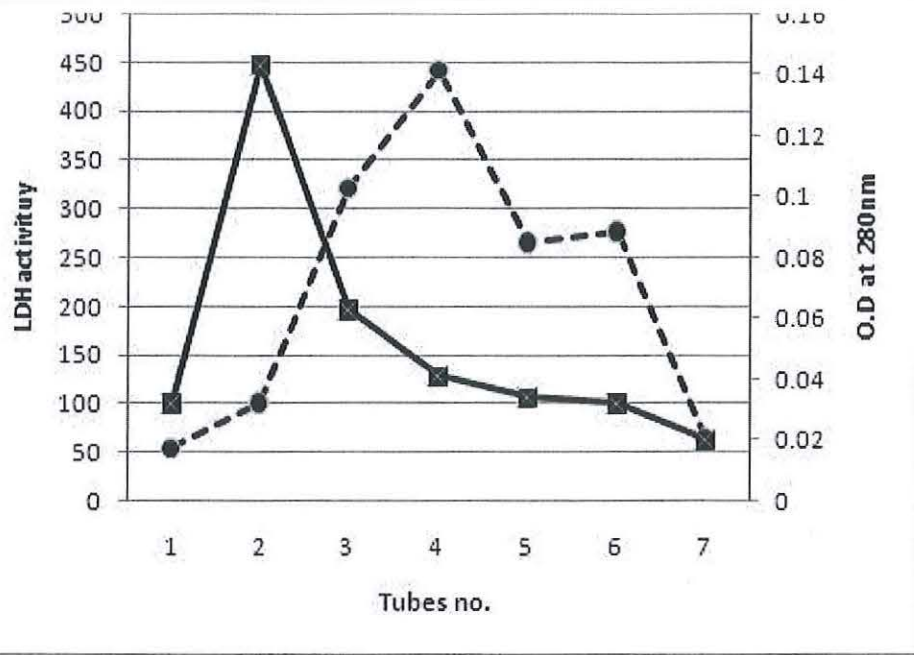


Fig 4

-175 KDa
- 95 KDa
- 51 KDa
- 42 KDa
- 29 KDa
- 22 KDa
- 14 KDa

Affinity

Sephacryl

M

Fig 5

U.hardwickii
Ac
Cl
Ii
Dm
Ol

---SQFKITVWE
-TLGQYKITVWE
-TLGQYKITVWE
-TLGQFKITVWE
EQMGQFKITVWE
-MQGQFRITVWE
. * : : * * * * *

Fig 6

500bp —
200 bp —
100 bp —



L PCR-B LDH LDH
Lens Liver

Fig 7

ctgaagggctacaccaactggggccattggcttaaggtgtgggctg
L K G Y T N W A I G L R C G L
atttgcttggaggaggcggcagttttgcgtggaggggagggcgct
I C L E E A A V L R G G E G A
tttgcggacagaggatggcggttttcggg
F A D R G W R F S

Fig 8

I.iguana
S.undulatus
u.h

CTGAAAGGCTATAACCAACTGGGCCATTGGCTTAAG-TGTTG-CTGAACTGCTAAAAACCA
CTGAAAGGCTATAACCAACTGGGCCATTGGCTTAAG-TGTTG-CTGACCTGCTAGAAACCA
CTGAAGGGCTACACCAACTGGGCCATTGGCTTAAGGTGTGGGCTGATTTGCTTGGAG---
*****.***** ***** ** * **** ***:..*.

I.iguana
S.undulatus
u.h

TCATGAAGAACCTTTGCCGAATTCATCCAGTATCCACCATGGTCAAGGGGATGTATGGCA
TAATGAAGAACCTTTGCCGAGTTCATCCAGTATCCACCATGGTCAAGGGCATGTATGGCA
-GAGGCGGCAGTTTTG-CGTGGAGGGGAGGGCGCTTTTGCGGACAGAGG-ATGGCGGTTT
* *..*.* **** **: . : . ..* . * : . **:***..** *** . * :

I.iguana
S.undulatus
u.h

TTGA-
TTGAA
TCGGG
* *.

Fig 9