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Extraction and Analysis of Protein from Unknown Species by SDS-PAGE and Immunobloting

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Abstract: *The study of proteins has influenced people's understanding of the phenomena and activities of life and the nature of life. According to the previous research, examining the proteins composition in the tissue the researchers can identify the unknown species based on its homology to other known proteins and its location within the cell. For this paper, Arctogadus glaciali and Spiniraja whitleyi are used to extract their unknown tissues as samples. Then, SDS-PAGE is used to identify the unknown tissues and the Immunobloting is developed for further detection of individual protein isolates. The result shows that the cod sample from lane 2, 5, 7, and 9 are highly concentrated with myosin light chain proteins, and skates sample from lane 3, 4, 8 and 10 does not contain a significant amount of myosin light chain proteins.*

Keywords: *SDS-PAGE; Immunobloting; Myosin light chain proteins; Tissue composition*

1. Introduction

Protein is an important component of various biological organisms, including human beings, and is one of the material bases of life. All life activities such as growth, development, heredity and reproduction of organisms are inseparable from proteins. With the deepening of molecular biology, structural biology, genomics and other studies, people have realized that only by studying the sum of all proteins from the perspective of proteomics can we grasp life phenomena and activity laws more scientifically and reveal the nature of life more perfectly. According to the Hohlfeld's study in 1994, the muscle system consists of large syncytial cells, including skeletal cells, cardiac cells, and smooth cells, specialized to generate mechanical force, where their cytoplasm consists of highly organized protein filament and interact with each other to generate the force of muscle contraction [1].

Skeletal and cardiac muscle are considered to be striated muscle, because of their organization of repeating units of contractile filaments, known as sarcomeres, into cylindrical bundles called myofibrils[2]. Sacromers are defined as spanning only few micrometers long from Z disk to Z disk, consists of an A band containing myosin (thick) filaments, which is between two half I-bands made up of actin (thin) filaments [2]. During muscle contraction, the myosin heads interact with actin filaments with rapid Myosin ATPase cycle, driven by ATP hydrolysis and causing conformational change in the myosin [3]. This conformational change will resulting in the Z disks being pulled toward each other and thus the shortening of the muscle [2]. Other than myosin and actin, other proteins also play an important role in muscle contraction, as if sarcomeres consisting of only thick and thin filaments and Z disk would be inherently unstable[4]. Although both myosin and actin are highly conserved proteins, the composition proteins may show more variety due to natural selection [5]. Therefore, the researchers can identify the unknown species based on its homology to other known proteins and its location inside the cell by examining the proteins composition in the tissue [6]. For example, Myosin light chain kinase (MLCK) plays a central role in regulating the actin-myosin interaction of smooth muscle. According to Cieri, Robert L [7], smooth muscles are primarily involved in tension regulation, therefore, it may serve to support lung emptying in lungfishes and aquatic amphibians, as well as maintain internal lung structure. Although direct exhalation of gas is not possible in physoclists, smooth muscle contraction and relaxation can regulate the amount of air in their swim bladders [8]. For chondrichthyes, however, they lack of a swim bladder, buoyancy is provided from lipids stored in their liver instead [9]. Thus it will have less MLCK composition compared to physoclists.

In this context, his paper conducts the experiment, which extractes the unknown tissue of Arctogadus glaciali and Spiniraja whitleyi and identify these tissues by SDS-PAGE. Then depending

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on the device of Bio-Rad ChemiDoc MP Imaging System and ImageLab software (Version 6.0), this paper uses western blot to examine individual separated proteins and analyze the image of samples.

2. Materials and Methods

2.1 Protein extraction

The researcher prepares two unknown tissue samples from cod fish (*Arctogadus glacialis*) and skates fish (*Spiniraja whitleyi*). The reseracher extractes unknown tissue from Arctogadus glacialis and Spiniraja whitleyi respectively to prepare two samples. 250μl Laemmli buffer are added into two 1.5ml microcentrifuge tubes. The prepared two samples are added into the microcentrifuge tubes respectively and then the researcher gently flicks the tubes so that the tissues can be stirred in the tubes. The tubes are incubated in room temperature for 5 minutes, then tubes should be shaken until tissues down to pellet loose tissue. Then, supernatant buffer should be transfered from each sample into two fresh 1.5 ml screw cap tubes, then boil the sample buffer at 95ºC for 5 min.

2.2 Sample loading

2*12% SDS--PAGE TGX gels should be prepared. One gel is stained with coomassie blue and the other is used for immunobloting. 10μl of samples are loaded in the protein ladder for coomasie blue staining gel, 5μl of samples for immunobloting. Then the gel electorphoresis should be ran for 30-40 min at 200 volt.

In this experiment, lane 2, 5, 7 and 9 are cods (*Arctogadus glacialis*), lane 3, 4, 8, and 10 are skates fish (*Spiniraja whitleyi*).

2.3 Gel removal and coomassie staining

When the gel electrophoresis is finished, the gel should be removed from the tubes and then placed into a clean tip box lid filling with deionized water. Rinse the gel in deionized water by a rocking platform for 1-2 min, then remove water, and add fresh deionized water, rock again. This process should be repeated 3 times. Water should be removed from the container and all of coomassie staining solution should be added into the container, then Prepare a larger Tupperware container should be prepared and deionized water are added into this container. The Coomassie stained gel is transfered into this larger container, a lid is put on this container and it is placed on a rocking platform for 5-10 minutes. Then all the deionized water is removed in this larger container and a new sample of deionized water is added into it and it should be stored at 4 degree centigrade.

3. Immunoblot

In order to transfer the blot on the other gel to the PVDF membrane, the gel blot should be assembled from top to bottom according to the following order : cassette top (-electrode), top ion reservoir stack, immunoblot gel, PVDF membrane, bottom ion reservoir stack, cassette bottom (+electrode), and place the cassette into the turbo blotter. Once electro-transfer is completeted, open the lid, peel the gel away from the membrane and store the membrane in a zip lock bag at -20ºC until the development of western blotting. The membrane should be placed in a container with the blocking solution (5% non-fat milk in 1x TBS-T) then the container is placed on a rocking platform for at least 1 hour. Then 5μl of the primary antibody (Mouse Anti-Myosin Light Chain Antibody) should be pipetted directly into the blocking solution in the container, the container should be swirled for 5 seconds and it should be placed back on a rocking platform for 20 min for incubation. Then 5μl of the primary antibody (Mouse Anti-Myosin Light Chain Antibody) should be pipetted directly into the blocking solution in the container, the container should be swirled for 5 seconds and it should be placed back on a rocking platform for 20 min for incubation. The blocking solution should be removed from the container and 15ml wash buffer should be used to wash off excess antibody. Then the container is placed on a rocking platform to incubate for 5 minutes. This step should be operated 3 times. Thenadd 15 ml of blocking solution to the container, and pipette 5μl of the secondary antibody (Goat Anti-Mouse IgG (H+L) HRP Conjugate Antibody) directly into the blocking solution. After 5 seconds of swirling, place it on the rocking platform and incubate for 15 min.. When the secondary antibody is completed, the blocking solution should be removed from the container and use 15ml wash buffer to

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wash off excess secondary antibody. Then the container is placed on a rocking platform to incubate for 5 minutes. This step should be operated 3 times.

After immunodetection, drain the membrane well and place the membrane protein side up on a clear surface inside of plastic paper protector. Then add 300µl of substrate mixture (Clarity Western ECL Substrate) across the midpoint of the membrane, cover the membrane with plastic protector and spread the substrate evenly across the membrane. Then the membrane should be incubated for 5 minutes.

4. Analysis

The immunobolted membrane is imaged by Bio-Rad ChemiDoc MP Imaging System and ImageLab software (Version 6.0). The two images of the membrane. Then 2 images are merged to have the ladder and band on the same image. The chemiluminescence is adopted to detect the bands that contains proteins bound by antibodies, then colorimetric setting is used to visualize the ladder.

Then the Coomassie gel should be imaged. The gel should be placed on the on a clean white porcelain plate moistened with a small amount of water to prevent the glue from being damaged. Then a Bio-Rad ChemiDoc MP Imaging System is used to image it and the ImageLab software (Version 6.0) is used to analyze the result.The whole process must be careful not to break the gel.

5. Results and images

For the Figure 1, lane 1 is the protein ladder with molecular weight markers (kDA) and lane 6 is the Actin/Mysoin Standard. Imaging is gathered from Bio-Rad ChemiDoc MP Imaging System and analysis is gathered from ImageLab software (Version 6.0). Protein fragments are separated on a 12% SDS--PAGE TGX gel electrophoresed at 200 volts for 40 min. According to the Figure 1, lane (L) 3, 4, 8 and 10 (skates fish/*Spiniraja whitleyi*) are a missing protein band (PB) between molecular weight of 15-20kDA, which is shown in lane 2, 5, 7, and 9 (cod/*Arctogadus glacialis*) (L2: PB20; L5: PB21; L7: PB17; L9: PB17). Sample from lane 2, 5, 7, and 9 are missing two protein bands between molecular weight of 10-15 which is present in lane 3, 4, 8 and 10 (L3:PB18&19; L4: PB18&19; L8: PB18&19; L10: PB18&19). There is no difference of protein between molecular weight of 20-25 kDA and 25-37kDA.In addition, Lane 2, 5, 7, and 9 are also shown proteins with higher molecular weight (>250kDA).

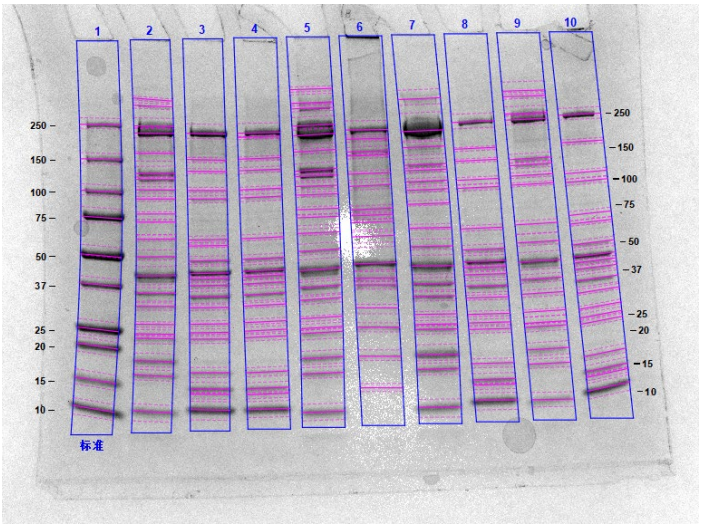

Figure 1: The Coomassie gel result

For the Figure 2, it is the merged figure of ladder and band of immunobloting results, which is tested with a primary anti body for myosin light chain. Lane 1 is the protein ladder with molecular weight markers (kDA) and Lane 6 is the Actin/Mysoin Standard. Imaging is gathered from Bio-Rad ChemiDoc MP Imaging System and analysis is gathered from ImageLab software (Version 6.0), Protein fragments are separated on a 12% SDS--PAGE TGX gel electrophoresed at 200 volts for 40 min. Based on the Figure 2, it shows that the myosin bands are corresponding to the protein with the

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molecular weight of 20-25kDA. This result indicates myosin light chain is highly concentrated in lane 2, 5, 7, and 9 but are insignificant in lane 3, 4, 8 and 10. However, from the coomassie gel result, there are no difference of protein between molecular weight of 20-25 kDA, which is inconsistent with the immunobloting results.

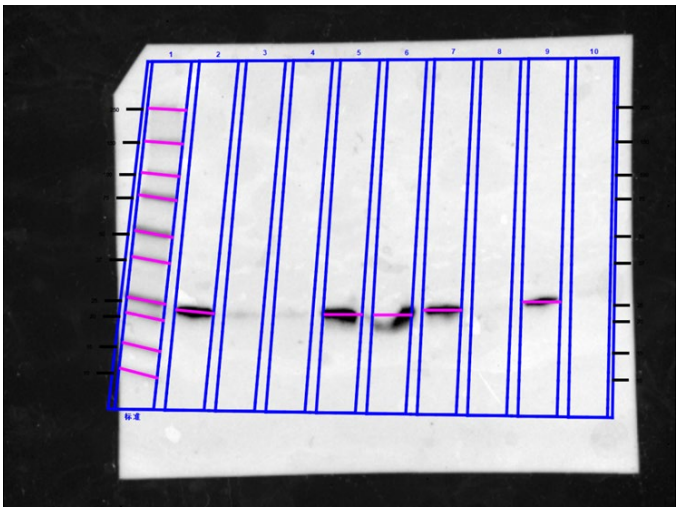

Figure 2: Merged figure of ladder and band of immunobloting results tested with a primary anti body for myosin light chain

6. Discussion

Based on our immunobloting results , sample from lane 2, 5, 7, and 9 (cod/*Arctogadus glacialis*) are highly concentrated with myosin light chain proteins with the molecular weight between 20-25kDA which is consistent with previous studies as smooth muscles in physoclists are critical for swim bladder regulation [8]. Sample from lane 3, 4, 8 and 10 (skates fish/*Spiniraja whitleyi*) does not contain a significant amount of myosin light chain proteins; which is consistent with previous studies, because Chondrichthyes, lack of a swim bladder, would have lower myosin light chain concentration in their tissues. However, our coomassie gel result (Figure 1) are inconsistent with immunoblotting results, as coomassie gel results have no difference of protein bands from the molecular weight between 20-25kDA. According to Table.1, one possible explanation of this inconsistency is there may be other muscle proteins with similar molecular weight present in the tissue sample, such as troponin.

Table 1: Characterized muscle proteins, in order of decreasing size, adapted from Alberts et al. 1994.

Protein	MW (in kD)	Function
titin	3000	centers myosin in sarcomere
dystrophin	400	anchoring to plasma membrane
filamin	270	cross-links actin filaments into a gel
myosin heavy chain	210	slides actin filaments
spectrin	265	attaches filaments to plasma membrane
M1/M2	190	myosin breakdown product
M3	150	myosin breakdown product
C protein	140	myosin breakdown product
nebulin	107	regulates actin assembly
a-actinin	100	bundles actin filaments
gelsolin	90	fragments actin filaments
fimbrin	68	bundles actin filaments
actin	42	forms filaments
tropomyosin	35	strengthens actin filaments
troponin (T)	30	regulates contraction
myosin light chains	15-25	slide actin filaments
troponin (I)	19	regulates contraction
troponin (C)	17	regulates contraction
thymosin	5	sequesters actin monomers

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7. Conclusion

In conclusion, the result of this study is consistent with previous studies, as cod sample from lane 2, 5, 7, and 9 are highly concentrated with myosin light chain proteins Skates sample from lane 3, 4, 8 and 10 does not contain a significant amount of myosin light chain proteins. While, further investigation is recommended to study and analyze other significantly different proteins contained in the coomassie gel.

This study has its limitation. In the experiment, other protein band which also showed significant differences between species, such as PB18&19 between molecular weight between 10-15kDA in lane 3, 4, 8 and 10 (skates fish/*Spiniraja whitleyi*) or proteins with higher molecular weight (>250kDA) presented in lane 2, 5, 7, and 9 (cod/*Arctogadus glacialis*) . Future studies should have the further investigation on protein presented in PB18&19 of skates fish (*Spiniraja whitleyi*), and proteins with higher molecular weight in this such as filamin, myosin heavy chain, and spectrin.

To enhance the comprehensiveness of the research, it would be valuable to explore these additional protein components and their potential implications in relation to species differentiation. This could contribute to a more comprehensive understanding of the protein composition and variations between cod and skates fish, shedding light on the molecular characteristics and functional differences in these species.

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