ABSTRACT

ONTOGENETIC CHANGES IN THE SKELETAL MUSCLE FUNCTION OF ZEBRAFISH (*DANIO RERIO*) LARVAE: IDENTIFICATION OF BIOCHEMICAL MARKERS FOR SLOW AND FAST CONTRACTILE BEHAVIOR

Zebrafish is an important model species to study vertebrate development. One particularly interesting aspect of fish ontogeny, which zebrafish larvae exhibit, is the high tail beat frequency up to 10 times higher than that of adult fish. Larvae therefore require a different muscle fiber type than adult fish. This study identifies the changes in muscle protein composition that might underlie this change in muscle contraction frequency. This question is interesting because contraction frequencies above 80 Hz require the so-called superfast muscles, a muscle type so far only been identified in non-locomotory muscles not required to generate significant forces. Superfast muscles are known to differ from normal fast muscles in several proteins, including myosin heavy chain. In this study we will focus on myosin, the largest protein fraction of a muscle that constitutes 40-50% of total muscle. This study identifies and validates a protocol for myosin protein extraction and identification of myosin isoforms. Fast myosin heavy chains (210 kD) were identified in hatchlings (not yet swimming actively) and in adult Zebrafish (tail beat frequency 10 Hz). In 6-d old larvae (swimming actively, tail beat frequency 100 Hz), we found an isoform (160kD) that was identified in cat masseter muscle, which is a muscle with superfast contraction characteristics. We identified isoforms by probing Western blots with primary antibody (MYH1/2/3) against myosin heavy chains. BCA and ImageJ analysis were performed to quantify the amount of total protein and the myosin content.

Christina Joyce Benjamin
May 2011

ONTOGENETIC CHANGES IN THE SKELETAL MUSCLE FUNCTION OF ZEBRAFISH (*DANIO RERIO*) LARVAE: IDENTIFICATION OF BIOCHEMICAL MARKERS FOR SLOW AND FAST CONTRACTILE BEHAVIOR

by

Christina Joyce Benjamin

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APPROVED

For the Department of Biology:

We, the undersigned, certify that the thesis of the following student meets the required standards of scholarship, format, and style of the university and the student's graduate degree program for the awarding of the master's degree.

Christina Joyce Benjamin
Thesis Author

Ulrike Müller (Chair) Biology

Joy June Goto Chemistry

Alejandro Calderon Urrea Biology

For the University Graduate Committee:

Dean, Division of Graduate Studies

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INTRODUCTION

Zebrafish (*Danio rerio*) are tropical freshwater fishes found in slow streams and rice paddies of the Ganges rivers of India and Burma (Mayden et al. 2007). Due to their popularity as good biological models they are now found in US and several other countries. They belong to the family of cyprinidae. They live at 27°C to 34°C and are maintained at a pH of 7.9–8.2. The Zebrafish adult can grow from 4 up to 6.5 centimeters in length. The life span of the fish is usually 5 years but is reduced to 2-3 years if it is held in captivity (Spence et al. 2008).

There are numerous advantages to using Zebrafish for research purposes. They are convenient to breed and maintain. They undergo external fertilization and reproduce regularly. A single female can lay up to 200 eggs during one reproductive cycle. They reach maturity at the age of 3 months. The entire genome of the Zebrafish has been sequenced. Genetic screening has led to the identification of hundreds of mutant phenotypes in Zebrafish that cause clinical disorders in humans like human skeletal muscular dystrophy, dilated cardiomyopathy and hypertrophic cardiomyopathy (Seeley et al. 2007). Knockout gene models can be constructed to study, phenotypical changes in known mutants (Bilotta and Saszik 2001). Hence it is an ideal model organism for human developmental genetics (Paquet et al. 2009). Egg and embryo are transparent, which greatly facilitates the study of developmental processes (Spence et al. 2008) and makes them an ideal model system to study vertebrate ontogeny (Kimmel 1993).

Zebrafish are also used to study muscle function and locomotion (Dou et al. 2008). Fish swim using skeletal muscles that provide adequate power at different swimming speeds and temperatures (Syme 2006). The muscles of fishes are

adapted to cruising (steady swimming) and high velocity bursts (unsteady swimming) locomotion behaviors (Syme 2006; Sanger and Stoiber 2001). Based on contraction speed and endurance, the muscles are classified into three main types slow, fast and superfast muscle fibers (Akster and Osse 1977).

Muscles used to power slower paced activities are typically activated and relaxed slowly. These muscles are aerobic and red in color. They are called slow-twitch red muscles. Muscles that are recruited for fast activities are used occasionally, since they consume a high amount of energy. These are anaerobic and white in color. They are called fast-twitch white muscles. There are also other types of fibers called pink muscles that are both aerobic and fast (Rome 2000; Sanger and Stoiber 2001). The white muscle makes up the major bulk of the muscle tissue. Red muscle make up to 0.5-30 % of trunk muscle depending on the locomotion mode of the fish (Syme 2006; Goldspink 2001). The red and white muscle fiber types together make up close to 90% of muscle mass in fishes (Johnston 1980). Apart from red, pink and white muscle fiber types, most fishes also have other fiber type according to their metabolic, histochemical, protein isoforms assessments (Sanger and Stoiber 2001).

One of the other fiber types found in few organisms is the superfast muscle fiber type. This fiber type has the highest contraction frequency of all the fiber types but produces very low force. Superfast muscle fibers exist in two different forms: aerobic and anaerobic. So far aerobic muscles that generate such high-frequency contractions have only been found in sound producing organs of animals (Young and Rome 2001). These muscles have been found in three organisms, namely the rattle snake (shaker muscles), the midshipman or humming toadfish (swim bladder muscle) and the oyster toadfish (swim bladder muscle) (Rome 2000). Anaerobic superfast muscles are more common and are found in

high-frequency contraction muscles like the eye muscle, syrinx muscles of song birds and masseter muscle (Young and Rome 2001; Elemans et al. 2004).

A study to understand the swimming behavior and the ontogenic development of larval Zebrafish, observed a crucial change in the tail beat frequency of Zebrafish larvae compared to that of adult (Müller and van Leeuwen 2004). The tail beat frequency of Zebrafish larvae at 3-6 d has been observed to be 100 Hertz and this frequency drops to 10 Hertz in adults (Müller and van Leeuwen 2004; Buss and Drapeau 2001) (Fig. 1).

Tail beat frequency is inversely proportional to the body length of a fish and directly proportional to the swimming speed of a fish. Hence the larvae exhibit low absolute swimming speed due to their smaller size. The larva needs to generate higher tail beat frequencies to reach absolute swimming speeds that are comparable to that of larger predators.

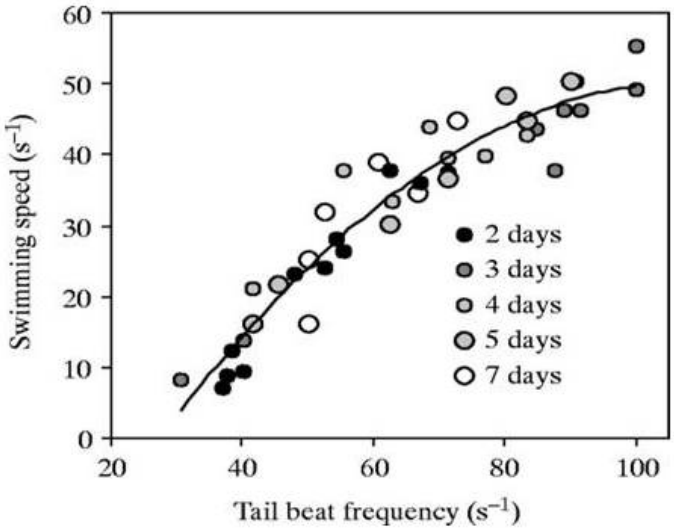

Fig. 1: Correlation between swimming speed and tail beat frequency for larval zebra fish (age 2 to 7 d post-fertilization) (Müller and van Leeuwen 2004)

If these muscles that generate such high tail beat frequencies are superfast muscles, then it contradicts the hypothesis that superfast muscles cannot have locomotory functions due to their low force production (Rome and Lindstedt 1998; Young and Rome 2001). At their maximum frequency, close to 200 Hertz in toadfish, these muscles only produce about a one-tenth of the force provided by locomotory muscles (Rome et al. 1999). The exact mechanism behind the low force is not known, but the low force could be attributed to either lower proportions of cross bridges and reduced myofibrillar region or low force per cross bridge than those of locomotory fibers (Rome and Lindstedt 1998; Rome et al. 1999) In order to determine if superfast muscles are responsible for the high tail beat frequency and locomotion in Zebrafish, study of the chemicals that characterize a muscle as superfast is necessary. The contraction and relaxation of muscles are controlled by myosin cross-bridges that alternately attach and detach. For contraction to occur, calcium-activated troponin undergoes a conformational change and moves the tropomyosin away from actin's binding sites, activating myosin head to be attached to actin. For relaxation to occur troponin does not undergo any conformational change, tropomyosin blocks the binding site on actin and relaxes the myosin cross-bridges from attaching to the binding sites on actin. Because both activation and relaxation are fast, we can expect that these processes of contraction and relaxation of the cross-bridges are extremely fast in the case of superfast muscles. In toadfish, three chemical variables that differ from the slow twitch fibers have been identified. These variables in the superfast twitch fibers are 1) a rapid release and uptake of Ca^{2+} by the pumps in the Endoplasmic reticulum, 2) a molecular modification of troponin to a lower affinity type and 3) a molecular isoform change in myosin that helps with rapid contractions (Rome and Lindstedt 1998).

I focus on the myosin protein not only due to its abundance in muscles but also since it plays an important role in the presence and functioning of superfast muscle. Myosin acts as a molecular motor that produces force for muscle contraction (Goldspink 2001). Myosin makes up to 7-10% of wet muscle mass, which accounts for 40-60% of the muscle proteins. Myosin isoforms present in the thick filaments plays an important functional role in generating mechanical force (Syme 2006). The relative content of Myosin Heavy Chain (MHC) and Myosin Light Chain (MLC) determines the shortening speed force velocity and contraction rate in muscles (Silva et al. 2010).

Together with the protein actin, myosin forms contractile elements of muscle and hence determines the contraction speed of muscles. Myosin along with actin and several other proteins, such as troponin, enables contraction of muscles. Twelve different classes of myosin have been discovered but only the class-2 myosin has been found to be involved in cardiac, smooth and skeletal muscle contractions (Syme 2006; Goldspink 2001; Weeds and Lowey 1971).

Myosin 2 is a hexamer that dissociates in alkaline medium into six polypeptide chains: two heavy chains (approximate molecular weight 220,000 Da each) and four light chains (approximate molecular weight of 17,000 - 20,000 Da each) (Weeds and Lowey 1971; Goldspink 2001) (Fig. 2).

The myosin heavy chains present in each of the three fiber types: white, red and superfast are responsible for generating muscle contraction. The heavy chains are bound at their carboxyl regions to form a α-helical coil that binds the molecule to the thick filament on the sarcomere. The N-region of the heavy chains form a globular head (containing actin binding domain and ATPase activity site), where force is generated to power a cross-bridge process in which the thin filament slides over the thick filament (Goldspink 2001). Myosin 2 generates force using a power

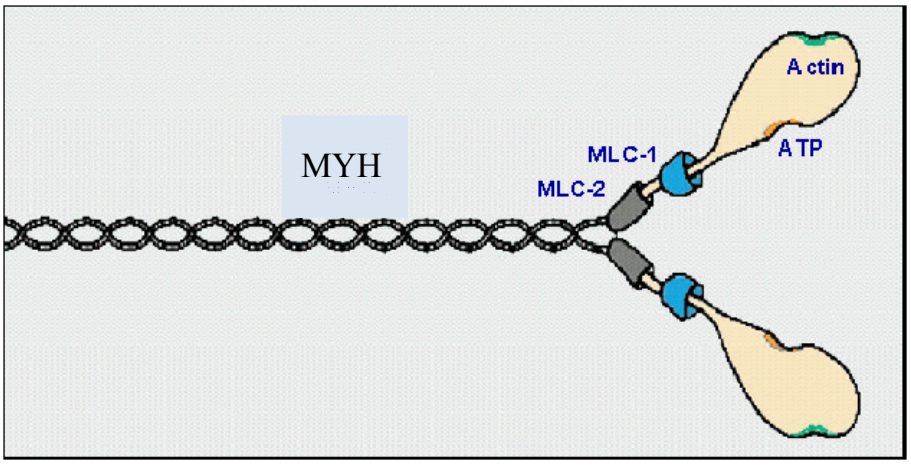

Fig. 2: Schematic representation of the myosin molecule (Sellers and Goodson 1995)

stroke mechanism fueled by ATP hydrolysis. The stroke occurs when a phosphate is released from the myosin molecule during ATP hydrolysis when the actin myosin bond is present. The pull of the phosphate molecule causes a conformational change in the myosin. The myosin is released from the actin once the hydrolysis is completed by formation of an ADP molecule. ATP hydrolysis proceeds as a cyclic process and causes myosin to bind to actin again (Tyska and Warshaw 2002).

Myosin heavy chain (MYH) is the predominant muscle protein and changes in its expression cause changes in other muscle proteins. Changes in MYH affect the cross-bridge formation and the ATP cyclic process, since MYHC contains the binding sites for actin and ATP (Coughlin et al. 2001; Johnston 1994).

The properties and functions of various muscles (formed by different fiber types) are influenced by the different myosin isoforms that confer functional heterogeneity on the muscles (Schiaffino and Reggiani 1996). This provides the possibility of myosin 2 heavy chain isoform being responsible for the higher tail beat frequency in Zebrafish.

During ontogenic development, fish muscle fibers undergo a lot of changes. Distinct isoforms present in larvae are not found in adults, as seen in Sea bass (Huriaux et al. 1999; Silva et al. 2010). There is also a possibility that the number of isoforms present in larvae might be increased as the fish reaches adult hood. This has been observed in rainbow trout , where the number of myosin isoforms increases between the parr phase and the juvenile phase (Coughlin et al. 2001). So on one hand different ontogenic stages might have different myosin isoforms that commensurate with the changing demands on the muscles during ontogeny. (Bottinelli 2001). On the other hand the changes in muscle demands might not be met by changes in myosin isoform but changes in other muscle proteins such as the aforementioned troponin and calcium channel proteins. If fiber type is based on the MYH content, then the change in the overall isoform content involves a developmental change in the muscle fiber type (Martinez et al. 1993). Hence it has been suggested that the abrupt changes in the overall ontogenic development might be induced by an unknown number of unidentified myosin heavy chains (Martinez et al. 1990)

.In a study on the masseter muscle of cat, dog and long-tailed macaque that contain super fast contracting muscles, antibodies were raised to detect myosin type 2M (superfast myosin in mammals) (Kirkeby 1995). The results revealed a presence of a 160 kD myosin band and three lighter myosin chains that are only found in fast twitch muscles like the masseter and not other limb muscles (Rowlerson et al. 1981; Whalen et al. 1979; Kirkeby 1995).

Rationale and Overarching Goals of the Project

It is reasonable to assume that we would identify a similar effect (change in myosin isoforms) when we analyze the myosin isoforms found in the 4-6 d

Zebrafish larva that exhibit the high tail beat frequency. We hypothesize that ***the high tail beat frequency in the Zebrafish larvae is due to the presence of a specific myosin isoform***. This hypothesis can be tested by looking at the different myosin isoforms at different ontogenic stages and correlating any possible isoform with the high tail beating frequency in larvae Zebrafish.

This study is also part of a bigger collaborative project on the genetic control of vertebrate and human development. It would be interesting to study correlation of genes and protein expression with that of developmental changes. This could pave way to study in detail the early developmental stages and how proteins help define functions of muscles. It would be particularly interesting to understand the role of mechanical stimuli in the development of locomotory structural tissue, such as muscle and bone. A better understanding of myogenesis and osteogenesis can lead to new insights into human muscle and bone diseases, such as cleidocranial dysplasia and achrondroplasia, which affect osteogenesis during fetal development.

Specific Objectives

One prediction of our hypothesis is that this myosin isoform will be found only in the Zebrafish larvae that exhibit a high tail beat frequency but not the adult Zebrafish. We will be analyzing the protein in 2-, 4- and 6-d old larvae and adult Zebrafish where we expect not to see the band found in the cat masseter muscles. This is because Zebrafish eggs hatch 48-72 h post fertilization and one d old embryo do not move around. They stay at the bottom and the tail beats only to refresh the water around. They have a yolk sac that provides nutrition and food. It is the larvae aged 4-6 d old that need to swim around for food and it is in this age group that we observe the superfast tail beat frequencies. This tail beat frequency

then drops down in the age groups 6 and up. We will be studying the age groups of 2- and 6-d old larvae and adult Zebrafish. We expect to see myosin bands corresponding to superfast muscles in the age groups from 4-6 d. This will provide us with further information that the high tail beat frequency is due to the superfast muscles. This will also suggest that superfast muscles could act as locomotory muscle in Zebrafish larvae.

As per these hypotheses the objectives in our study are:

To develop a protocol for extracting myosin protein

To quantify the total amount of protein in the 4-6 d old larvae and adult Zebrafish samples using BCA assay techniques

To identify the band of myosin isoform present in the 4-6 d old larvae and adult stages using SDS-PAGE and the band corresponding to the cat masseter muscles in the 160 kD region

To identify and confirm the presence of the specific myosin heavy chain isoform using Western blot techniques.

To determine the overall myosin present by ImageJ analysis on the bands that correspond to the myosin isoform

MATERIALS AND METHODS

Muscle Specimen Collection

Initial experiments were carried out using Tilapia due to its bigger size and higher protein concentration compared to that of the smaller Zebrafish. It allows for manipulations while validating the protocols and to avoid wasting precious samples of Zebrafish larvae and adult during the trial experiments. Myosin extracted from adult Tilapia muscle (5g) was studied for comparison.

Zebrafish larvae (sample size 300 per d) were harvested at 2-, 4- and 6-d. The larvae were obtained from the Zebrafish International Resource Center (ZIRC), Eugene, Oregon (http://Zebrafish.org) and were maintained in the animal facility at California State University, Fresno. Larvae (1 g) and adult fish (1 g) were used for protein extraction and the subsequent experiments. The maximum possible amount of water was drained off the larvae before the subsequent experiments.

Protein Extraction

The most suitable and reliable protocol for extraction of Zebrafish myosin protein was determined from three different procedures with various buffers. The first protocol (Westerfield 1993) was the SDS sample buffer extraction that consists of 1 M Tris-HCl pH 6.8, glycerol, 20% SDS, β-mercaptoethanol and DI water. Buffer 1 was stored at -20°C. The second protocol, (Westerfield 1993) protein extraction buffer consists of 10 mM Tris, pH 7.4, 2% Triton-X 100, 1 mM PMSF and 0.010 % of protease inhibitor (Roche, 11836170001). The fish were chemically anaesthetized before carrying out these two protocols in 10% formalin.

The third procedure used the high-ionic gradient extraction buffer consisting of various concentrations of KCl to isolate myosin (Martone et al.

1986). The samples extracted using this buffer yielded a consistent amount of myosin and this procedure was found to be the most suitable for the extraction. All samples were placed on ice. All chemicals were reagent grade. The composition of the buffers used in the extraction protocol was as follows. Solution A: 0.10 M KCl (Fisher Scientific), 1 mM phenylmethylsulfonylfluoride (PMSF), 0.02% Sodium azide (J.T. Baker Co) and 20 mM Tris-HCl buffer, pH 7.5 (Fisher Bioreagents). Solution B: 0.45 M KCl (Fisher Scientific), 5 mM β-mercaptoethanol (β-MCE) (Fisher Scientific), 0.2 M Magnesium acetate (Spectrum Corp), 1 mM ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) and 20 mM Trismaleate buffer pH, 6.8 (Aldrich Inc.). Solution C: 0.5 M KCl (Fisher Scientific), 5 mM β-MCE (Fisher Scientific) and 20 mM Tris-HCl buffer, pH 7.5 (Fisher Bioreagents).

The adult and larvae fish samples were flash freezed using dry ice and ethanol bath and stored at -80 °C. The samples were homogenized in a Potter-Elvehjem homogenizer with 10 volumes of solution A (0.10 M KCl). Samples were then incubated at 4°C for 30 min and then centrifuged at 1,000 x g for 10 min. The pellet (Pellet 1) obtained was resuspended in 5 volumes of solution B (0.45 M KCl). The final concentration was made up to 10 mM with ATP. The samples were incubated at 4°C for 1 h and centrifuged at 10,000 x g for 15 min. The supernatant (Sup 2) was collected and suspended in 25 volumes of sodium bicarbonate solution. It was incubated at 4°C for 15 min and centrifuged at 12,000 x g for 10 min. The pellet was resuspended in 5 volumes of buffer C and homogenized in a Potter-Elvehjem homogenizer. The pellet was incubated at 4°C for 10 min and then diluted with 2.5 volume of 1 mM sodium bicarbonate. The solution was then made up to 10 mM using magnesium acetate (Pellet 3). The solution was then incubated overnight at 4°C then centrifuged at 22,000 g for 15

min. The resulting pellet (Pellet 4) was diluted with 1 volume of buffer C, homogenized and saved for further use. The samples were stored at -80°C.

Estimation of Protein Concentration

The concentration of the protein obtained after extraction was determined using the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). This method uses the reduction of Cu^{2+} to Cu^{1+} by the sample protein, in an alkaline medium, coupled with the highly sensitive colorimetric detection of cuprous cation using the bicinchoninic acid. The presence of protein was confirmed by the purple color of the solution. The absorbance was read at 562 nm in a plate reader (Molecular Devices) in the lab of Dr. Jason Bush, Department of Biology, California State University, Fresno. A standard protein, bovine serum albumin (BSA), with a linear absorbance range for varying concentration of 0.25-2 µg/µl was used to plot a standard curve. The standard plot was then used to determine the concentration of the samples.

Protein Separation

The samples were separated on a 10 or 12-well, 4-20% Tris-Glycine gradient SDS-PAGE gel (Invitrogen). Samples were prepared in a 6X sample buffer dye (1.2 ml of 0.5 M Tris–HCl, 1.2 g SDS, 0.006 g of bromophenol blue, 2.1 ml of DI water, 0.93 g of dithiothreitol and 4.7 ml of glycerol) by boiling for 10 min The SDS-PAGE electrophoresis was run at 30 mA/gel for 90 min. The gel was stained with Coomassie blue stain (2.5 g of Comassie blue R-250 stain (Sigma), 450 ml of ddH_2O, 450 ml of methanol, 100 ml of glacial acetic acid) for 30 min and then placed in de-stain solution (10% glacial acetic, 20% methanol and de-ionized water) overnight. Samples were run with a Precision Plus protein standard molecular weight marker (10 kD to 250 kD) (Bio-Rad, cat.161-0373) and

a myosin-actin standard (Bio-Rad, cat.1660010). The gel was then visualized using a gel documentation system (Kodak Gel Logic imaging system 1500) in the lab of Dr. Larry Riley, Department of Biology at California State University, Fresno.

Protein Identification

SDS-PAGE analysis identified a band corresponding to myosin. In order to confirm the presence of the myosin isoform a more specific method had to be used to confirm the presence of the myosin in the band observed using Coomaasie stain.

A Western blot analysis of the samples was carried out. Another gel containing a duplicate of the samples, from the SDS-PAGE analysis, was transferred on a polyvinylidene fluoride (PVDF) membrane for Western blot analysis. The PVDF membrane with the proteins were probed using the MYH2 (Santa Cruz, cat. 53095) or MYH1/2/3 antibody (Santa Cruz, cat. 53092). The MYH2 is a mouse monoclonal antibody raised against adult skeletal muscle myosin of human origin detecting myosin heavy chain 2 (MYH2) of mouse, rat, human and rabbit origin at 200 kD. The MYH1/2/3 primary antibody is a mouse monoclonal antibody raised against skeletal muscle myosin of human origin, detecting the myosin heavy chains encoded by MYH1, 2 and 3 of fish origin at 210 kD. The Precision Plus protein standard, molecular weight marker (250 kD to 10 kD) (Bio-Rad, cat.161-0373), and the myosin-actin standard (Bio-Rad, cat.1660010) were used as reference. The PVDF membrane was first rinsed in methanol solution and then in 1X Tris-buffered saline Tween-20 (TBST) buffer. Filter paper soaked in 1X TBST buffer on the bottom of the gel transfer apparatus was followed by the membrane, the gel and another filter paper soaked in 1X TBST buffer on top of the gel. The transfer was then performed at 110 mA for 60

min using a semi-dry transfer unit (Hoefer TE77, Amersham Biosciences). The membrane was blocked to avoid non-specific binding by placing it in a 5% blocking solution (1g of non-fat milk powder (Kroger) in 20 ml of 1X TBST) on an orbital shaker overnight at 4°C. The membrane was washed 3X for 10 min with 1X TBST buffer. The membrane was then probed with the primary antibody MYH1/2/3 (Santa Cruz, cat. 53092) of dilution 1:10,000 for 3 h. The membrane was then washed 3X for 10 min with 1X TBST buffer to remove unbound antibody. The membrane was then probed with a goat anti-mouse IgM-HRP secondary antibody (1:1,000) (Pierce, cat.31430) for 2 h. The membrane was then washed 3X for 10 min with 1X TBST buffer.

This membrane was developed using a chemiluminescent substrate and detection kit (Thermo Scientific, cat.34075). The membrane was then visualized using a gel documentation system (Kodak Gel Logic imagining system 1500) in Dr. Larry Riley's lab, California State University, Fresno.

Image Analysis and Quantification of the Gel and Western Blot, Band Intensity

Measurement of the band intensity was carried out using the ImageJ software (Ver.1.60.0-23) available at the NIH website (http://rsbweb.nih.gov/ij/). JPEG images of the gels and blots, with a resolution of 1392 X 1040 were used for this analysis. ImageJ is a Java-based image processing program helpful in analyzing the intensity of a region of interest (ROI). In the case of this study, the region of interest is the protein bands on the SDS-PAGE gels and the myosin heavy chain band in the Western blots. JPEG files of 205 X 309 pixels were converted to 8-bit gray-scale image for analysis. The gel analysis routine was then carried out by choosing the ROI with a *Rectangular* selection tool from the ImageJ toolbar. The images of the SDS-PAGE gels were rotated using the *Image* >

Transform > *Rotate* option to straighten the image so as to be able to analyze entire lanes of sample protein. The images of the Western blots were processed as is, since only the myosin heavy chain band was of interest. In the Western blots the standards were marked as the first lane using the *Analyze* > *Gels* > *Select first lane* option and then the sample lanes were marked as *Analyze* >*Gels* > *Select next lane*. Once the lanes were marked the *Analyze* > *Plot lanes* was used to obtain a profile plot. A profile plot shows the relative density of the region marked by the rectangular box. The plots are arranged according to the order in which the rectangles were selected, top to bottom. The peaks in the plots represented dark bands in the original image. Due to background signal the peaks do not reach the baseline of the plot and have to be manually drawn using a *Straight* line selection tool from ImageJ toolbar to be able to measure the size of the peak. The area of each peak was then measured using the *Wand* (tracing) tool, the measurement are displayed in the *Results* pop-up window. Once the peak area is measured the peaks are then labeled with its size using the *Analyze* > *Gels* > *Label peaks* option and expressed as a percentage of the total size comparison to all the selected peaks. This value was then stored for further analysis.

The data from the results window was saved to an Excel file and then further computed to get the relative density of each of the bands. The percent value of the myosin actin standard was used throughout the analysis of SDS-PAGE and Western blot images. The percent values of all the other samples were divided by the percentage of the standard to give the relative density. This relative density is a measurement using pixels to determine the concentration of protein loaded in each of the wells. These values were then used to determine the concentration of proteins in the SDS-PAGE gels and the concentration of myosin loaded on the Western blots.

RESULTS

Validating a Procedure for Myosin Protein Extraction by SDS-PAGE Analysis

The extraction of myosin protein from Zebrafish was carried out with different procedures to compare and maximize the yield of protein. The various procedures were identified based on their ability to extract proteins efficiently from Zebrafish samples and to yield a high amount of myosin protein for detection. The extraction was carried out using three different buffers: 1) SDS extraction buffer, 2) protein extraction buffer and 3) high-ionic gradient buffer (KCl). The SDS extraction buffer and the protein extraction buffer have been used by other studies to extract protein from Zebrafish embryo. These procedures did not produce myosin band (Fig. 3). The procedure involving the high-ionic gradient buffer, used increasing concentrations of KCl (0.1, 0.45 and 0.5 M) in solutions A, B and C, to lyse the cell and solubilize myosin. This procedure yielded the 6maximum amount of myosin protein from the Zebrafish samples. This procedure was used to extract myosin for all subsequent studies.

SDS-PAGE Analysis of Protein Sample Using the First Procedure SDS Extraction Buffer

The extraction of protein from adult Zebrafish was carried out using SDS extraction buffer (Procedure 1) (Westerfield 1993) (Appendix. A). Samples were diluted to determine and validate the concentration of myosin. Actin and other bands corresponding to lower molecular weight protein like tropomyosin (8-50 kD) were visualized. The bands observed were not distinct and the high molecular weight proteins including myosin were not visualized (60-200 kD). This could be due to denaturation of the highly sensitive myosin in the high concentration of

SDS and EDTA in the buffers (Fig. 3). Procedure 2 yielded similar results and was not found to be suitable for the extraction of myosin (data not shown).

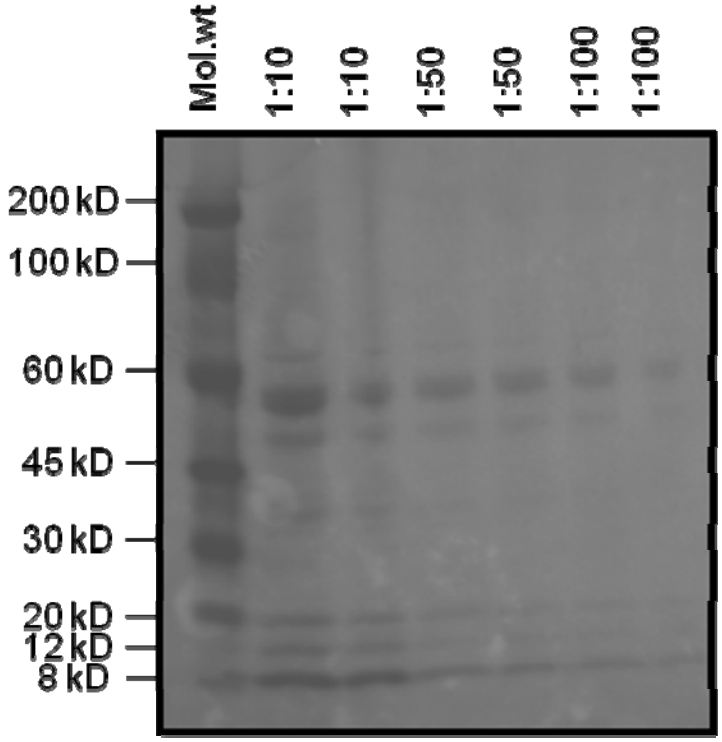

Fig. 3: SDS-PAGE gel displaying bands of the extracted adult Zebrafish total protein, using protein extraction buffer (Procedure 2).

SDS-PAGE Analysis of Protein Sample Using High-Ionic Protein Extraction Buffer

The extraction of protein from adult Tilapia was carried out using high-ionic protein extraction buffer which lyses the cell, dissociating the myosin filaments into monomers and solubilizing myosin. Initially, 5g of Tilapia abdomen muscle was used to validate the Procedure 3. This procedure was then used to extract myosin from Zebrafish adult and larvae for all experiments reported (Fig. 4).

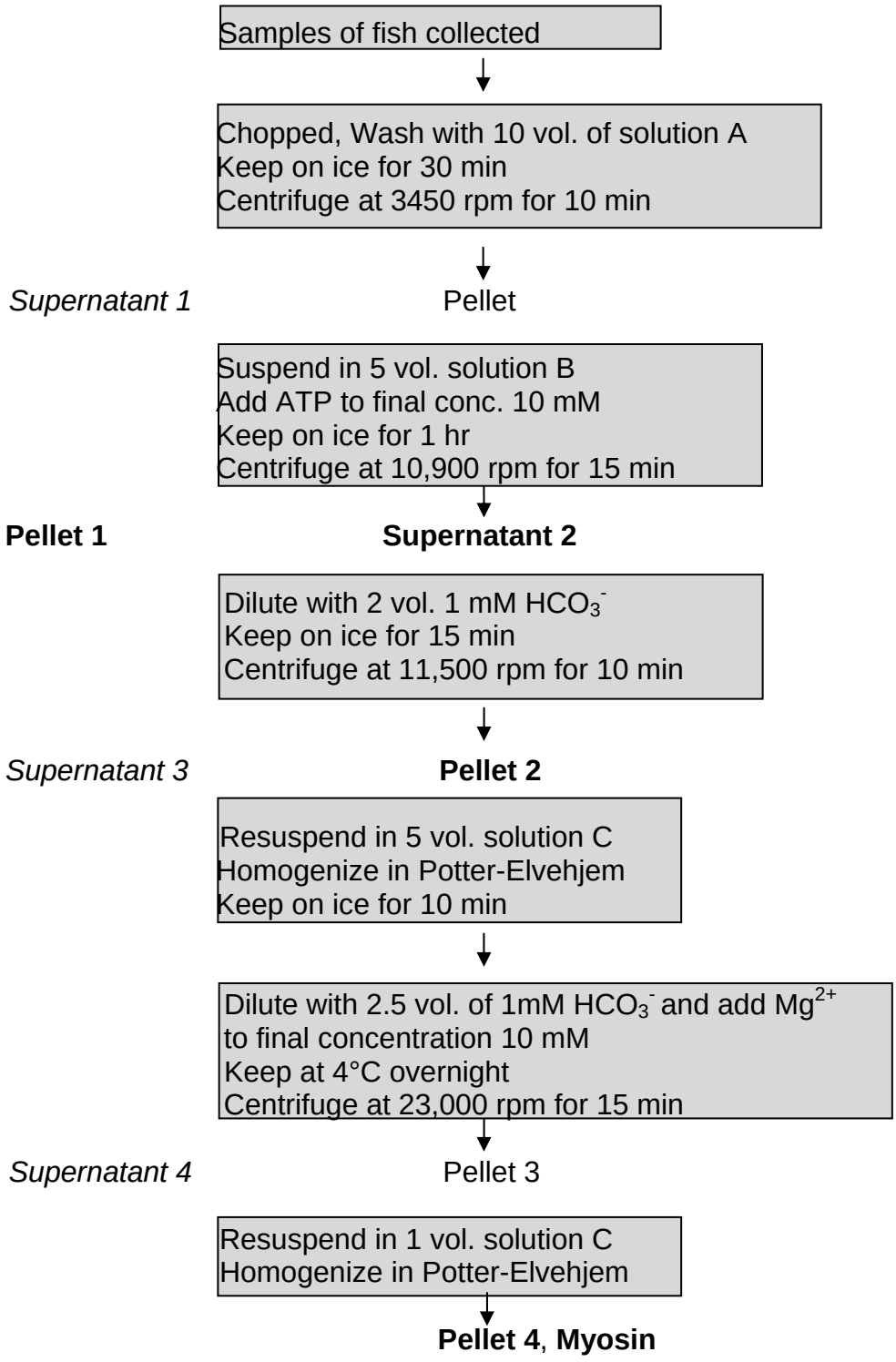

Fig. 4: Flow chart of Procedure 3, the high ionic gradient buffer for extraction of myosin from Tilapia and Zebrafish samples. The pellet and supernatant samples important in this extraction that contain myosin are indicated in bold. The discarded samples are in italics.

Extraction of Protein from Tilapia Samples

The protein extraction from Tilapia (5g) was initially carried out using the high-ionic protein extraction buffer (Martone et al. 1986) (Fig. 5.). The SDS-PAGE experiment verified that the procedure 3 did extract myosin protein from the abdomen muscle of the Tilapia adult. Bands were visualized corresponding to proteins to: gelsolin (90 kD) actin (42 kD), tropomyosin (35 kD), troponin (30 kD) and myosin light chains 1(25 kD) , 2 (19 kD), 3 (15 kD) and troponin. All of the above proteins are associated with myosin in skeletal muscles and are involved in the mechanism of muscle contraction. In the purified final pellet 4, the myosin band is visualized corresponding to a 200 kD band observed in the last two lanes (Fig. 5).

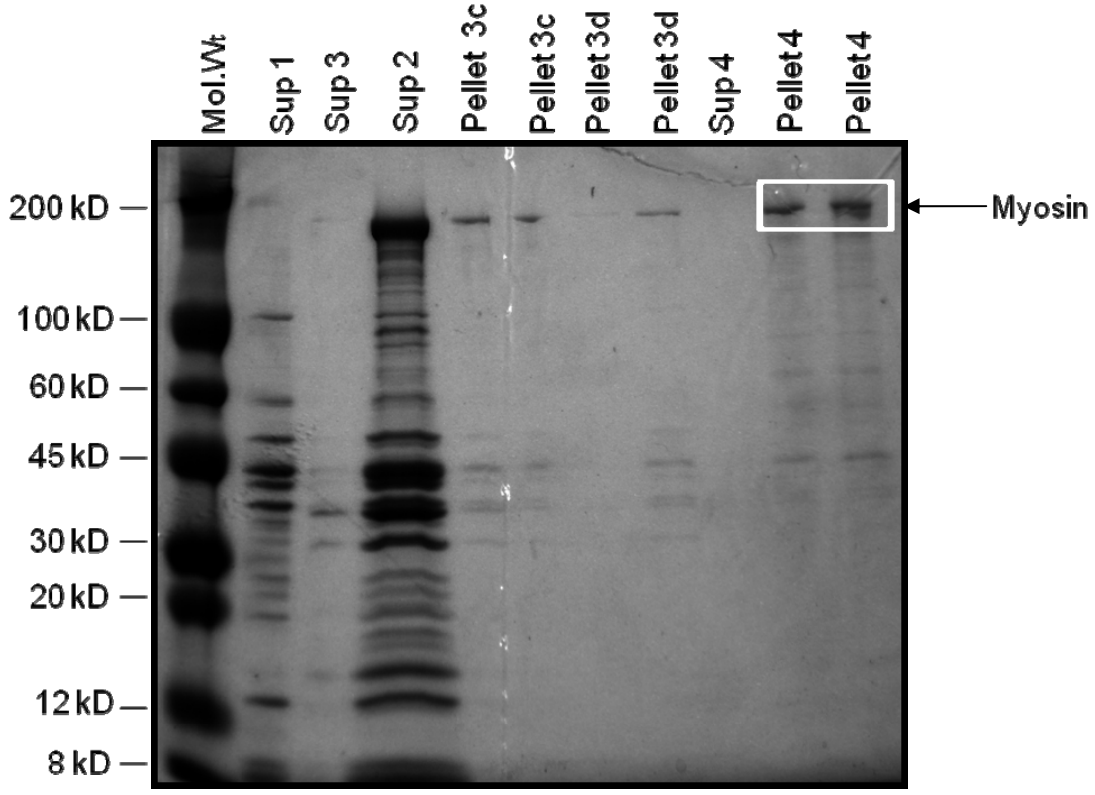

Fig. 5: SDS-PAGE gel displaying bands of the extracted protein from Tilapia abdomen section using extraction Procedure 3. Myosin band visualized at 200 kD.

Extraction of Protein from Zebrafish Samples

The extraction of protein from adult Zebrafish (5g) was then carried out using high-ionic protein extraction buffer (Martone et al. 1986) (Fig. 6). Pellet 4 sample of Tilapia and standard actin-myosin protein were run as positive control. The samples at different stages in the protocol were run to check the amount and presence of protein at each step of extraction, and to determine the loss of protein at the intermediate steps.

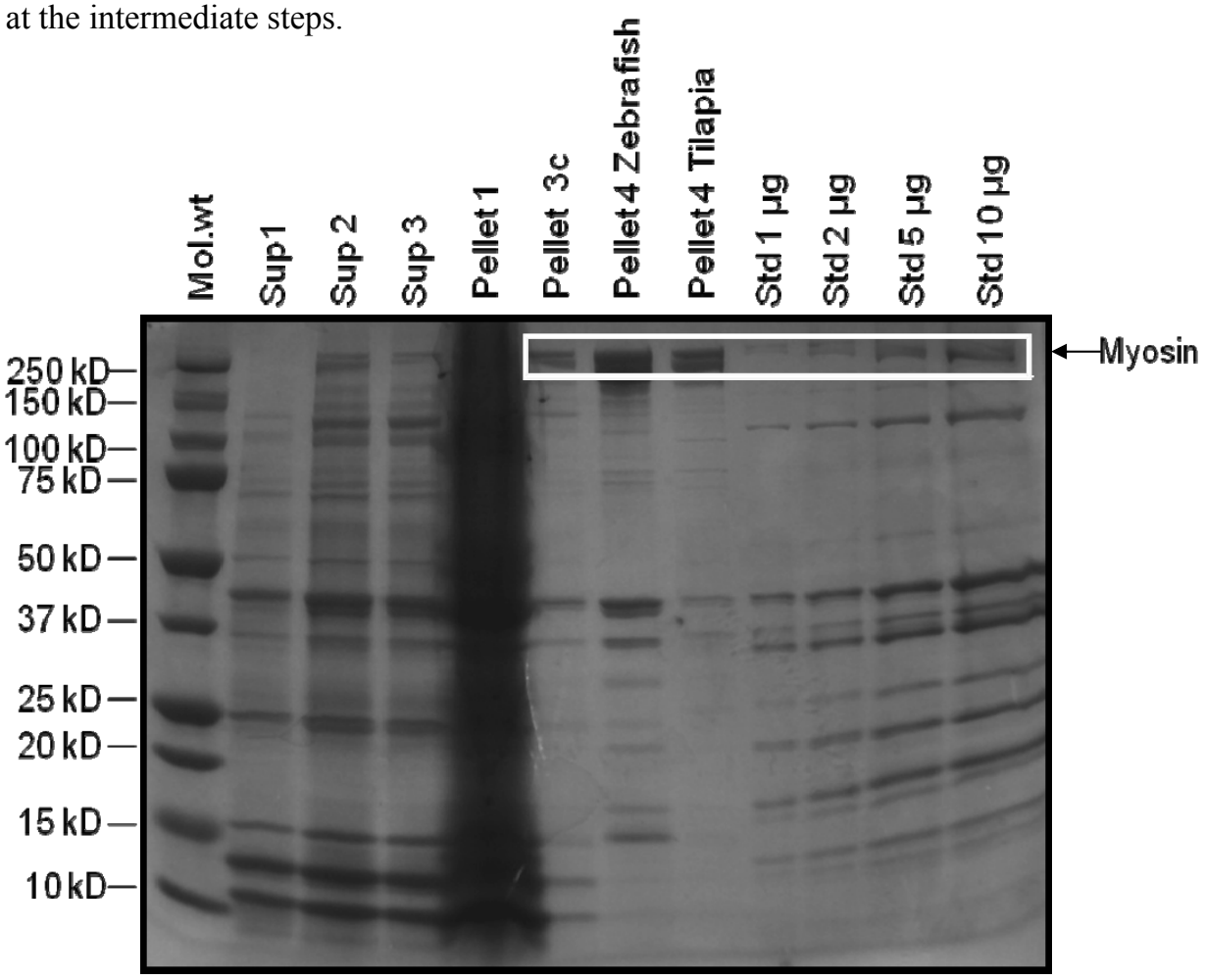

Fig. 6: SDS-PAGE gel displaying bands from adult Zebrafish samples and final pellet of Tilapia, extracted using Procedure 3. The bands from the extracted samples were compared against several concentrations of standard myosin-actin protein. Myosin band was visualized at 200 kD in pellet 4 and standard myosin-actin lanes.

Pellet 1 had a very intense band, indicating that a high amount of protein was extracted from the fish. The intensity of the bands was less in the preceding samples Sup 2, Pellet 3c. The final Pellet 4 corresponded with a distinct band for myosin. The intensity of bands corresponding to actin (42 kD) and tropomyosin (35 kD) was lower in pellet 4 (lane 7) than in pellet 1 (lane 5), indicating that a good amount of myosin protein was purified from the Zebrafish and Tilapia samples. A distinct band corresponding to the protein myosin was visualized in the standards and the samples.

Bicinchoninic Acid (BCA) Protein Assay for Analysis of Total Protein Concentration

The total concentration of the extracted protein samples was estimated using the BCA protein assay. Bovine serum albumin (BSA) protein was used as the standard and various known concentration of the BSA protein was prepared. Several known concentrations of BSA protein were prepared. The absorbance measurements of the various concentrations of the BSA protein were read at 562 nm. The absorbance measurements of the standard protein BSA, was plotted against its known concentrations (µg/µl) to prepare a standard curve. The standard curve was used to determine concentration of the samples. Various dilutions of the samples were prepared and their mean average was used to determine the final concentration of the sample. The concentration of the samples was calculated in µg/µl.

The Pellet 1 in all the samples had relatively high total protein as expected (2.4 mg). The protein concentration level gradually decreased and was minimal in the final Pellet 4 for all the samples (Table 1). The total amount of protein across the various age groups was also different, the 2-d old larvae had a lower amount of total protein (1 mg) than the 6-d old larvae (1.4 mg). Though the muscle mass

used was 1 g for all the age groups, the total protein extracted from the 2 and 6-d larvae groups was considerably low when compared to that of the adult (Table 1). The reason for this lower concentration observed in the 2-and 6-d old larvae samples could be due the inability to account for differences in the wet mass values , which have included in the initial weights measurements of the two larvae samples.

Table 1: BCA analysis of 2-, 4-, 6-d old and adult Zebrafish protein samples

	Protein concentration by BCA			
		µg/ul	Total volume (ml)	Total protein (mg)
2- day old zebrafish larvae	Pellet 1	2.00	0.50	1.00
	Sup 2	1.70	0.10	0.30
	Sup 3	0.13	7.50	1.00
	Pellet 4	0.48	0.50	0.24
6-day old zebrafish larvae	Pellet 1	2.80	0.50	1.40
	Sup 2	0.75	0.10	0.08
	Sup 3	0.20	7.50	1.50
	Pellet 4	0.54	0.50	0.27
Adult zebrafish	Pellet 1	2.14	1.00	2.14
	Sup 2	2.00	0.10	0.20
	Sup 3	0.14	7.50	1.05
	Pellet 4	0.60	1.00	0.60

SDS-PAGE Analysis of Zebrafish Adult and 2-, 4-, 6-d Old Larva

The extraction was carried out with 1 g each of 2-, 4 and 6-d old Zebrafish larvae. The extraction procedure was then repeated with 1g of Zebrafish adult samples to ensure that the initial sample weight for the extraction of protein was the same for the adult as well as the larvae. Distinct bands corresponding to myosin were observed in the samples from 1g of Zebrafish adult (Fig. 7).

The protein samples pellet 1, supernatant 2 and pellet 4, extracted from 1 g of adult Zebrafish, was loaded on the gel. Actin-myosin standard 10 µg was loaded as and run as a control. The visual intensity of the bands after Coomassie stain were correlated with the protein concentration analysis by BCA assay and the extraction was successful, yielding bands corresponding to myosin at 210 kD.

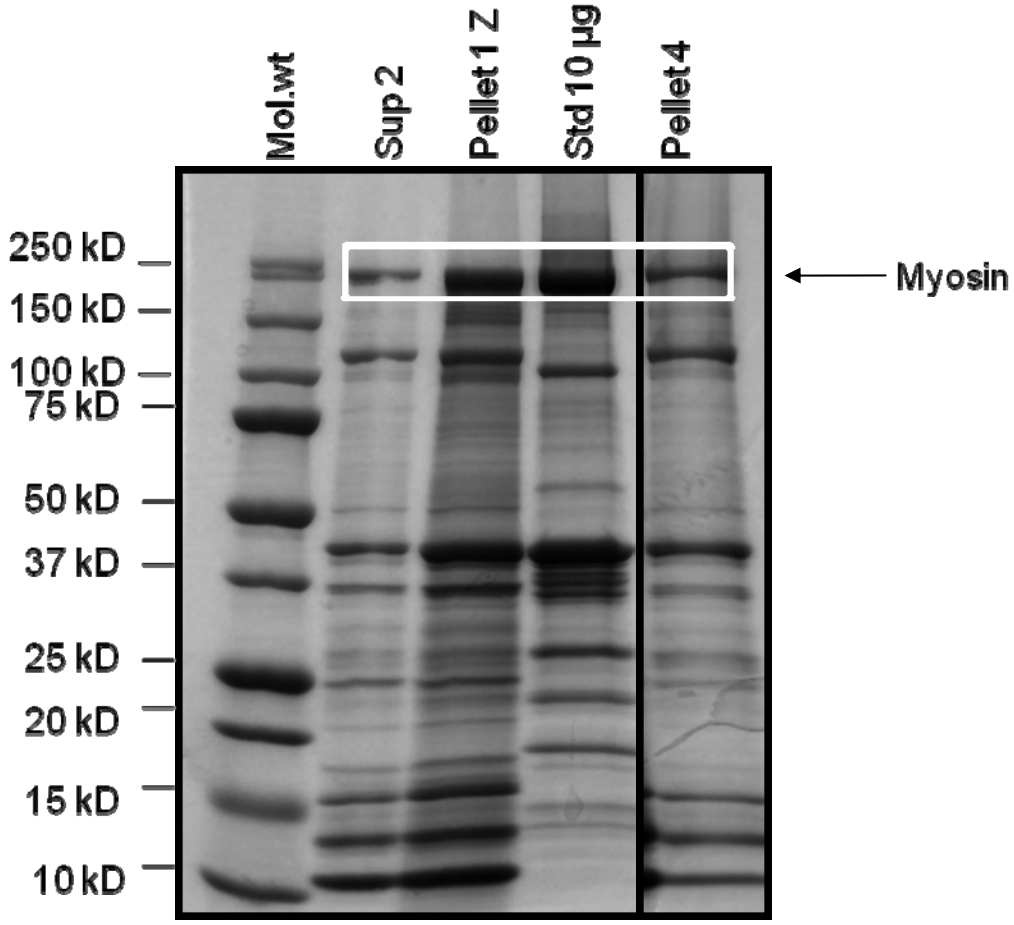

Fig. 7: SDS-PAGE analysis of adult Zebrafish protein samples extracted using Procedure 3. The initial amount of Zebrafish sample used was 1 g.

The extraction using 1g of fish sample also yielded bands corresponding to proteins like actin, troponin, tropomyosin and myosin light chains 1, 2, 3, as in the extraction using 5g of fish sample (Fig. 6 and Fig. 7 for comparison). This confirmed that the extraction was successful for the 1g of initial sample weight

used. The extraction was then carried out on the 2-, 4- and 6-d old larvae. The protein extracted from 4-d old larvae did yield a pellet but SDS-PAGE analysis did not identify any unique bands. The extraction of the 2- and 6-d old larvae were carried out since they were found to be most pertinent for this study.

The protein extracted from the 2-d and 6-d old larvae samples of 1g each were run on the gel and stained with Coomassie. The bands observed in both of these samples were sharp and consistent with the total amount of protein extracted, when correlated with results from the BCA assay and later with ImageJ analysis data. Standard actin-myosin (5 µg) was loaded with these samples as a positive control. The bands observed in the 2-d old larvae corresponded to myosin (210 kD), actin (42 kD), tropomyosin (35 kD) contractile proteins (Fig. 8A).

The protocol was then used to extract protein from the 6-d old larvae. The bands observed were lighter in visual intensity compared to those observed on Coomassie stained adult samples. This observation was later correlated with data from BCA assay confirming that the total amount of protein extracted was lower in the 6-d old larvae when compared to that of the adult Zebrafish. However, a unique band corresponding to 160 kD was noted in both pellet 1 and pellet 4 of the 6-d old larvae sample (Fig. 8 B). This band at ~160 kD was higher in molecular weight than similar bands at 140 kD which corresponded to the protein C, which is a myosin binding protein. The intensity of this band is higher compared to any of the other bands in the same lane. This 160 kD band is of primary interest since a similar band at 160 kD was observed in the superfast cat masseter muscles using a monoclonal anticarbohydrate antibody (Kirkeby 1995). The study on masseter also revealed that this band was only found in the superfast muscle fibers. However, the exact isoform was not identified in this research (Kirkeby 1995) and several identified and unidentified myosin isoforms could correspond to this band.

In this study, we analyzed the protein sample of 6-d old larvae using MYH1/2/3 antibody to confirm the presence or absence of this band. This band that was detected could be an isoform of any one of the myosin heavy chains 1, 2 or 3.

Western Blot Analysis for Identification of the Myosin Heavy Chain Isoform

The presence of myosin heavy chain was confirmed using a primary antibody reactive to fish. The primary antibody chosen and essential for this study is the myosin heavy chain 2 (MYH2). The MYH2 antibody was initially used for the experiment but had to be rejected since the ones available in the market were not reactive to fish species. We then used the primary antibody MYH1/2/3 to confirm the presence of the myosin heavy chain 2 in the adult and larvae samples.

Western Blot Analysis on Adult Zebrafish Using Primary Antibody MYH2

Since MYH2 is the most important isoform for this study, adult Zebrafish protein extracted and transferred on Western blot membranes was probed with MYH2 primary antibody (Fig. 9). This primary antibody probed only the myosin-actin standard (rabbit host) but failed to identify the myosin band (200 kD) in the samples. The myosin band in the rabbit myosin-actin standard was visualized but the antibody was not reactive to fish myosin in the samples. Hence it was found to be unsuitable to be used in this experiment on fish. Further studies were carried out with a more general primary antibody.

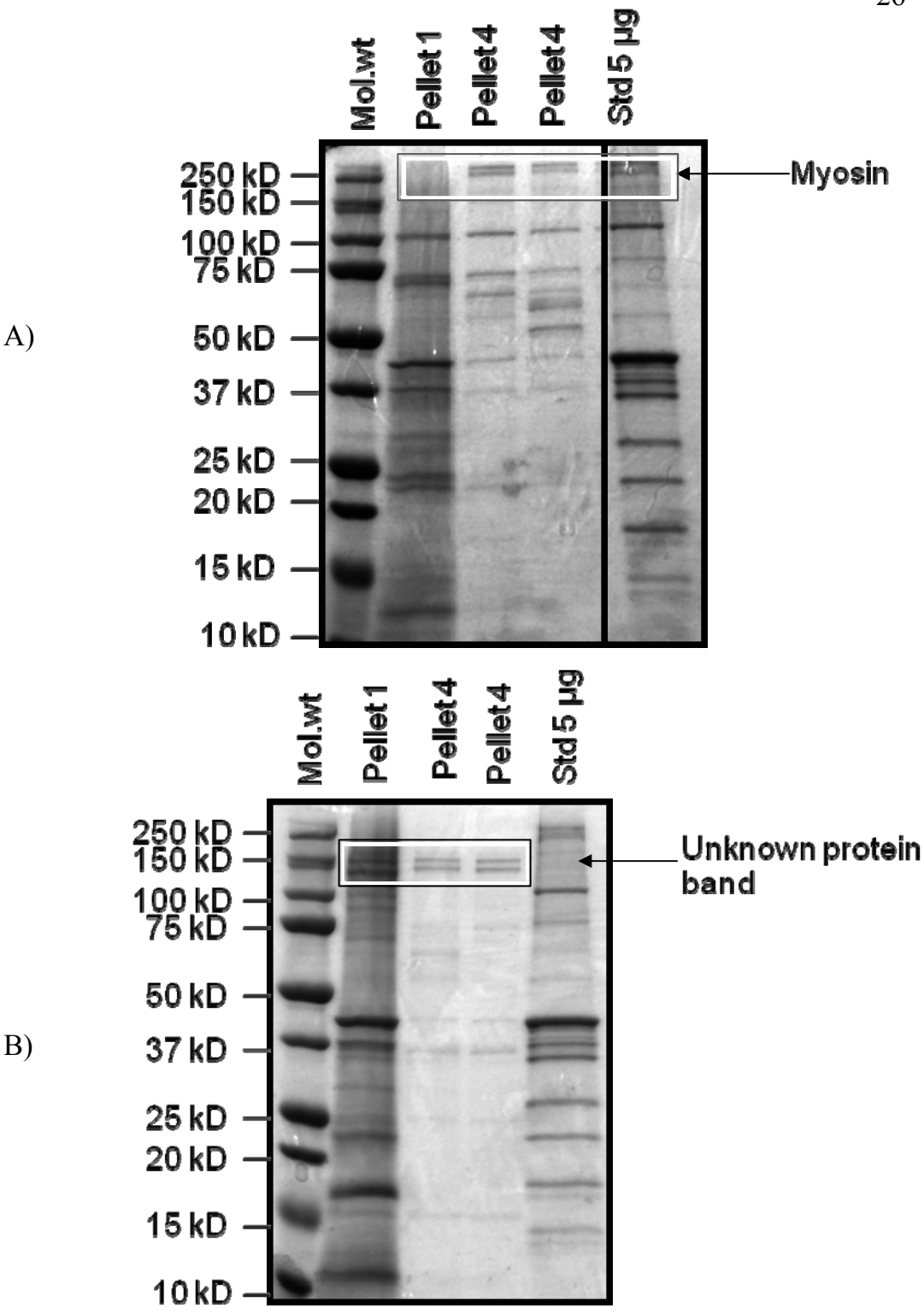

Fig. 8: SDS-PAGE analysis of protein samples extracted using buffer 3 from 2-d old (A) and 6-d old (B) Zebrafish larvae. Myosin was visualized in Pellet 1 of both samples.

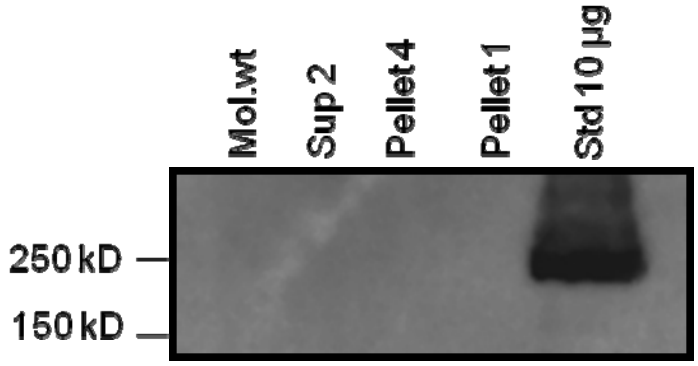

Fig. 9: Western blot analysis of the extracted protein using MYH2 primary antibody.

Western Blot Analysis on Adult Zebrafish Using Primary Antibody MYH1/2/3

The Western blot membranes containing the extracted Zebrafish protein, were probed with MYH1/2/3 primary antibody. This primary antibody was compatible with the standard actin-myosin protein. It was also able to identify proteins like actin (42 kD), tropomyosin (35 kD), myosin heavy chain (210 kD) and the myosin light chains 1, 2 and 3 (21, 19 and 16 kD). MYH1/2/3 antibody was used to probe a membrane with different concentrations of the standard myosin-actin to confirm if low concentrations of myosin protein could be detected using the antibody (Fig. 10). Various concentrations of the standard myosin-actin were probed with MYH1/2/3 primary antibody to determine the lowest concentration of protein that could be identified by Western blot analysis. The analysis was done to detect the myosin heavy chain in the larvae samples of total protein content: 1 mg (2-d) and 1.4 mg (6-d). The larvae have a total protein concentration of approximately 1-1.4 mg and will have lower concentration of myosin in the samples. The antibody was able to probe concentration of 1 µg/µl and so will detect even lower concentrations of myosin heavy chain isoforms in the Zebrafish protein samples (Fig. 11).

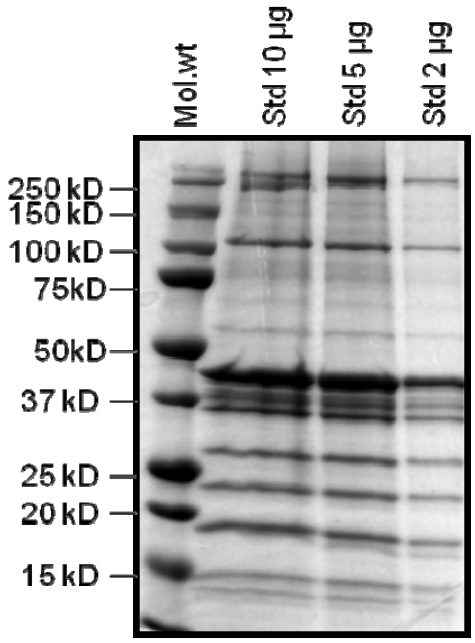

Fig. 10: SDS-PAGE gel analysis of different concentrations of actin-myosin standard (A).

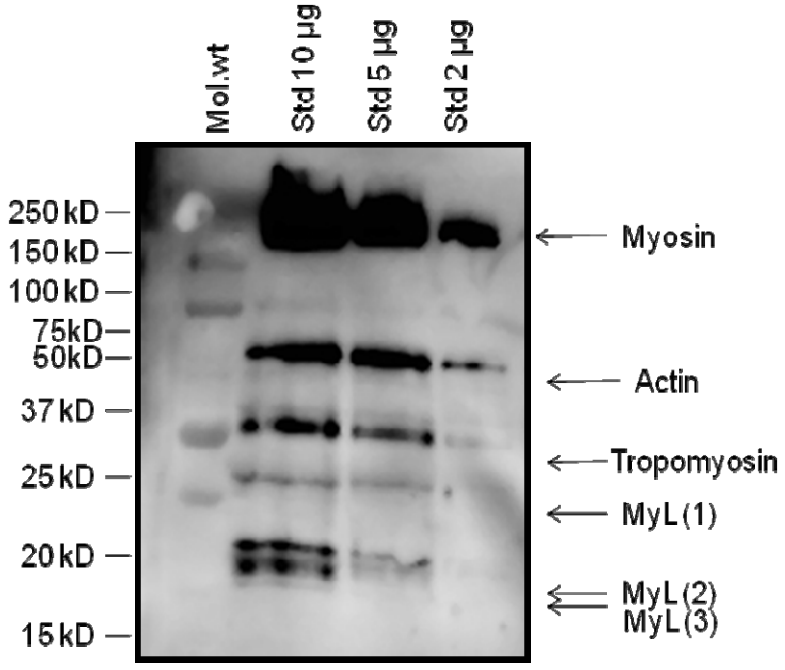

Fig. 11: Western blot analysis of the actin-myosin standard expressing bands of myosin, actin, tropomyosin and myosin light chains 1, 2 and 3 (B).

The Western blot was carried out with the adult Zebrafish to check if the myosin isoform can be identified in the extracted protein. The blot was probed with the primary antibody and an intense band corresponding to the myosin heavy chain was visualized. A band corresponding to myosin heavy chain was also observed in the purified pellet 4 confirming the presence of MYH2 in the adult Zebrafish (Fig. 12).

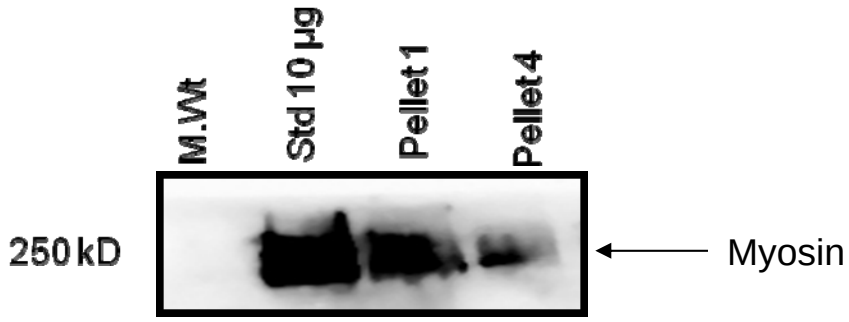

Fig. 12: Western blot analysis of protein sample from adult Zebrafish showing the presence of myosin heavy chain 2 detected using MYH1/2/3 antibody. Standard myosin-actin was used as a positive control.

The membranes containing protein samples from 2- and 6-d old larvae were then probed with the MYH1/2/3 antibody. The blot containing the 2-d old larvae samples expressed a band corresponding to the myosin heavy chain 2 but was the intensity was very less compared to that of the 5 µg of standard used for comparison. The band was in the pellet 1 sample but not in pellet 4 suggesting the myosin concentration was too low to be identified (Fig. 13). The absence of intense bands in the pellets also correlate with the fact that 2-d old larvae do not move around much and do not require high amount of myosin heavy chain for muscle contractions.

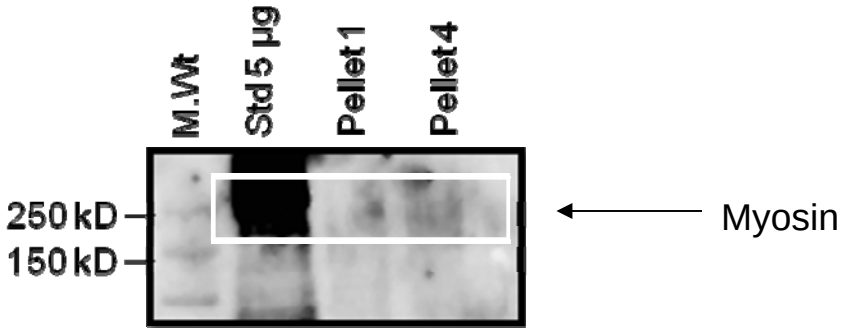

Fig. 13: Western blot analysis of protein sample from 2-d old Zebrafish larvae showing the presence of myosin detected using MYH1/2/3 antibody. Standard myosin-actin was used as a positive control.

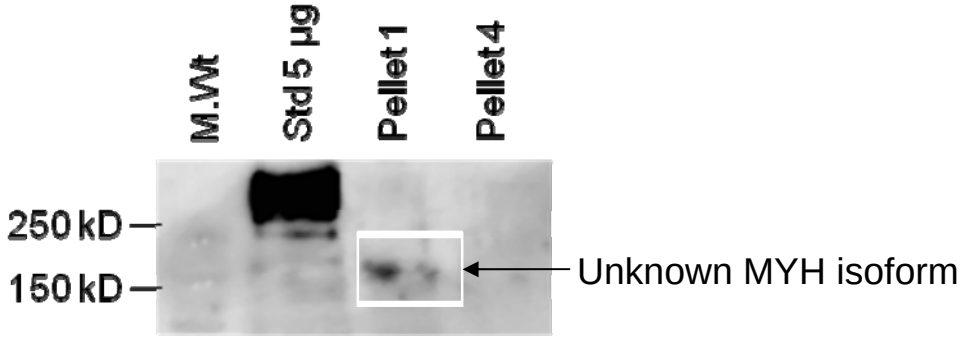

Fig. 14: Western blot analysis of protein sample from 6-d old Zebrafish larvae showing the presence of unique myosin heavy chain isoform detected using MYH1/2/3 antibody. Standard myosin-actin was used as a positive control

The 6-d old larvae was also probed with the MYH1/2/3 antibody to check for myosin heavy chain bands and also to possibly identify the band corresponding to 160 kD observed in the SDS-PAGE gel (Fig. 14). The analysis did not identify any myosin heavy chains at 210 kD but a blot corresponding to 160 kD band was observed. This suggests that the protein isoform reacts to the MYH1/2/3 antibody, which could mean it is a myosin heavy chain isoform yet to be determined or a different isoform of myosin heavy chain 2.

ImageJ Analysis of the SDS-PAGE Gels

Image analysis of the SDS-PAGE gels and Western blots was carried out using ImageJ software. The intensity of the bands was measured as previously mentioned in the methods section. The bands of several gels and Western blots were measured and an average of these values is being reported.

In the case of the SDS-PAGE gels the entire lanes of samples were compared against the myosin-actin standard (Table 2 and Table 4). The bands in the Western blot were individually measured and compared against the myosin-actin standard (Table 3 and Table 5).

Table 2: ImageJ analysis and measurement of total protein concentration from SDS-PAGE gel image of adult Zebrafish sample with standard myosin-actin.

Samples	Area	Percent	Relative density	µg/µl	µg	mg
Std (10 µg)	95083.235	33.214	1.000	10.000	500.000	0.500
Pellet 1	94045.113	32.852	0.989	9.891	1648.502	1.649
Sup 2	40707.051	14.220	0.428	4.281	71.355	0.071
Pellet 4	56436.729	19.714	0.594	5.935	494.621	0.495

Table 3: ImageJ analysis and measurement of total protein concentration from Western blot image of adult Zebrafish sample with standard myosin-actin.

Samples	Area	Percent	Relative density	µg/µl	µg	mg
Std (10 µg)	15669.99	42.79	1.00	10.00	500.00	0.5
Pellet 1	14239.23	38.88	0.91	9.09	1514.5	1.514
Pellet 4	6710.31	18.32	0.43	4.28	356.85	0.357

The intensity of the bands was measured and the total amount of protein was calculated. The concentration values obtained in the SDS-PAGE gel images were the total concentration of the sample. The concentration values of the

Western blots are the total concentration of myosin heavy chain protein in the sample. The results from the ImageJ analysis also suggest similar conclusions to that of the BCA assay namely, that the amount of protein extracted from the adult is higher than that of larval Zebrafish. The advantage of this method is that concentrations of the unknown band of myosin isoform were also determined to be 0.086 mg (Table 5). The total protein concentration of the myosin heavy chain band in the 2-d old larvae was 0.012 mg compared to the total protein content in Pellet 1, 0.944 mg.

Table 4: Total protein concentration of 2-d, 6-d old larvae and adult Zebrafish determined by ImageJ analysis of SDS-PAGE gel images

	Total protein in mg		
	Adult	2-day larvae	6-day larvae
Pellet 1	2.432	0.944	1.140
Pellet 4	0.614	0.219	0.253

Table 5: Total protein concentration of 2-d, 6-d old larvae and adult Zebrafish determined by ImageJ analysis of Western blot images.

	Total protein in mg		
	Adult	2-day larvae	6-day larvae
Pellet 1	1.500	0.012	0.086

These results conclude that there is a presence of a 0.086 mg of protein in the 6-d larvae that is an isoform of myosin heavy chain. This study concludes that there is a possibility that this isoform that has been detected in the 250-150 kD region could have superfast muscle properties since it is a isoform of myosin heavy chain that is responsible for superfast muscle contractions.

DISCUSSION

The aim of this study was to identify superfast muscles in the Zebrafish larvae aged 4-6 d. Analysis was done on the 2-, 4-, 6- d old larvae and adult Zebrafish for comparison between the different age groups and identifying the protein corresponding to the superfast muscle only in the 6-d old larvae.

Several protocols were experimented with to determine the suitable method to yield a purified myosin from the samples for good analysis in Western blot and SDS-PAGE gels. The extraction procedure involving the SDS-extraction and the protein extraction buffer did not yield myosin bands, but bands corresponding to other contractile proteins like actin, tropomyosin were visualized. It was then concluded that these procedures were not suitable to extract myosin protein from the sample size of 1g since it was possible that myosin was being denatured or was not precipitating.

Research on the sarcoplasmic proteins in porcine muscle tissue suggests that the sarcoplasmic proteins partially denature and deposit on myofilaments causing reduced extraction of the myofibrillar proteins. Since the sarcoplasmic proteins and myofibrillar proteins denature at the same temperature range, it is difficult to extract the myofibrillar proteins (Stabursvik et al. 1984; Bendall and Wismer-Pedersen 1962).

The susceptibility to myosin denaturation was found to be very high in liquid-air interphases. Another study on postmortem glycolysis in pork found that when the pH of the cell drops lowers than 7 to 5.5-5.6, at a temperature of > 35°C, there is rapid degradation of myofibrillar proteins. It was also suggested that different myosin isoforms respond differently to denaturing conditions (Van Laack and Lane 2000; Cheng and Parrish 1978). Extraction of myosin required a more

specific buffer that lyses the cell and solubilize the protein without denaturing it. Three types of buffers were found to be suitable to solubilize myosin: sucrose, pyrophosphate and high-ionic strength buffers. The pyrophosphates like sodium pyrophosphate and high-ionic strength buffer containing KCl or KHPO4 were previously studied on skeletal muscles (Pollard 1982; Kielley and Bradley 1956). The buffers also contain a pH buffer, a sulfhyrdyl reducing agent, low concentrations of ATP to dissociate actin from myosin, and a detergent to solubilize the membranes (Pollard 1982). Hence, a method with high-ionic gradient buffer (Martone et al. 1986) that yielded high myosin content from fish sample was experimented on to check if it would yield a sufficient amount of myosin for our analysis. This method used high-ionic gradient concentrations of KCl, with a slightly acidic pH of 6.8 or 7.5, to solubilize and extract myosin. The procedure also suggested that all steps be carried out at 4°C to prevent denaturation of the protein samples.

The high-ionic gradient buffer procedure yielded a good amount of total protein concentration as determined by BCA assay. The original procedure mentioned in Martone *et.al*. had used 15g of sample whereas in our study the procedure was modified to extract protein from 1g sample (Martone et al. 1986). The yield of myosin in the original study was 10mg from 15g. In our study, the yield of myosin was in the range of 1.0-2.4mg from 1g of initial mass.

The protein extraction and analysis was carried out on an entire adult Zebrafish and 300 larvae samples. The SDS-PAGE analysis of the 2-, 4-, 6-d old larvae and adult Zebrafish were carried out to detect the presence of myosin heavy chain 2 isoform. Myosin heavy chain isoform 2 was observed in the 2-, 4-d old larvae and adult Zebrafish corresponding to the 210 kD band. The Western blot analysis of the 2-d old larvae detected a band of lower intensity, which was

determined to be 0.012 mg using ImageJ analysis (Table 5). This value is less than that of the total protein concentration for the same 2-d old Pellet 1 sample, which the BCA assay determined to be 1 mg (Table 1), and ImageJ analysis determined to be 0.94 mg (Table 4).

Zebrafish hatchlings tend to be stationary and do not seek out food, therefore a high concentration of myosin heavy chain 2 (MYH2), which is responsible for muscle contraction, was not expected in the 2-d old larvae Zebrafish. The nourishment for the hatchlings is provided by a yolk sac that is twice the head size of the larvae in width. The tail of the 2-d old larvae (48 h after fertilization) is still undergoing morphogenesis and differentiation to be able to develop swimming activities. The 3-6-d old larvae on the other hand, have completed their morphogenesis and their yolk sacs have depleted. (Kimmel et al. 1995). Due to their need for survival, the 3-6 d old age group of larvae produces swift escape responses and start to seek out food (Kimmel et al. 1995). It is in this age group of 3-6 d old larvae that we expect to see a higher concentration of MYH2, and the unique myosin isoform corresponding to superfast muscles.

Though previous studies have shown that the superfast tail beat frequency of 100 Hertz has been observed in the 3-4 d old larvae, our results indicated the presence of a unique band only in the 6-d old larvae (Müller and van Leeuwen 2004; Buss and Drapeau 2001).The strong binding of this protein to the primary antibody MYH1/2/3 confirms the presence of an unknown isoform that is not present in the 2-d old and adult Zebrafish, making it a special interest for this study (Fig. 14). This discrepancy in the data could possibly be explained by considering two facts. Firstly the 72 h early larva is just out of its hatching period and it is possible that the development of the larvae in our facilities was slowed, as noticed by the length of the larvae and its body size. Secondly the length of the 3 d

old larvae is roughly 3.7 mm and that of the 6-d old larvae is 4.2 mm, a less than 1 mm difference so it is possible that the larvae still has its superfast tail beat frequency contractions (Kimmel et al. 1995). However the presence of the myosin isoform corresponding to the region between 150-250 kD corresponds with the fact that this age group does posses a myosin heavy chain isoform that is responsible for its muscle contraction property. This 160 kD band was also observed in the cat masseter muscle; in this study an anticarbohydrate antibody detected a carbohydrate that is associated with the myosin heavy chains in superfast muscle fibers from jaw-closing muscles. However, the function or significance of this band was not determined in the cat masseter study.

Our current study demonstrated that myosin heavy chain at 210 kD was found in all of the larvae and adult Zebrafish except for the 6-d old larvae. Since the 6-d old larvae is known to power swim the absence of the myosin heavy chain at 210 kD and the presence of another band of lesser molecular weight in the 250-150 kD suggest the presence of another myosin heavy chain isoform that could play the muscle contraction role. This is of primary interest since we postulated that there would be an isoform of myosin that would correspond to this unusually high tail beat frequency of 100 Hertz observed in Zebrafish larvae. Though this research did not identify the exact myosin isoform, the results from the SDS-PAGE, Western Blot, BCA and ImageJ analysis suggests the presence of such an isoform, which has properties of a myosin heavy chain, though its exact function was not identified in our study.

CONCLUSIONS

The results from our study suggest that the adult zebrafish has a higher total protein content in both Pellet 1 and Pellet 4 compared to that of the 2-and 6-d old larvae according to BCA assay and ImageJ analysis. The presence of a band corresponding to 210 kD was visualized in the 2-, 4-, 6- d old larvae and adult zebrafish during SDS-PAGE analysis. The presence of a unique band in the 210-150 kD region was observed only in the 6-d old larvae Zebrafish. Western blot analysis was carried out on the 2-, 6- d old larvae and adult Zebrafish using the MYH1/2/4 primary antibody. The results from the 2-d old larvae and the adult Zebrafish indicated the presence of myosin heavy chain 2 (MYH2). The total amount of MYH2 was 0.086 mg in the 2-d old larvae and 1.5 mg in the adult Zebrafish, indicating that the 2-d old larvae has lower total amount of the MYH2 protein. The Western blot results on the 6-d old larvae indicated a band in the region between 210 kD-150 kD not corresponding to the 210 kD band observed for MYH2. The strong binding of the primary antibody suggested that it was a heavy chain isoform of myosin with a concentration of 0.086 mg which is greater than the MYH2 content in the 2-d old larvae. Our findings conclude that the protein band corresponding to 210-150 kD could help determine if a myosin isoform is responsible for the high tail beat frequency exhibited by the zebrafish larvae. Further studies have to be carried out in order to identify and understand the specific function and role of this protein isoform in ontogenic development of Zebrafish larvae.

FUTURE DIRECTIONS

Future studies will include the extraction of myosin protein from 3-, 4-, 5-d old and juvenile zebrafish. Identification of MYH2 would be essential in the above age groups to verify that the band identified in the 6-d old larvae is not present. The growth of the fish under lab conditions were reexamined and measure the length of the larvae groups if possible to precisely determine the age group with the superfast tail beat frequency. A Tris-acetate or a resolving gel with a different gradient concentration can be used to identify precisely the region of the band corresponding to 210-150 kD. Analysis of the superfast muscles of rattle snake, toadfish and cat masseter muscles could aid in identifying the protein isoform found in the 6-d old larvae. Homology studies on the 6-d old larvae and the three species mentioned above could also help in identifying any other similarities. A MALDI-TOF analysis could be performed to identify the protein band found in the 6-d old Zebrafish larvae. These results could then be further used to understand if proteins responsible for locomotion could also function to assist superfast tail beat frequency.

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APPENDIX: EXTRACTION BUFFERS

SDS sample buffer (Westerfield 1993)

Composition

0.63 ml 1M Tris-HCl, pH 6.8

1.0 ml glycerol

0.5 ml - mercaptoethanol

1.75 ml 20% SDS

6.12 ml H_2O

(10 ml total)

Store at -20°C in aliquots.

Preparation:

Place fish samples in 200 µl of SDS extraction buffer and homogenize.

Boil 5 min in a water bath.

Centrifuge 2 min at 5000 x g.

Discard pellet and homogenize again if pellet present.

Transfer supernatant to a new tube and freeze at -70°C.

Protein sample buffer (Westerfield 1993)

Composition

10 mM Tris, pH 7.4

2% Triton-X 100

0.010 % of protease inhibitor

Preparation:

Place fish samples in 200 µl of extraction buffer and homogenize.

Incubate overnight at 4°C.

Centrifuge 20 min at 5000 x g

Transfer supernatant to a new tube and freeze at -70°C.

Discard pellet.

High-ionic extraction buffer (Martone et al. 1986)

Samples of fish collected **(5g)**

↓

Chopped
Wash with 10 vol. of solution A **(50 ml of Sol A)**
Keep on ice for 30 min
Centrifuge at 3450 rpm for 10 min

↓

(□25 ml) Supernatant 1 Pellet

Suspend in 5 vol. solution B (5 ml)
Add ATP to final conc. 10 mM
Keep on ice for 1 hr
Centrifuge at 10,900 rpm for 15 min

↓

(□ 1ml) Pellet 1 **Supernatant 2 (22.5 ml)**

Dilute with 5ml of 1 mM HCO_3^-
Keep on ice for 15 min
Centrifuge at 11,500 rpm for 10 min

↓

Supernata **Pellet**

Resuspend in 5 vol. solution C – **5 ml**
Homogenize in Potter-Elvehjem
Keep on ice for 10 min

↓

Dilute with 2.5 vol. of 1mM HCO_3^- and add Mg^{2+} to final concentration 10 mM **-(5 ml)**
eep at 4°C overnight
Centrifuge at 23,000 rpm for 15 min

↓

Supernatant 4 **Pellet 3**
Resuspend in 1 vol. solution C **(1ml)**
Homogenize in Potter-Elvehjem

↓

Pellet 4, Myosin **(1 ml, 25 mg)**

Samples of fish collected **(1g)**

↓

Chopped
Wash with 10 vol. of solution A **(10 ml of Sol A)**
Keep on ice for 30 min
Centrifuge at 3450 rpm for 10 min

↓

(9 ml) Supernatant 1 Pellet

Suspend in 5 vol. solution B – (5 ml)
Add ATP to final conc. 10 mM
Keep on ice for 1 h
Centrifuge at 10,900 rpm for 15 min

↓

38 mg (1ml) Pellet 1 **Supernatant 2 (4 ml) 33.98 mg**

Dilute with 5ml of 1 mM HCO_3^-
Keep on ice for 15 min
Centrifuge at 11,500 rpm for 10 min

↓

Supernatant 3 (**8 ml, 1.06 mg**) **Pellet (1 ml)**

Resuspend in 5 vol. solution C – **5 ml**
Homogenize in Potter-Elvehjem
Keep on ice for 10 min

↓

Dilute with 2.5 vol. of 1mM HCO_3^- and add Mg^{2+} to final concentration 10 mM **-(5 ml)**
eep at 4°C overnight
Centrifuge at 23,000 rpm for 15 min

↓

Supernatant 4 **Pellet 3 (50 µl)(**
(10 ml)

Resuspend in 1 vol. solution C **(1ml)**
Homogenize in Potter-Elvehjem

↓

Pellet 4, Myosin **(1 ml, 25 mg)**

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Date