Optimization of SDS PAGE Analysis of Parvalbumin Protein in Fish

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Abstract

SDS PAGE technique plays an essential role in the study of protein. However, this analytical method occasionally suffers from limitations such as poor separation, signal reduction, or total absence of target bands. This research aims to develop a protocol to improve the consistency of the technique while quantifying 10-12kDa parvalbumin proteins. It is a major fish allergen associated with neurodegenerative diseases, yet, the amount of parvalbumin in different kinds of fish has not been published. In this research, proteins in flounder, white, catfish and tilapia were extracted with PBS and quantified with Bradford assay and SDS PAGE. The results supported that the 0.5g fish muscle/ 1750uL PBS extraction was the most optimized and that white fish had the highest amount of proteins, followed by tilapia, flounder, and catfish.

Background Information

Proteins are macromolecules that play essential roles in most biological processes. Parvalbumin is a small protein of 12 kDa containing 108-109 amino acid residues (Houhoula, 2015). It is well-known as the major and sole allergen for 95 % of patients suffering from IgE- mediated fish allergy (*Fish Allergens*, 2020). Parvalbumin is extremely abundant in fish muscle, which can be as high as 2g per kg fish muscle (Xu et al., 2006). Like many other calcium- binding pro-

teins, parvalbumin contains three EF-hand motifs with two high-affinity calcium ion- binding sites and plays an important role in muscle relaxation (*Fish Allergens*, 2020).

It has been supported that there is a relationship between parvalbumin protein and neurodegenerative diseases such as Parkinson and Alzheimer's disease (AD). For example, research published in 2018 suggested that parvalbumin can bind to alpha-synuclein protein, which inhibits its transformation to amyloid form and prevents its accumulation in Parkinson patients' brain (Werner et al., 2018). Moreover, a study mentioned that parvalbumin-containing neurons demonstrated vulnerability in AD (Solodkin et al., 1996) while another showed enhancing parvalbumin interneuron activity in a mouse model of Alzheimer's disease can decrease the accumulation of amyloid peptides in the brain (Iaccarino et al., 2016). In addition, it was shown that among those who follow a Mediterranean diet, with more fish, one sees lower rates of Parkinson's and Alzheimer's (*Chalmers University of Technology*, 2018). As parvalbumin passes into the body of the person eating fish, it made sense to study its interaction with human proteins (*Chalmers University of Technology*, 2018). These research supported that parvalbumin protein obtained from fish can be beneficial to the degenerative brain.

Bradford Assay, which is the most sensitive colorimetric method, measures the concentration of protein based on the absorbance of Coomassie Brilliant Blue G-250 dye (*Bradford Protein Assay*, 2015). Normally, the dye exists in its unstable cationic form with a red color. If proteins are present in the solution containing the dye, their hydrophobic and ionic interactions stabilize the dye and convert it into its stable anionic form. This process turns the dye from red color into blue. Therefore, the more proteins there are, the darker blue the samples will be. To measure the absorbance of the standards and the unknown samples, a light spectrophotometer is needed. A series of absorbance and concentration values of the known protein standards

is used to generate the standard curve. As the absorbance of the unknown samples can be measured, their protein concentrations are determined based on the standard curve. PBS, or phosphate buffered saline, is commonly used buffer in biological research for its ability to maintain constant pH and its non-toxicity to cell. In this Bradford assay experiment, it was used to extract the proteins from fish muscle based on the research by Ma and colleagues (Ma et al., 2017).

SDS PAGE, which stands for sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a common analytical technique to separate proteins based on their molecular mass (*SDS PAGE*, 2012). In this method, proteins are first denatured by SDS-containing Laemmli sample buffer, which provides the overall negative charge to the polypeptide chains. The negatively- charged proteins are then separated on a polyacrylamide gel matrix by means of electric current. The polyacrylamide gel is used because they can restrain large molecules from migrating as fast as the small ones. As the polypeptide chains have the negative charge, they will move towards the positive electrode once the current is applied. The smaller the proteins, the more quickly and further they will travel toward the end of the gel, which means different bands will reflect different molecular masses of the proteins. Once the electrophoresis is completed, Coomassie Brilliant Blue dye is added to the gel to allow for its binding to the proteins. The gel is then rinsed and incubated in a de-stain solution so that proteins bands can be visualized in UV light. A protein ladder with known molecular weights is used to determine the molecular weight of the proteins of interest (*SDS PAGE*, 2012).

Problem & Purpose

Despite the potential value of fish to human brain health, there has been little knowledge and data about the amount of parvalbumin in fish, especially the fish that humans might include in their diets.

Therefore, this research aimed to quantify the amount of parvalbumin in catfish, white, tilapia, and flounder, which might illuminate the nutrition value of these four kinds of fish and contribute to future research in parvalbumin protein.

Despite the techniques commonly being used in the laboratory, SDS-PAGE suffers from many limitations such as poor separation, unstable power supplies (weak or no current during electrophoresis), and even a total absence of target bands. Hence, the research set the goal to optimize the SDS PAGE technique in many aspects such as protein loads and visualization of protein bands.

Hypothesis

As tilapia are well-known for its higher protein value compared to flounder, white, and catfish, we hypothesized that the percentage of parvalbumin proteins in tilapia will be the highest and thus it is the most beneficial for patients with Alzheimer's disease. We also hypothesized that the optimized fish samples should undergo a series of dilutions which had the maximum of 0.5g fish muscle /5000uL extraction solution PBS, before being loaded in the SDS PAGE gel.

Procedure

A. Protein Extraction

The protein was first extracted from four fish samples: Flounder, White, Catfish and Tilapia. First, 0.5g fish white muscle samples from the four types of fish were obtained. They were then added to the tubes with different amount of 1X PBS, which is indicated in **Table 1.** In addition, scallop was used as a negative control sample, as parvalbumin is known as a fish allergen and there has been no research that reported the presence of parvalbumin in

shellfish (Kuehn et al., 2014). After 0.5g of scallop muscle was obtained, its proteins were extracted in 1750μL of 1X PBS. The tubes were then flicked multiple times and let sit for one hour to make sure all the proteins in fish muscle were extracted.

Table 1: The amount of fish muscle and 1X PBS in four protein extraction trials

	First Trial	Second Trial	Third Trial	Fourth Trial
Amount of each fish example	.5g	.5g	.5g	.5g
Amount of 1X PBS	1000uL	1750uL	3500uL	5000uL

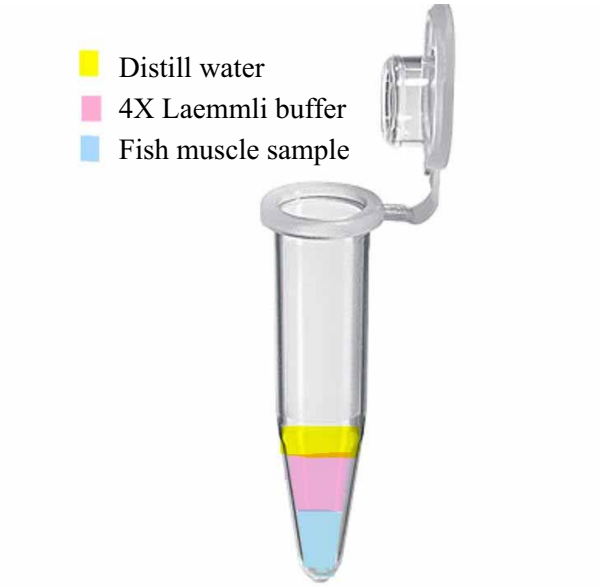
B. Protein Quantification (Bradford Assay)

The Bradford Assay experiment included the preparation of seven standard and four fish samples. The cuvettes were labeled blank and number 1-7 for the standards and F, T, W, C for the corresponding fish samples. The 1X PBS solution was prepared by adding 100mL of the purchased 10X PBS solution to 900mL of distill water. Next, the blank cuvette was added with 20ul of 1X PBS. The 1-7 standard cuvettes were pipetted with 20ul of each standard (0.125, 0.250, 0.500, 0.750, 1.000, 1.500 and 1.7500 mg/mL). The sample cuvettes were added with 20μL of the protein- extracted samples in part A. Next, all cuvettes were pipetted up and down five times to mix. They were then let sit in room temperature for ten minutes. While there is no color change in the blank cuvette, the sample cuvettes were observed to turn blue. Finally, the absorbance of the samples in the cuvette were then measured with the spectrophotometer at 595 nm. The data was recorded in **Table 2** (see in Results).

C. SDS PAGE

The SDS PAGE experiment included three main steps: assembling the gel cassette, preparing the fish samples and loading buffer, and run the electrophoresis assembly. The gel cassette was first assembled as instructed in the manual. The 4–20% Mini-PROTEAN Precast Protein Gel, purchased from Bio-Rad, was unpacked and removed with its green plastic strip at the bottom. The gel was assembled in the cassette and then placed in the assembly with the short plate facing inward. Next, the electrophoresis buffer 1X TGS was prepared by adding 100mL of the purchased 10X TGS buffer to 900mL of distill water. After the comb was removed from the cassette, the inner chamber and mini tank were filled with 1X TGS buffer to the 2-gel mark on the chamber. The next step was to prepare 4X Laemmli buffer and the fish samples following the method by Ma and colleagues (Ma et al., 2017). The reducing agent, 2-mercaptoethanol, was obtained with the amount 100 μL and then added to 900 μL of the Bio-Rad's Laemmli sample buffer. As the protein concentration for each type of fish was different, there were different samples, Laemmli buffer and distill water prepared for each fish sample. Eight microtubes were obtained and added with the components illustrated in **Figure 1** and **Table 4**. The total protein loading volume for each well was 10uL, whose components was calculated based on the example on the Research Gate website (*Western Blot/ SDS-PAGE- Help with Protein Loading Calculation*, 2018). After the microtubes were mixed and heated in 5 minutes at 95°C, 10uL of each fish sample was pipetted to the wells with the order indicated in **Figure 3**. Finally, the assembly was powered at 200V for thirty minutes. It was stained for 1 hour in a rocking machine and de-stained two times overnight. The gel was then visualized under the UV light.

Figure 1: Components of a fish sample prepared for SDS PAGE



Results

Table 2: Absorbance of standards in Bradford Assay

	Blank	S1	S2	S3	S4	S5	S6	S7
Measured	1.93	-0.05	-0.02	0.17	0.21	0.3	0.57	0.61
Calculated	0	1.88	1.91	2.1	2.14	2.23	2.5	2.54

Figure 2: Concentration Curve of protein concentration vs absorbance of standards

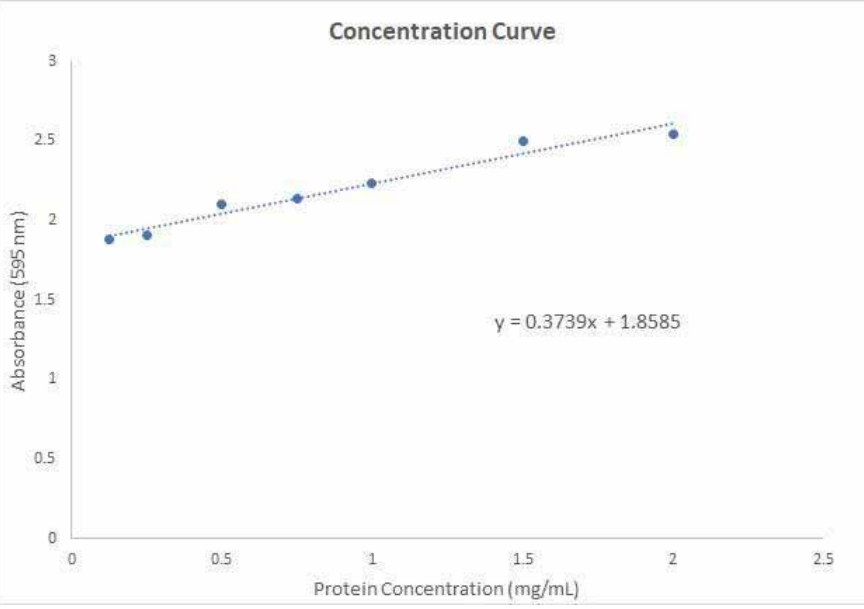


Table 3: Absorbance and protein concentration of fish samples in the second and fourth trials

Second trial (0.5g fish muscle/ 1750uL PBS)

	Flounder	White	Catfish	Tilapia
Absorbance	2.73	2.94	2.53	2.75
Calculated concentration (mg/l)	2.33	2.89	1.80	2.38

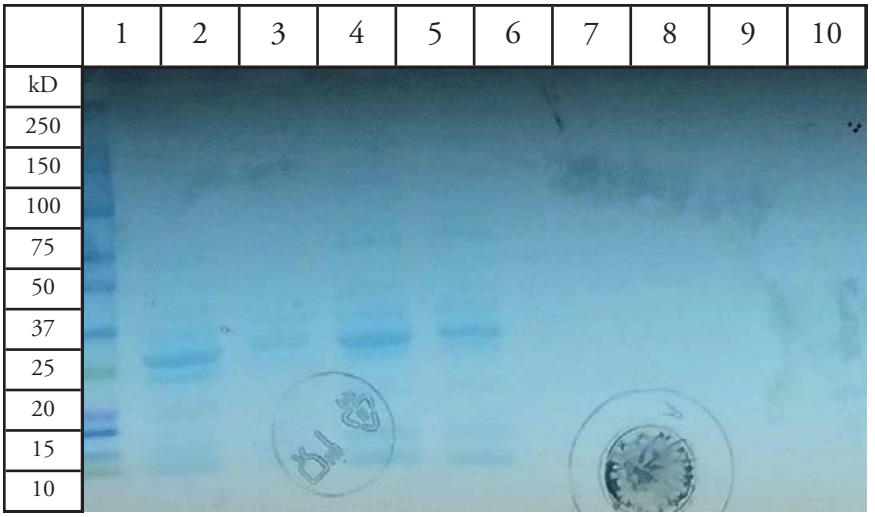
Fourth trial (0.5g fish muscle/ 5000uL PBS)

	Flounder	White	Catfish	Tilapia
Absorbance	1.89	2.08	2.26	1.89
Calculated concentration (mg/l)	0.084	0.59	1.07	0.084

Table 4: Calculated components for SDS PAGE experiment

	Second Trial				Fourth Trial			
	Flounder	White	Catfish	Tilapia	Flounder	White	Catfish	Tilapia
Protein amount expected in each well	13 µg	13 µg	13 µg	13 µg	1.3 µg	1.3 µg	1.3 µg	1.3 µg
Volume of protein added	5.6 µL	4.5 µL	7.2 µL	5.5 µL	15.5 µL	2.2 µL	1.2 µL	15.5 µL
Laemmli buffer	2.5 µL	2.5 µL	2.5 µL	2.5 µL	2.5 µL	2.5 µL	2.5 µL	2.5 µL
Distill Water	1.9 µL	3 µL	0.3 µL	2 µL	0 µL	5.3 µL	6.3 µL	0 µL
Total volume added to each well	10 µg/µL	10 µg/µL	10 µg/µL	10 µg/µL	10 µg/µL	10 µg/µL	10 µg/µL	10 µg/µL

Figure 3: SDS PAGE gel



Well 1: Protein ladder and their molecular weight (Precision Plus Protein Kaleidoscope standard)
Well 2-5: Fish samples in Flounder, White, Catfish and Tilapia in the second trial
Well 6-9: Fish samples in Flounder, White, Catfish and Tilapia in the fourth trial

Analysis

In the Bradford Assay experiment, the absorbance of the standards and samples were measured. Figure 2 showed the expected relationship between protein concentration and absorbance of the standards: the more proteins there are in the samples, the higher their absorbance. According to Table 3, white fish had the highest amount of protein concentration, following by flounder and tilapia which had quite the same protein concentration. The data of catfish was inconsistent in the two trials. Only the second and fourth trials were included in the data, because the first one was too concentrated to be measured while the third one yield quite the same data as the second one. The equation from the best fit line was used to calculate the protein concentration of the fish samples, based on their measured absorbance.

In the SDS PAGE experiment, the protein ladder was loaded in well #1; the fish samples diluted with the amount 0.5g/ 1750uL PBS was loaded in the wells #2-5 while the ones diluted with the amount 0.5g/ 5000uL was loaded in wells #6-9. Figure 3 illustrated that many protein bands were observed on the wells #1-5 of the gel while the rest did not produce any observable bands. The protein ladder's bands on the first well were shown on the gel as expected. The observable bands on wells #2-5 suggested that the protein extraction with 0.5g/ 1750uL PBS was optimized for SDS PAGE gel. The absence of bands on wells #6-9 supported that the protein extraction with 0.5g/ 5000uL was not suitable for running SDS PAGE gel.

Conclusion

This research produced data about protein quantification and the optimized protein loadings for fish protein analysis on SDS PAGE. Research supported that white fish had a highest amount of proteins, followed by tilapia, flounder, and catfish. For the future direction, the quantification of parvalbumin in white fish muscle can be further confirmed with different protein quantification tests such as: Western Blotting, ELISA, and Mass Spectrometry. More research should be conducted in the future to quantify the parvalbumin protein in other types of fish and examine the relationship between parvalbumin and human brain health.

Abbreviations

AD .....Alzheimer's disease
ELISA.....Enzyme-linked immunosorbent assay
PBS .....Phosphate-buffered saline buffer
SDS PAGE Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
TGS.....Tris-Glycine-SDS Buffer

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