CHAPTER 2

MATERIAL AND METHODS

MODEL ANIMALS

Hemidactylus flaviviridis, (Rüppell, 1835), commonly known as Northern House Gecko, of both the sexes were collected from their natural habitats located in Baroda district Gujarat, India. They were kept in well-ventilated wooden cages of dimension 45×35×60 cm in the animal house (827/ac/04/CPCSEA) of Department of Zoology, The M.S. University of Baroda. The front panels of the cages are fitted with clear glass sliders and a photo-period of 12 hours day and night cycle was maintained. Moreover, a constant room temperature and humidity of 30 ± 2^{o}C and 70 ± 5% respectively were also maintained during the experimental regime since these climatic conditions are reported to yield optimal regenerative response (Shah and Hiradhar, 1973). After acclimatization period of one week, healthy lizards with normal intact tail weighing 10 ± 2gm were selected for each experiment. All through the housing period, the animals were fed twice a week with insects and had access to sufficient UV irradiated potable water.

Sailfin-white molly, *Poecilia latipinna* (Lesueur, 1821) of both the sexes (size 4-5cm), having regular caudal fins, weighing around 4-5gm were purchased from a commercial supplier (Yash aquarium, Vadodara, Gujarat) and maintained in glass aquaria containing sterile dechlorinated water with constant aeration. All fishes were monitored daily and the healthy ones were used for the experiments. The water in which the fishes were maintained was periodically checked for routine physiochemical parameters that were maintained as follows: temperature range of 26±2°C, pH 7.0 to 7.4, salinity of 0.25ppt, dissolved oxygen 7.8 ± 0.2mg/L, phosphate 0.36 to 2.38 mg/L, total nitrate 1.0 to 2.2 mg/L. Photoperiod was kept as 12:12 hour light to dark cycles. About 10% of the aquarium water was changed every day and

replaced with fresh charcoal-UV purified water from a water purification system (Aquaguard, Eureka Forbes, Mumbai, India).

The animal care was in full compliance with the guidelines of Schedule Y-Appendix-III of Drugs and cosmetics rules (1945). The experimental protocols were approved by the Institutional Animal Ethics Committee (No. ZL/IAEC/15), prior to the commencement of experiment.

EXPERIMENTAL PROTOCOLS

Induction of Autotomy and collection of Regenerates from Lizards

For each experiment, 16 lizards were selected and autotomy of the tail was induced by pinching on the third tail segment with thumb and index figure. The animal therefore shed the tail from the second tail segment placed at the vent of the animal i.e. a segment proximal to the one on which pressure was applied (Figure 1). Three autotomised tails were quickly collected and the first segment from each was excised and processed for ascertaining the basal levels of the parameters studied, further expressed as values for resting tail. Subsequent collections were made as the autotomised lizards achieved the following milestones, which were treated herein as stages, of epimorphosis namely wound epithelium, blastema and differentiation.

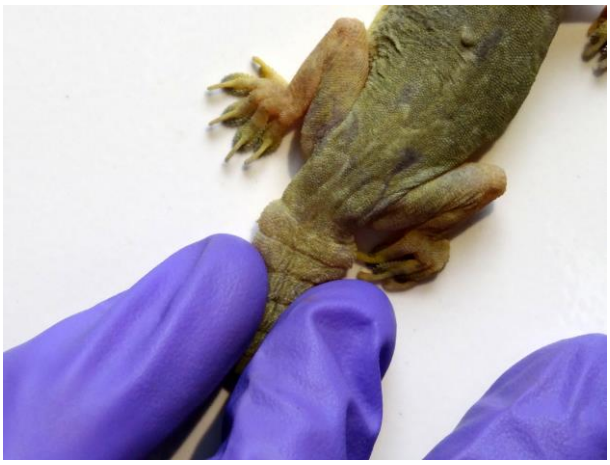

Figure 1. Induction of autotomy in lizard by pinching on the third tail segment starting from the base.

Morphologically, the wound epithelium stage is characterized by an epithelial covering across the site of amputation which is recognized as a smooth shiny surface

against the former rough scab on the wound surface. Blastema stage is distinguished from the wound epithelium stage by the conical protrusion at the growing tip of the tail, which appears due to the aggregation of blastema cells between the mesenchyme and the wound epithelium. Finally, the differentiated (regenerated) tail can be recognized by the epithelial scales on the surface the growing tail that greatly reduces the otherwise dark shade of an undifferentiated regenerate (Figure 2). Three samples for each stage were collected from the lizards that were achieved on a given day as described previously (Buch *et al*., 2017).

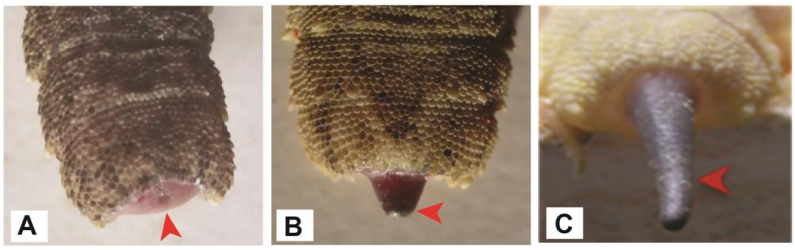

Figure 2. Stages of lizard tail regeneration. A. Wound Epithelium stage; B. Blastema stage and C. Differentiation stage. Arrowhead indicates the stage-specific characteristics.

Method of Amputation and Collection of Regenerating Fin of *Poecilia latipinna*

A fresh set of six fishes per group was used for each stage or time point studied. Distal 1/3 of the tail fin was amputated from each fish under hypothermic anesthesia using a sterile surgical blade. The amputated part of the tail fins from six fishes were collected expeditiously and processed to obtain the basal values of parameters studies and are expressed in this text as resting stage. For the regeneration stage-specific studies, once the regenerating tail attained a particular stage of inertest, tail tissues were harvested as per standard procedure under hypothermia (Rajaram *et al*., 2016) and processed as per the requirement. A diagrammatic representation of wound epithelium, blastema and differentiation stages of fish was made in the figure 3 for visual comprehension.

In the time scale study of MMPs activity, the collection of the tissues was carried out at various time intervals from 1-hour post-amputation (hpa) to 7th day post-amputation (dpa) in addition to resting stage. It includes 0hpa, 1hpa, 2hpa, 3hpa, 4hpa, 5hpa, 6hpa, 12hpa, 24hpa, 60hpa and 5dpa.

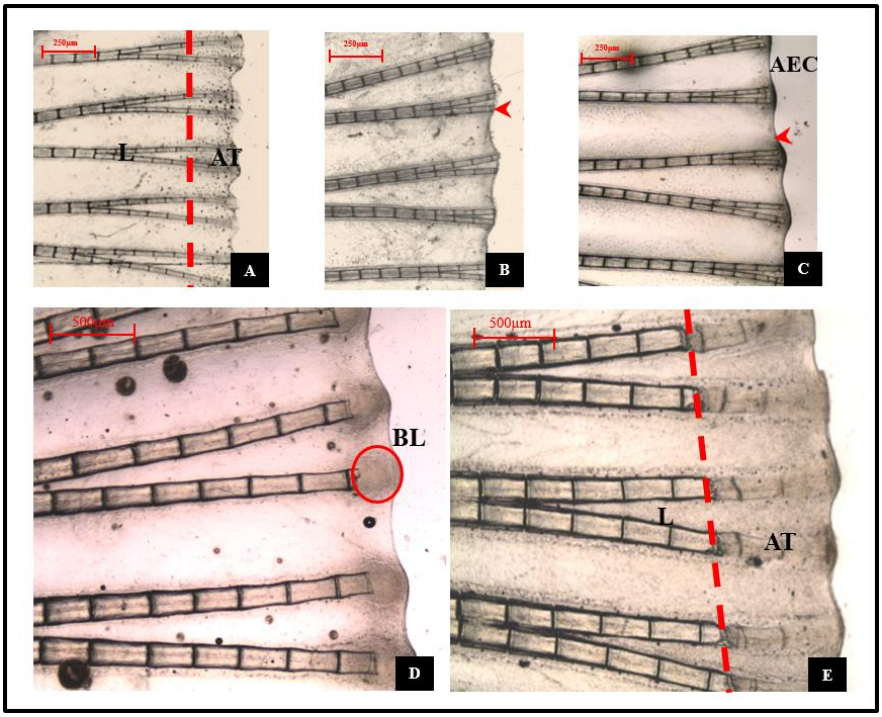

Figure 3. Stages of epimorphic regeneration in *Poecilia latipinna*. A. Resting caudal fin: Red dotted line represents amputation plane; B. Cut fin: Shows rough margin (red arrow head) following amputation of fin; C. Wound healing (24hpa): The edges of the cut fin becomes smooth, a thick layer of epithelial cells (AEC) cover the wound; D. Blastema (60hpa): A bulge of proliferating cells (BL) distal to each lepidotrichium; E. Differentiation (5dpa): newly formed lepidotrichia (L) distal to amputation plane and actinotrichia (AT) formed towards to the distal end of lepidotrichia. Figure A, B, C the magnification was 25X and for figure D and E the magnification was 40X.

EXPERIMENT - I

Estimation of Protein Content

Protein content was estimated by Bradford method (Bradford, 1976). This method involves a colorimetric protein assay based on the acidic dye Coomassie Brilliant Blue (CBB) G-250 where there is a shift in absorbance when CBB binds to protein. CBB donates its free electrons to ionizable group on proteins, which disrupts proteins' native state and exposes its hydrophobic pockets to bind with it non-covalently. They possess red shift in the absorbance and develop a blue colour on binding with proteins. The amount of dye-protein complex present in the solution is measured for the protein amount added and is read as absorbance at 595nm.

Tissues of regenerates were homogenized with (1:1) PBS: Lysis buffer in pre-chilled mortar pestle. 10% homogenate was prepared and supernatant was collected from each samples by centrifugation. To find out the protein amount of the samples,

known amount of BSA (Bovine serum albumin) ranging from 1-10µg was prepared. 10 µl of diluents were added in the microtiter plate and 200µl of Bradford reagent was added. Same procedure was followed for unknown protein samples. All the samples were incubated for 5 minutes in dark at room temperature. Absorbance was taken on ELISA reader (Metertech Σ960). A standard curve from the known concentration of BSA was plotted and regression equation was obtained from which unknown concentration of protein was calculated.

Materials used.

- Lysis buffer
  - 10mM Tris.Cl (pH 8.0)
  - 150mM NaCl
  - Triton X-100
  - 0.1M $CaCl_2$
  - 1X protease inhibitor
- Bradford reagent
  - 100mg coomassie brilliant blue G 250
  - 50ml ethanol (95%)
  - 100ml orthophosphoric acid (85%)

The reagent was kept overnight for incubation and next day the volume was adjusted to 1000ml with autoclaved distilled water. The prepared reagent was stored in an amber coloured bottle.

10% Bovine Serum Albumin (BSA)

- 10X Phosphate Buffer Saline (PBS)
  - 80g of NaCl
  - 2.0g of KCl
  - 14.4g of Na_2HPO_4
  - 2.4g of KH_2PO_4

pH was adjusted to 7.4. To make up the volume to 1000ml, sterilized water was added.

Protein estimation was used for the loading of equal amount of proteins in the one-dimensional gel electrophoresis, two-dimensional gel electrophoresis, for western blot analysis and for Gelatin zymography during the study.

SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) ANALYSIS OF THE PROTEINS

Gel electrophoresis is used in molecular biology for the separation of various macromolecules on the basis of their confirmation, size and charge they carry. In this method sodium dodecyl sulphate is an anionic detergent which unfolds the proteins quaternary and tertiary structures to its denatured linear state and gives negative charge to the proteins. Even distribution of SDS on protein mass unit results into fractionation by approximate size of the polypeptide. Heating up to near boiling will add up in the denaturation in the presence of reducing agent (β-mercaptoethanol) by reducing disulphide bonds during sample preparation. Proteins will be separated in the gel matrix formed due to crosslinking of bis-acrylamide between two acrylamide molecules. To initiate polymerization for formation of gel matrix, APS (ammonium persulphate); known to form free radicals and TEMED (*N*, *N*, *N*′, *N*′-tetramethylethylenediamine); that stabilizes free radicals, are used which further improves polymerization, are used. Proteins were separated on a discontinuous gel (Laemmli 1970). Proteins were resolved optimally by using stacking gel on top of the resolving gel which allows a tight band formation at lower pH (6.8), large pore size of gel (4%) and different ionic content. For the electric conductivity gels are formed in the buffers.

Procedure is as follows:

Gel plates were assembled using a gel assembly apparatus as per the instruction manual given with the apparatus (GeNei, Merck). Resolving gels were cast as per the protocol. Gels were allowed to polymerize for 30 to 45 minutes. Stacking gel was prepared and overlaid on a resolving gel with a comb. Gels were kept for polymerization for another 30 minutes. Samples were prepared with 5X loading dye and boiled for 10minutes. Samples were loaded in the wells. Standard molecular weight marker was also loaded in one of the wells as reference for the sample. Gels were allowed to run at constant voltage ~100V and stained with 0.5% coomassie brilliant blue R-250 overnight and destained with methanol: glacial acetic acid: water (40:10:50) solution.

Materials used

- 10% resolving gel (5.0 ml)

  2.0 ml ddH_2O

1.3 ml	1.5 M Tris-Cl (pH 8.8)
1.6 ml	30% Gel stock (29:1 Acrylamide: bisacrylamide)
50 µl	10% SDS
50 µl	10% Ammonium Persulphate
5.0 µl	TEMED

- 4% stacking gel (2.0 ml)

1.4 ml	ddH_2O
0.25 ml	1.0 M Tris-Cl (pH 6.8)
0.32 ml	30% Gel stock (29:1 Acrylamide: bisacrylamide)
15 µl	10% SDS
15 µl	10% Ammonium Persulphate
3.0 µl	TEMED

- 5X Tank Buffer

125mM	Tris buffer
1250mM	Glycine
10%	SDS

Solution A: Dissolve 5g SDS in 50 ml sterile ddH_2O. Solution B: Dissolve Tris and glycine in 450 ml autoclaved ddH_2O. Mix solution A & B. Make up to 1000ml with autoclaved ddH_2O.

- 5X Sample loading buffer

250mM tris-Cl (pH 6.6)
50% Glycerol
10% SDS
0.5% Bromophenol Blue (BPB)
500mM 2-mercaptoethanol

One-dimensional gel electrophoresis was used to check the quality of the protein samples before subjecting it to two-dimensional gel analysis, western blot and gelatin zymography.

TWO-DIMENSIONAL GEL ELECTROPHORESIS (2D-GE)

Proteomics is generally used to study the abundance and presence of proteins. This measurement tool is required for the measurement of proteins expressed

conditionally and their post-translational modification. The aim of proteomics study is the characterization of complex network of cell regulation at a definite time point. Neither genomic DNA nor transcript mRNA can clearly state the cell alteration in various conditions. Qualitative and quantitative changes of proteins can be identified by comparing two or more different state of the organism or cells. Reproducible fractionation of the isolated proteins or peptides from the complex mixture is the greatest challenge for the proteomic study. To resolve this issue, best method used is 2D-PAGE which can separate more than 1000 proteins in a single gel (Choe and Lee 2000).

Two-dimensional polyacrylamide gel electrophoresis (2D-GE) is used for the separation of protein mixture on the basis of their isoelectric point (pI) and molecular weight (MW). This technique was first introduced in 1975 by O Farrell and Klose. Firstly, proteins separate according to their pI linearly in a pH gradient. Proteins' isoelectric point separation is called as Isoelectric focusing (IEF). Each protein is focused at its pI when it moves under an electric field where it has no net charge. Other than its pI, protein will be having either positive or negative charge. After focusing, gel is equilibrated in the urea and non-ionic detergent for the sodium dodecyl sulphate polyacrylamide gel electrophoresis. In the first equilibration step, Dithiothreitol (DTT) is used as reducing agent which reduces di-sulphide bond of the native proteins. Prior to reduction, protein samples should be denatured using urea or SDS. Second equilibration step involves the alkylation of proteins with iodoacetamide (IAA), which binds covalently with the thiol group of cysteine molecule of any peptide and prevents the reformation of disulphide bond which is already reduced by DTT. Followed by equilibration IEF gels are immediately placed for SDS-PAGE. Different techniques can be used for the visualization of protein spots with different staining methods.

Procedure is as follows:

Rehydration and sample application

Samples of each stage were collected according to the protocol, by using 2D lysis buffer. Samples were homogenized & centrifuged at 4°C. Samples were precipitated using methanol-chloroform method and pellets were dissolved in 6M urea solution. Each sample was estimated and checked on the one-dimensional gel electrophoresis for the sample integrity. Equal amount of protein samples of each stages was diluted

in the BIO-RAD rehydration buffer and required volume of buffer was applied to immobilized pH gradient (IPG) strip with the broad range of pH 3-10 overnight (BIO-RAD, USA) in the rehydration tray. All the instructions were followed according to the Readystrip™ IPG instruction manual (BIO-RAD, USA). To avoid drying and evaporation, after 1 hour all the gels were covered with mineral oil (BIO-RAD, USA).

Isoelectric Focusing

A clean, dry Protean™ IEF focusing tray was placed for focusing with the rehydrated IPG strips. For the proper electric conductivity IPG strip was placed on a distilled water wetted paper wick. Program used for isoelectric focusing is as follows.

Steps	Voltage	Mode	Time	Unit
1	250	Rapid	0:15	HH:MM
2	4000	Gradual	1:00	HH:MM
3	4000	Rapid	15,000	Volt hour
4	500		Hold	

When the run was completed, the strip was transferred into a clean rehydration tray. (note: before transfer hold the strips on paper for the removal of mineral oil, gel side should be up).

Equilibration and second-dimensional electrophoresis

Each IPG strip was placed in the rehydration tray and 2.5ml of DTT containing equilibration buffer 1 was added and incubated for 10 mins. Further buffer 1 was replaced with 2.5ml IAA containing equilibration buffer 2 for 10 mins. 10% resolving gel was cast according to the protocol mentioned in the SDS-PAGE. Strips were washed thoroughly into the 1X tank buffer and placed it on the top of resolving gel. Strips were sealed with low melting agarose gel. All gels were run at 200V. Gels were stained according to the manual of the pierce silver staining kit (Thermo scientific, USA). Spots were fixed with 5% acetic acid solution and Images were taken using gel doc imager (Biorad, USA).

Materials

- **Rehydration buffer (Biorad-ReadyPrep™ 2D starter Kit Rehydration/ sample buffer)**

  8 M Urea

  2% CHAPS

50mM DTT

0.2% Bio-Lyte® 3/10 ampholyte

0.001% Bromophenol Blue

- ***Equilibration buffer 1***

  6M Urea

  2% SDS

  0.375M Tris-Cl

  20% Glycerol

  130mM DTT

  Water

- ***Equilibration buffer 2***

  6M Urea

  2% SDS

  0.375M Tris-Cl

  20% Glycerol

  135mM Iodoacetamide

  Water

- **2D lysis buffer**

  7M Urea

  2M Thiourea

  4% CHAPS

  18mM Tris.Cl

  0.2% Triton X

  50mM DTT

  10% Isopropanol

  12.5% Isobutanol

  5% Glycerol

  1X Protease Inhibitor

2-dimensional gel electrophoresis was conducted to study the differentially expressed peptides among stages of both the animal model *H. flaviviridis* and *P. latipinna* during their tail and fin regeneration respectively.

Nano LC-MS/MS

Liquid chromatography-mass spectrometry (LC-MS) is an analytical chemistry technique. Higher sensitivity of this powerful technique is useful in many applications like separation, detection and identification of potentially important molecule from the complex mixture that are important in research, pharmaceuticals, agrochemical, food and other industries.

For sample introduction in the instrument, generally high pressure is used and this liquid chromatography is called as high-performance liquid chromatography (HPLC). Two types of phases are there - one is sample (in liquid), which will pass with the mobile phase through stationary phase. On the basis of use of different phases of solvent and stationary materials, liquid chromatography is divided into two types one is reverse phase chromatography and second is normal phase liquid chromatography. In reverse phase liquid chromatography (RP-LC), octadecylsilyl (C18) and related organically-modified particles are used as stationary phase with pure or pH-adjusted water-organic mixtures such as water-acetonitrile and water-methanol while silica gel with pure or mixed organic mixtures is used in normal phase liquid chromatography(NP-LC).

Moreover, Liquid chromatography is coupled with mass spectrometry which measures the mass to charge ratio of charged particles and has been used for determining composition and structure of a molecule. MS instrument contains three sources to provide ionized based separation; an ion source, implies charge to the molecule, a mass analyzer sorts the ions by their masses in electromagnetic fields and a detector measures the value of an indicator quantity thus providing data for calculating the abundances of each ion present.

LC-MS is also used in proteomic studies for the detection and identification of a peptide from a complex mixture. The bottom-up approach of LC-MS is used for most of the proteomics studies where it involves protease digestion and denaturation followed by LC-MS or tandem MS (Wysocki *et al*., 2005).

In the current study, selected spots from the 2D gels of *H. flaviviridis* tail were cut with the sterile scalpel and sent to the Center for Cellular and Molecular Platform (C-CAMP) Bangalore, for the peptide identification and further characterization. They have used, high-performance liquid chromatography (Agient 1200 HPLC) which enables to separate complex tryptic peptide mixture with 1D nano-LC setup

consisting of a capillary pump for loading the sample onto a trap column and a nano pump (operated at 300nL/min) for the separation on the analytical column. Further, Nanomate Triversa was used as an automated nano-ESI source to generate ions. These ions were transferred through a specially designed LC coupler connects to the mass spectrometer. Detection of the ions was achieved by LTQ Orbitrap Discovery which is a hybrid type MS system with the ability to determine accurate m/z of intact precursors in the orbitrap analyzer.

Protocol which was used is as follows,

- Samples were subjected to in-gel digestion as per protocol no. 1: (http://www.ccamp.res.in/mass_spectrometry_services/sites/default/files/Digest%20Protocols.pdf)
- Digested peptides were reconstituted in 15µL of 2% ACN with 0.1% formic acid and 3 µl of the same was injected onto the column.
- Reconstituted peptides were then subjected to 70-minute RPLC gradient, followed by acquisition of the data on LTQ-Orbitrap-MS.
- Generated data was searched for the identity on MASCOT as search engine using SWISS-PROT, TrEMBL, *Anolis carolinensis* and *Hemidactylus flaviviridis* combined databases.

Database was searched against the *Anolis* database due to lack of peptide database for *Hemidactylus*.

Label free Protein Analysis

Tissues of regenerates were homogenized with 2D lysis buffer in pre-chilled mortar pestle. 10% homogenate was centrifuged and supernatant from each sample was collected in the new tube to send it for in solution digestion followed by MS analysis. To find out the protein quantity present in each stage of lizard regeneration, each sample were subjected to the in solution digestion as per the protocol mentioned in the site (http://www.ccamp.res.in / mass_spectrometry_services/sites/default/files/Digest%20Protocols.pdf). Trypsin buffer was re-dissolved in 1.5ml ice cold 1mM HCl (13ng/µl Trypsin prepared) and 100µl aliquots stored at -20˚C. Also, 100mM ammonium bicarbonate in water was freshly made and 5% Formic acid (vol/vol) was made in water.

In brief, the procedure steps of in sloution digestion are as follows; pH of the protein sample was adjusted to 8.5 by adding 50mM ammonium bicarbonate. After vortex mixing with short spin 13ng/µl ice cold trypsin was added in 1:30 ratio. Mixed well. Tubes were places into thermostat and incubated at at 55^{o}C for 2 hours/37^{o}C for overnight. Tubes were chilled to roomtemperature and 5% formic acid was added till the pH reaches nearly 3. Digested peptides were subjected to MS analysis. (http://www.ccamp.res.in/mass_spectrometry_services/sites/default/files/Digest%20P r otocols.pdf) (with additional alkylation and reduction). Digested peptides were vacuum dried and reconstituted in 15 µL of 2% ACN with 0.1% formic acid and 1 µL of the same was injected on to the column. These were then subjected to 180 minute RPLC gradient, followed by acquisition of the data on LTQ-Orbitrap-MS. Generated data was searched following standard approach for the identity using MASCOT 2.4 as search engine on Proteome discoverer 1.4. The data was searched against UniprotSwiss-Protdatabase (non redundant database with reviewed proteins), *Hemidactylus flaviviridis* database downloaded from NCBI. Minimum of two High confident peptides were used as a prerequisite to identify the proteins. 20 fmoles of Standard BSA digest was analyzed in parallel to the sequence to check the performance of the instrument. 20 fmoles of standard BSA was successfully found back with area of 2.001E8 and 2.618E8 and sequence coverage of 38.71% & 48.11% respectively. Due to insuficient database of *H. flaviviridis* corresponding search against different databases was made.

EXPERIMENT-II

Gelatin Zymography

Zymography is an electrophoretic technique used to evaluate the activities of hydrolytic enzymes such as MMPs. In non-reducing condition proteins are being separated on a polyacrylamide gel. Protein renatures after separation and recover its activity upon incubation in the activation buffer. Gelatin was used as substrate. The gelatinases digest the gelatin resulting as a clear zone of band against stained background. MMP2 and MMP9, both are gelatinases and their activity during regeneration process was checked.

Briefly, 7.5% gelatin zymogram gel was prepared containing 2.5% gelatin. Equal amount of protein samples from each extract was loaded on the gel and allowed to run at 4˚C on 100 volts. After running, gels were washed with 2.5% Triton-X 100

solution for 30 mins which was repeated twice and incubated with incubation buffer containing 50mM Tris-HCl, 5mM $CaCl_2$, 0.2M NaCl and 1% triton X-100 for 18 hours. Gels were stained with 0.5% Coomassie Brilliant Blue R-250 and destained with methanol: glacial acetic acid: water destaining solution. Bands appeared in the form of clear area against the dark blue background of gelatin. Relative intensities were determined using Doc-ItLs software (GeNei, Merck, USA).

Material used

- 7.5% resolving gel

  1.4ml ddH_2O

  1.0 ml Gelatin

  1.25 ml 1.5 M Tris-Cl (pH 8.8)

  1.25 ml 30% Gel stock (29:1 Acrylamide: bisacrylamide)

  50 µl 10% SDS

  50 µl 10% Ammonium Persulphate

  5.0 µl TEMED

- Incubation buffer

  50mM Tris-HCl

  5mM$CaCl_2$

  0.2M NaCl

  1% Triton X-100

Gelatin zymography was used to check the temporal activity of MMP2 and MMP9 during *P. latipinna* fin regeneration.

EXPERIMENT-III

Western Blot

For the detection of specific protein in a sample, western blot, a molecular biology technique is widely used. Method for this technique originated in the laboratory of Harry Towbin at the Friedrich Miescher Institute (Towbin *et al*., 1979). The name *western blot* was given by W. Neal Burnette in 1981(Burnette, 1981) from the name of Southern blot, a technique for DNA detection developed earlier by Edwin Southern. First step for the western blot is separation of native proteins or denatured proteins by gel electrophoresis. They are transferred in the electric field on nitrocellulose or PVDF (polyvinylidene difluoride) membrane, where proteins bind with hydrophobic as well as charged interaction. Detection of the specific peptide is

achieved using antibodies (Renart et al., 1979; Towbin *et al*., 1979). Different detection techniques like fluorescent tagged antibodies, radiolabeled antibodies and substrate-enzyme reaction based techniques have been used.

Procedure is as follows:

Samples were prepared according to the protocol of SDS-PAGE. Equal amount of proteins was loaded and proteins were separated on 10% gel. Gels and 0.45 µm thick nitrocellulose membranes were washed prior to transfer with the transfer buffer containing tris-glycine-methanol. Transfer was achieved at 100V for 1hr 40 mins. The uniformity and overall transfer had been checked with 0.5% ponceau S stain (prepared in 2.5% acetic acid). Non-specific binding of antibodies was eliminated by using blocking reagent PBS-MT for 1 hour. Membranes were incubated separately with the diluted primary antibodies; anti-rabbit MMP2 (Sigma-Aldrich, USA), anti-goat MMP9 (Sigma-Aldrich, USA) and anti-mouse β-actin at 4°C overnight. For the removal of the excess unbound antibodies, membranes were washed with PBS-T thrice each for 10 mins and incubated further with diluted secondary antibodies biotinylated anti-IgG (Genei, USA) for 30mins at room temperature. Membranes were washed with PBS-T for 10mins thrice followed by incubation with alkaline phosphatase bound streptavidin complex which covalently bind with biotin. Development of the specific protein band was achieved using BCIP (5-bromo-4-chloro-3-indolyl-phosphate)/NBT (nitro blue tetrazolium) (Genei, Merck, USA) substrate. Desired bands appeared in a dark purple colour against clear background. Here, β-actin was used as internal control.

Material used,

- Transfer buffer

  25mM Tris

  192mM Glycine

  Buffer was prepared in 20% methanol solution.

Western blot analysis was used for MMP2 and MMP9 with both the animal models *H. flaviviridis* and *P. latipinna* during the study.

EXPERIMENT-IV

Real-time PCR

A molecular biology technique, based on polymerase chain reaction, has been used to determine the targeted DNA quantitatively. For the detection two common methods

have been used- first, non-specific fluorescent dyes intercalate with double-stranded DNA and second, sequence-specific DNA probes labelled with a reporter which upon binding to the complementary sequence can be detected. For gene expression studies, small level of mRNA transcripts need to be amplified and polymerase chain reaction (PCR) method is used for this amplification. First step for this detection is the reverse transcription of the RNA to complementary DNA (cDNA) with reverse transcriptase followed by PCR.

Real-time PCR is performed with a thermal cycler which has the capacity to detect each sample individually at a specific wavelength and detect the excited fluorophore emitted light. Advancement of the thermocycler of the rapid heating and chilling in the real-time PCR is making it more reliable due to the properties of the nucleic acids and DNA polymerases. Any PCR includes three steps; denaturation at 95 °C for the separation of double-stranded DNA, annealing between 50-60 °C where primers binds to specific sequence on the template DNA and elongation between 68-72 °C allows polymerization of the fragment. These three steps are repeated 25-50 times to achieve the amplification of the specific gene. At the end of each cycle, an additional step of higher temperature at 80 °C for the detection of fluorescent signal is included which lasts for few seconds for the elimination of the non-specific detection of primer dimers. For non-specific binding, different fluorescent dyes like SYBR green are used for reporting the PCR products. Specific amplification can be seen by analysis of melting curve of amplified DNA fragments by addition of the melting step at the end of the cycle where amplified products gets separated from double stranded to single stranded. Specificity of amplification has been achieved by a single peak in the melting curve at a particular melting temperature (Tm). DNA quantification by real-time PCR can be achieved by plotting fluorescence against the number of cycles on a logarithmic scale. During the reaction, a threshold for detection of DNA-based fluorescence is set 3-5 times of the standard deviation of the signal noise above background. For each sample, a quantification cycle (Cq) or threshold cycle (Ct) represents the number of cycles at which the fluorescence exceeds the threshold value. To quantify gene expression, normalized value called ΔCq value (Cq of RNA or DNA of a sample is subtracted from the Cq of a housekeeping of the same sample) (Schefe *et al.,* 2006) is used between the samples.

This technique is used in many applications like in quantification of gene expression, in diagnosis of many disease state and infections, for the detection of

phytopathogens, genetically modified organisms and Clinical quantification & genotyping etc.

Experimental protocol for the real-time PCR is as follows:

Tissues from lizard and fish tail/fin regeneration stages were collected in TRIzol reagent (Applied biosystems, USA). RNA isolation was achieved using TRIzol method. RNA purity was checked by ratio of absorbance at 260 nm and 280 nm and quality was ascertained with 1% agarose gel electrophoresis. To avoid contamination of genomic DNA, RNA samples were treated with RNase-free DNase (Thermo Fisher Scientific, USA). 1µg RNA was subjected for reverse transcription with cDNA synthesis kit (Applied Biosystems, USA).

Sequences were used for qRT-PCR are as follows:

K12 Forward; 5'- GAGGCTGACATCAACGGTCT -3'

K12 Reverse; 5'- TGACTTCGTACTGTCCCCTCA -3'

MYL-1 Forward; 5'- GCAACCCCAGCAATGAGGAA-3'

MYL-1 Reverse; 5'- GAGTTCAGCACCCATGACTGT -3'

MMP2 Forward; 5'- GTCTCCTGGCTCATGCCTTT-3'

MMP2 Reverse; 5'-TTTCACCACTTGGCCCTCTC-3'

MMP9 Forward; 5'-GCCTTCAAGGTGTGGAGTGA-3'

MMP9 Reverse; 5'-ATCCCCGTGGTCAGCTTTTC-3'

18S Forward; 5'-GGCCGTTCTTAGTTGGTGGA-3'

18S Reverse; 5'-TCAATCTCGGGTGGCTGAAC-3'

cDNAs were subjected to qRT-PCR analysis using select SYBR green mix (Applied Biosystems, USA) and specific primers with QuantStudio 12K flex (Applied Biosystems, USA) and StepOnePlus™ System (Applied Biosystems, USA). Samples were measured in triplicate with 18S endogenous control. $\Delta\Delta$Cq values were calculated in reference to 0dpa i.e. resting stage sample. The fold change in the target gene relative to the 18S rRNA endogenous control gene was calculated using Livak method ($2^{-\Delta\Delta Cq}$) (Livak and Schmittgen, 2001). Annealing temperatures for each gene and their amplicon size in base pair (bp) were used in this study are as follows.

Gene Name	Annealing temperature	Product length
krt-12	58.3°C	189bp
myl-1	61.0°C	170bp
mmp2	60.9°C	101bp
mmp9	59.6°C	102bp
18S rRNA	57.0°C	144bp

STATISTICAL ANALYSIS

All data were subjected to One Way Analysis of Variance (ANOVA) followed by Bonferroni's multiple range tests using GraphPad Prism 5.0, statistical analysis package. A 'p' value of 0.05 or less was accepted as being statistically significant.