Analysis of proteins in fish muscle tissue

Introduction

In vertebrates, the muscular system is an anatomical organ system controlled through the nervous system. Derived from the mesodermal layer of embryonic germ cells, these contractile tissues-of skeletal, smooth, or cardiac origin-are responsible for blood circulation, internal organ function, heat production, and organ protection. [1] With the skeletal system integrated, voluntary and reflexive movement, as well as posture and body position, become possible. Surrounded by an epimysium, skeletal muscles are composed of many long muscle fibers lined with endomysium, which are bound together by perimysium into bundles called fascicles. [2] Within these myocytes, there are smaller strands of myofibrils that contain myofilaments *(or sarcomeres)* – the basic unit of a striated muscle tissue. These repeating sarcomeres contract in response to nerve signals by means of sliding filaments: actin and myosin. The thin filaments consist of two chains of spherical actin proteins twisted in a helical conformation and troponin as a contraction regulator. [2] Each actin molecule has a myosin-binding site that is covered by tropomyosin during muscle relaxation. Having a head and tail region, myosin II proteins generally form the thick filaments with its six polypeptide chains and can cross bridge with actin filaments due to their elasticity and contractibility properties. Specifically, the motor domain of its two heavy chains adopt an Î±-helical coiled coil configuration and couple ATP hydrolysis with its motion while its two light chains-which wrap around the neck region of each heavy chain at the IQ sequence motif-have regulatory roles [1] . Although this major multi-subunit protein has remained greatly stabile across the animal kingdom over time, myosin light chains have

undergone evolutionary divergences for different species; however, the essential structure and functions have remained highly conserved. [3] Caused by genetic mutations, only favorable variations are passed through – this process allows for specialization, speciation, and evolution that eventually increases survival ability: *DNA (genes) ® RNA ® Protein ® Trait ® Evolution.* Protein gel electrophoresis and western blotting can be used to compare myosin light chains of different species by identifying any commonalities or alterations in specific subunits. Since proteins reflect changes in the gene pool, the phenotype and function as well as form of an organism can be identified, allowing for the study of their physiological adaptations to the environment. Through comparative proteomics-defined as the analysis of differentially expressed proteins with comparison between at least two protein profiles-changes in the proteome that have been caused by development, diseases, and the environment can be identified – allowing for assessment of biological variability and dataset comparability. [4]

The objective of this lab was to extract proteins from unknown samples of fish muscle tissue and then qualitatively analyze this protein mixture by performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) twice. The protein bands of the first gel-representing the total amount of proteins found in the tissue homogenate-were stained and visualized at 595nm with the Bio-Safe Coomassie Blue G-250 dye at 595nm while the fractionated proteins of the second gel were electroblotted onto a nitrocellulose membrane via Western blotting – where the specific protein of interest was selectively immuno-detected by chemiluminescence with a horseshoe radish peroxidase-linked secondary antibody. [3, 4] Accordingly,

the goal of this report is to identify the different types of proteins found in fish muscle-specifically of shark, tilapia, skitter, and salmon-required for muscle contraction and movement and to establish whether they are highly conserved or variable across all animal species. Consequently, information about the environment, niche, or physiological stresses faced by the organism can be elucidated as specific protein modifications that alter muscle function and performance work to increase their fitness and adaptiveness. [2] Differences in proteins may reveal information about the evolutionary relationships among various organisms and by understanding this diversity in the natural world, many biological problems can be solved to improve the quality of human life.

Materials and Methods

First , unknown tissue samples from two different fish species were prepared for protein extraction: in a 1. 5mL microcentrifuge tube, 250Î¼L of Laemmli (1x SDS) sample buffer was added as well as the minced tissue. After gently agitating the contents by flicking the tube, it was left to incubate at room temperature for five minutes. Next, the tube was centrifuged to pellet the tissue; this allowed for transfer of the supernatant buffer to a new 1. 5mL screw cap tube, which was then boiled at 95°C for five minutes. *Second* , SDS PAGE was performed on two separate precast TGX gels (purchased from Bio-Rad) since both Coomassie Blue staining and Western blotting were required. Refer to the BIO314 experiment 7 lab manual for instructions on how the gel apparatus was assembled with the Mini-Protean gels and tetra cell. When this was completed, the loading scheme for Coomassie staining involved pipetting the protein ladder (Biorad cat #161-0375) in lane 1 (at 7

Î¼L/line) and the actin/myosin standards in lane 6 (at 5 Î¼L/line). The rest of the lanes were used to load the samples (at 10Î¼L/line). The same set-up was done for the immunoblotting gel, except only 5Î¼L/line of each boiled sample was loaded. Refer to the BIO314 experiment 7 lab manual for instructions on how these solutions were loaded. After all of the samples have been loaded, the gel box lid was connected to the electrode assembly by matching the red and black leads with their corresponding electrodes. Then, the leads were plugged into the power supply, which was subsequently turned on and set to run at a constant voltage of 200V. This process was terminated at 30 minutes when the loading dye started to exit the gel. Refer to the BIO314 experiment 7 lab manual for instructions on how the gels were removed.

Third , Bio-Safe Coomassie staining was done on the appropriate gel-with samples loaded at 10Î¼L/line-which was peeled from the plate: it was then inserted into a container of deionized water and washed for 5 minutes on a rocking platform. Afterwards, the gel was transferred to another container with Coomassie staining solution – again, this was left on a rocking platform for 15 minutes. Upon completion, the stained gel was put in deionized water (destaining solution) and the lid was capped onto this container, which was placed onto the rocking platform for 15 minutes. *Fourth* , the immunoblot was prepared and transferred: with blunt-ended tweezers, the PVDF membrane and bottom stack was placed on the cassette base; the membrane was left facing up. Any air bubbles seen were immediately removed with a blot roller. Since one mini gel was employed, the stack was centered in the cassette. Then, the second gel-with samples loaded at

5Î¼L/line-was peeled from the plate (from the SDS-PAGE step) and stacked over-top of the PVDF membrane. Any air bubbles present were subsequently removed using a blot roller. Next, a second wetted top-ion transfer stack was placed above this gel. This assembled sandwich was rolled thoroughly with a blot roller to prevent any air bubbles from being trapped. Finally, the lid was closed and locked onto the cassette and this was set inside the turbo blotter to initiate the transfer. When the electro-transfer process was finished, the blots were dismantled and stored (at -20°C) according to the instructions written in the BIO314 experiment 7 lab manual. After one week, the Western blot-that had been rocked on a platform with block solution A for 1 hour-was placed into 10mL of blocking solution B and 5Î¼L of primary antibody was added on that solution with swirling; this was incubated for 20 minutes. Upon completion, the gel was washed with 15mL of wash buffer (three times, each with 10 minutes of incubation); then 15mL of blocking solution B and 5Î¼L of secondary antibody was added and incubated at 15 minutes. The three wash steps were repeated. With the wash buffer drained, the membrane was put on a plastic paper protector (with the protein side up) and 400Î¼L of substrate (made by mixing reagent A and B in 1: 1 ratio, 200Î¼L each) was spread evenly across the middle of the blot. A plastic protector was then added over it and this was imaged with a digital imager for chemiluminescence detection and analyzed using the *BioRad ChemiDOC-MP Imaging System* for the molecular weight and signal intensity of the protein bands (refer to the instructions posted on blackboard on how this program was operated).

Results and Discussion

According to the Coomassie-stained gel, the variability in the staining intensity of the protein bands in lanes 2, 3, 4 and 5-for skeletal muscle tissue samples from shark, tilapia, skitter, and salmon-signify the difference in the relative abundance of individual polypeptides in each organism (note that lane 5, band 11 was used as the reference). Influenced by factors such as protein expression and control, these species have generated different quantities of proteins with similar masses in their muscle tissues as they have adapted to specific environmental and biochemical interactions. [5] In *figure 1* , the potential mass and intensity values of myosin-light chain *(MLC)* are as follows: shark (15. 43kDa at 0. 37, 17. 65 at 1. 71, 20. 64 at 1. 09, 21. 60 at 0. 25, 23. 05 at 0. 69, 23. 79 at 0. 92, and 25. 54 at 1. 02); tilapia (15. 33kDa at 1. 34, 16. 42 at 0. 75, 19. 02 at 0. 35, 20. 37 at 1. 56, 21. 47 at 0. 34, and 23. 79 at 0. 36); skitter (15. 92kDa at 2. 09, 17. 99 at 0. 94, 20. 12 at 0. 48, and 23. 75 at 0. 55) and salmon (16. 07kDa at 1. 13, 20. 12 at 0. 31, 21. 08 at 0. 64, 21. 76 at 0. 26, and 24. 92 at 0. 34). Due to selective immunodetection of MLC proteins in Western blotting by a primary antibody, the various protein bands lying in the general MLC range of 15-25kDa in the Coomassie gel can be narrowed to: shark (23. 94kDa at 1. 33); tilapia (24. 47 at 0. 70); skitter (24. 47 at 0. 36); salmon (24. 47 at 0. 22) and myosin marker (24. 47 at 2. 40) – all of which resemble the myosin light chain isoform I (> 20kDa) as isoforms II (20kDa) and III (15kDa) have lower masses; with a greater variability of myosin, tilapia has an additional band of 20. 68kDa at 0. 39 that resembles isoform II. [5] The other bands were dismissed as non-specific background interferences (note that lane 4, band 5 was used as the reference for the immunoblot). The high specificity of

primary antibodies in probing their target allows for its wide-use in proteomic research as a reliable immunodetection technique; since proteins can indicate evolutionary relatedness or the presence of genetic diseases, their role as biomarkers has allowed for measurements of physiological changes as well as their quantifications. [6] In the *appendix* , all of the protein bands for the four species have been assigned a protein that corresponds to its molecular weight. From this, it can be denoted that sharks are more closely related to salmons than tilapia and skitters, both of which are tied for second place. However, based on fish phylogeny: sharks and skitters-belonging to the same class called Chondrichthyes-have diverged prior to the class of Actinopterygiis, which include both salmon and tilapia. [7] In terms of classification relative to the *" order",* sharks (of Elasmobranchii) have the greatest evolutionary relationship with skitters (of Rajiformes), then salmons (of Salmoniformes), and lastly tilapia (of Perciformes). [7]

As a hexameric ATPase cellular motor protein, myosin is composed of four light chains *(MLC)* -two non-phosphorylatable essential alkali chains, two phosphorylatable regulatory chains-and two heavy chains *(MHC).* Specifically, the protein bands of these light chains have a molecular weight as a range from 15 to 25kDa; this diversity in the masses occur largely from alternative RNA splicing mechanisms that generate multiple tissue-/developmental stage-specific isoforms. [7] Although these polymorphic variations do not significantly alter the actin-activated ATPase activity of the myosin-heavy chain, they affect the actin-filament sliding velocities and kinetics-leading to different force-generating abilities. [8] In an

evolutionary context, the existence of these hybrid molecules has been adopted by muscles-in response to changing functional demands-to shorten this translocation time in order to increase their overall fitness. Consequently, numerous variants of slow and fast light chains were developed despite the underlying plasticity of striated muscles. [7]

Voluntary muscles are divided into slow twitch and fast twitch muscles. The main difference is that the former *" red"* muscle contracts for longer periods of time with little force, require an oxygen-rich operating environment, and contain only two distinct light chains while the latter *" white"* type contracts quickly and powerfully for only short bursts of anaerobic activity as they become exhausted due to lactic acid buildup, have glycogenolytic capacity, and possess three different light chain subunits. [8] Over 90% of swimming muscles from sharks are composed of myotomes that can create massive propulsive forces by contracting their high numbers of white fibres; only a few such as the Great White incorporate bands of red muscle to elevate endurance over strength. [9] Accordingly, this explains why the MLC band on the Western blot has the greatest intensity of 1. 33 relative to the other species. Conversely, fish species are generally composed of endothermic red-segmented muscles in their trunk musculature-allowing for their stiff-bodied, slow undulatory swimming motions. [6] Due to their decreased mass of white muscles, MLC bands of tilapia, skitter, and salmon are of lower intensity at 0. 70, 0. 36, and 0. 22 – respectively. Relative to mammals, fish myosins share the same light chain patterns but have higher variability in MLC mass and quantity due to adaptive differences in movement between

red and white myofibrils. [6] Since they have larger phylogenetic diversity, there is an enormous range of contraction speeds and swimming styles among homologous muscles. [6] For example, fast twitch muscles of rabbit, sheep, and chicken have three light chain components at 250kDa-whereas only one is found homologous at 180kDa among pike, dogfish, mackerel, angler-fish, and carp. [5] Moreover, their poikilothermic-nature may have contributed to these light chain divergences as they were forced to adjust to fluctuating environment temperatures that required specific muscle responses for survival. [9]

Sources of errors with the techniques employed contributed in hindering the accuracy of the results. *First* , the amount of protein stained with Coomassie dye varied greatly between the sample replicates since the dye may complex with the anionic detergent in its free cationic form – interfering with protein concentration estimates. Moreover, this dye selectively targets amino acid resides arginine, tryptophan, tyrosine, histidine, and phenylalanine; however, the assay performed responds primarily to arginine residues – eight-times higher than other ones listed above. [2] *Second* , reproducibility of the sample preparation and protein extraction steps was an issue due to variability among the skills of the student, which may have caused the quantity differences seen among the replicates. For example: if more tissues were added for one specie, the increased concentration of proteins loaded into the lane would be misled for a true difference in expression among or between the species. To overcome these problems: *one* , an automated protein extraction systems should be employed since its robotic liquid handing technology can control for errors and contaminations –

leading to greater reproducibility and accuracy; *two* , silver staining can be substituted for Coomassie due to its higher sensitivity (0. 2ng versus 7ng – respectively); *third* , adjustable single-/multi-channel Rainin electronic pipettes should be used as its fully automated and repetitive micro-pipetting has superior consistency – allowing for higher throughput work. [4, 5, 6, 9]

Overall, it has been discovered that-irrespective of muscle tissue origin-myosin light chain molecules are heterogeneous in mass and intensity and the existence of phasically active fast muscles versus slow tonic muscles has led to characteristic light chain patterns among different fish species. Based on similarities and divergences in the overall protein content and intensities of the different fish species mentioned above, sharks are deemed to be more closely related to salmons than tilapia and skitters – both of which are tied for second place. However, according to fish phylogeny, sharks and skitters have diverged before salmon and tilapia, leading to an *" order"* classification of sharks *(Chondrichthyes, Elasmobranchii)* having the greatest evolutionary relationship with skitters *(Chondrichthyes, Rajiformes),* then salmons *(Actinopterygiis, Salmoniformes),* and lastly tilapia *(Actinopterygiis, Perciformes).* Radical alterations in their muscle proteome may have originated from adaptive responses to environmental stresses-i. e. osmotic, anaerobic, and thermal condition changes- or during symbiosis and development since cells can make different sets of proteins based on its specific spatial-temporal conditions. [5] The inferences made in this lab come with great uncertainty due many accuracy and reproducibility problems. Thus, fluorescence two-dimensional differential gel electrophoresis can be substituted for SDS-PAGE; high-throughput proteomic technologies like micro

arrays, mass spectrometry-based methods, protein chips, and reverse-phased protein-microarrays can be used for protein profiling and detection; and hybrid separation-analysis techniques such as reversed-phase chromatography-ESI ionization online analysis systems can be utilized for greater sensitivity, accuracy, and precision – all of which allow an experimenter to draw firmer conclusions.

References

1. Bandman, E. et al. Developmental Appearance of Myosin Heavy and Light Chain Isoforms in-Vitro and in-Vivo in Chicken Skeletal Muscle. *Developmental Biology* . 1982, 2, 508-518.
2. Chatfield, S. Experiment 7: Extraction and Electrophoresis of Proteins: Immunoblot Preparation. BIO 314 Laboratory Manual. 2017.
3. Chatfield, S. Experiment 8: Development of Immunoblots (Western Blots). BIO 314 Laboratory Manual. 2017.
4. Focant, B. et al. Subunit Composition of Fish Myofibrils: The Light Chains of Myosin. *Journal of Biochemistry.* 1976, 110-120.
5. Lowey, S. et al. Function of Skeletal Muscle Myosin Heavy and Light Chain Isoforms by an in Vitro Motility Assay. *The Journal of Biological Chemistry.* 1993, 268, 20414-20418.
6. Lowey, S. et al. Light Chains from Fast and Slow Muscle Myosins. *Nature.* 1971, 81-85.
7. Syme, D. et al. Red Muscle Function in Stiff-Bodied Swimmers: There and Almost Back Again. *Philosophical Transactions of the Royal Society B: Biological Sciences.* 2011, 1507-1515.

8. Tomanek, L. et al. Environmental Proteomics: Changes in the Proteome of Marine Organisms in Response to Environmental Stress, Pollutants, Infection, Symbiosis, and Development. *Journal of Animal Science.* 2003, 373-390.
9. Young, R. et al. Structural Analysis of Myosin Genes Using Recombinant DNA Techniques. *Journal of Animal Science.* 1968, 259-268.