23 chapter

Proteins: Extraction, Quantitation, and Electrophoresis

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23.1 INTRODUCTION

23.1.1 Background

Electrophoresis can be used to separate and visualize protein banding patterns. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins are dissociated into subunits and then separated based on size within a gel matrix by applying an electric field. It is usually necessary to apply a sample volume to the gel that contains a known amount of total protein to allow comparisons between samples. While it is possible to use an official method (e.g., Kjeldahl, *N* combustion) to determine total protein, it is often convenient to use a rapid colorimetric method of protein analysis that requires only a small amount of sample. The bicinchoninic acid (BCA) assay method will be used for this purpose.

In this experiment, sarcoplasmic muscle proteins are extracted with a 0.15*M* salt solution, the protein content of the extract is measured by the BCA colorimetric assay, and the proteins in the fish extracts are separated and visualized by SDS-PAGE. Visualization of the protein banding patterns makes it possible to distinguish among different types of fish since many fish have a characteristic protein subunit pattern. For example, one might use this technique as part of a scheme to detect economic adulteration when an inexpensive fish species is substituted for a more expensive fish in the wholesale or retail marketplace.

23.1.2 Reading Assignment

Chang, S.K.C., and Zhang Y. 2017. Protein analysis. Ch.18, in *Food Analysis*, 5th ed. S.S. Nielsen (Ed.), Springer, New York.

Smith, D.M. 2017. Protein separation and characterization. Ch. 24, in *Food Analysis*, 5th ed. S.S. Nielsen (Ed.), Springer, New York.

23.1.3 Objective

Extract proteins from the muscles of freshwater and saltwater fish, measure the protein content of the extracts, separate the proteins by electrophoresis, and then compare the different protein banding patterns that result based on subunit size and relative quantity.

23.1.4 Principle of Method

Sarcoplasmic proteins can be extracted from fish muscle with 0.15 *M* salt. Protein content of the extract can be determined by the colorimetric BCA assay method. In this assay, protein reduces cupric ions to cuprous ions under alkaline conditions. The amount of cuprous ions formed is proportional to the amount of protein present. The cuprous ions react with the BCA reagent to form a purple color that can be quantified spectrophotometrically and related to the protein content by comparison to a standard curve. Proteins in the extract can be separated by SDS-PAGE. Proteins become negatively charged when bound to SDS, so they move through the gel matrix toward the anode (pole with positive charge) at a rate based on size alone. The molecular mass of a given protein subunit can be estimated by comparing its electrophoretic mobility with proteins of known molecular weight using a standard curve. A linear relationship is obtained by plotting the logarithm of the molecular mass of standard proteins against their respective electrophoretic mobilities (R_f).

23.1.5 Notes

This experiment may be done over two laboratory sessions. The protein can be extracted, quantified, and prepared for electrophoresis in the first session. The prepared protein samples can be frozen until electrophoresis is completed in the second laboratory session. Alternatively, in a single laboratory session, one group of students could do the protein extraction and quantitation, while a second group of students prepares the electrophoresis gels. Also, different groups of students could be assigned different fish species. Multiple groups could run their samples on a single electrophoresis gel. The gels for electrophoresis can be purchased commercially (e.g., Bio-Rad, Mini-PROTEAN TGX precast gels, 12% resolving gel) or made as described below.

Some fish species work better than others for preparing the extracts and comparing differences in protein patterns. Catfish (freshwater) and tilapia (saltwater) work well as extracts and show some differences. Trout gives very thick extracts. Freshwater and saltwater salmon show few differences.

23.1.6 Chemicals

	CAS No.	*Hazards*
Sample Extraction:		
Sodium chloride (NaCl)	7647-14-5	Irritant
Sodium phosphate, monobasic ($NaH_2PO_4 \cdot H_2O$)	7558-80-7	Irritant
Protein Determination (BCA method):		
Bicinchoninic acid		
Bovine serum albumin (BSA)	9048-46-8	
Copper sulfate ($CuSO_4$)	7758-98-7	Irritant
Sodium bicarbonate ($NaHCO_3$)	144-55-8	
Sodium carbonate (Na_2CO_3)	497-19-8	Irritant
Sodium hydroxide (NaOH)	1310-73-2	Corrosive
Sodium tartrate	868-18-8	

Electrophoresis:		
Acetic acid (CH_3COOH)	64-19-7	Corrosive
Acrylamide	79-06-1	Toxic
Ammonium persulfate (APS)	7727-54-0	Harmful, oxidizing
Bis-acrylamide	110-26-9	Harmful
Bromophenol blue	115-39-9	
Butanol	71-36-3	Harmful
Coomassie Blue R-250	6104-59-2	
Ethylenediaminetetraacetic acid, disodium salt ($Na_2EDTA \cdot 2H_2O$)	60-00-4	Irritant
Glycerol ($C_3H_8O_3$)	56-81-5	
Glycine	56-40-6	
Hydrochloric acid (HCl)	7647-01-0	Corrosive
β-Mercaptoethanol	60-24-2	Toxic
Methanol (CH_3OH)	67-56-1	Highly flammable
Protein molecular weight standards (e.g., Bio-Rad 161–0374, Precision Plus Protein Dual Color Standards, 10–250 KD)		
Sodium dodecyl sulfate (SDS, dodecyl sulfate, sodium salt)	151-21-3	Harmful
N, N, N′, N′-Tetramethylethylenediamine (TEMED)	110-18-9	Highly flammable, corrosive
Tris base	77-86-1	

23.2 REAGENTS

(** It is recommended that these solutions be prepared by laboratory assistant before class.)

23.2.1 Sample Extraction

- Extraction buffer, 300 mL/fish species **
  Buffer of 0.15*M* sodium chloride, 0.05*M* sodium phosphate, pH 7.0 (Students asked to show the calculations for this buffer later in Questions.)

23.2.2 Protein Determination (BCA Method)

Commercial BCA test kits can be purchased, such as the Pierce BCA Protein Assay Kit (cat. No. 23225, Rockford, IL) which includes procedures to complete either test tube (2 mL working reagent/tube) or microplate assays (200 uL working reagent/well).

This kit includes:

- Bovine serum albumin (BSA) standard ampules, 2 mg/mL in 0.9% saline and 0.05% sodium azide as a preservative
- BCA Reagent A: Contains sodium carbonate, sodium bicarbonate, BCA detection reagent, and sodium tartrate in 0.1 M sodium hydroxide
- Reagent B: 4% cupric sulfate solution

23.2.3 Electrophoresis

(*Note*: Running buffers (Tris/glycine/SDS), sample buffers (Laemmli sample buffer), and precast gels can be purchased commercially or made in the laboratory. β-Mercaptoethanol may need to be added to a commercial sample preparation buffer.)

- Acrylamide: bis-acrylamide solution**
  29.2 g acrylamide and 2.4 g methylene bis-acrylamide, with dd water to 100 mL
- Ammonium persulfate (APS), 7.5%, in dd water, 1 mL, prepared fresh daily**
- Bromophenol blue, 0.05%
- Coomassie Brilliant Blue stain solution**
  Purchase premixed or prepare: 454 mL dd water, 454 mL methanol, 92 mL acetic acid, and 1.5 g Coomassie Brilliant Blue R-250. Note: Bio-Rad offers Coomassie G-250 stains that do not require traditional destaining with methanol or acetic acid. A water wash stops the staining process.
- Destain solution**
  850 mL dd water, 75 mL methanol, and 75 mL acetic acid
- EDTA, disodium salt, 0.2*M*, 50 mL **
- Glycerol, 37% (use directly)
- Electrophoresis sample preparation buffer **
  Purchase premixed or prepare: 1 mL of 0.5*M* Tris (pH 6.8), 0.8 mL glycerol, 1.6 mL 10% SDS, 0.4 mL β-mercaptoethanol, and 0.5 mL 0.05% (wt/vol) bromophenol blue, diluted to 8 mL with dd water.
- Sodium dodecyl sulfate, 10% solution in dd water, 10 mL**
- TEMED (use directly)
- Tray (running) buffer, 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3**
  Purchase premixed or prepare
- Tris buffer, 1.5*M*, pH 8.8, 50 mL (separating (resolving) gel buffer)**
  Purchase premixed or prepare
- Tris buffer, 0.5*M*, pH 6.8, 50 mL (stacking gel buffer)**
  Purchase premixed or prepare

Gel preparation: Use the formula that follows and the instructions in the procedure to prepare two 8.4×5.0 cm SDS-PAGE slab gels, 15% acrylamide, 0.75 mm thick. Actual formulation will depend on size of slab gels.

Reagent	*Separating gel 15% gel*	*Stacking gel 4.5% gel*
Acrylamide: bis-acrylamide	2.4 mL	0.72 mL
10% SDS	80 µL	80 µL

Reagent	*Separating gel 15% gel*	*Stacking gel 4.5% gel*
1.5 M Tris, pH 8.8	2.0 mL	–
0.5 M Tris, pH 6.8	–	2.0 mL
dd water	3.6 mL	5.3 mL
37% glycerol	0.15 mL	–
10% APS[a]	40 µL	40 µL
TEMED	10 µL	10 µL

[a]APS added to separating and stacking gels after all other reagents are combined, solution is degassed, and each gel is ready to be poured

23.2.4 Hazards, Precautions, and Waste Disposal

Acrylamide monomers may cause cancer and are very toxic in contact with skin and if swallowed. β-Mercaptoethanol is harmful if swallowed, toxic in contact with the skin, and irritating to eyes. Adhere to normal laboratory safety procedures. Wear gloves and safety glasses at all times. Acrylamide and β-mercaptoethanol wastes must be disposed of as hazardous wastes. Gloves and pipette tips in contact with acrylamide and β-mercaptoethanol also should be handled as hazardous wastes. Other waste likely can be washed down the drain with a water rinse, but follow good laboratory practices outlined by environmental health and safety protocols at your institution.

23.3 SUPPLIES

(Used by students)

23.3.1 Sample Extraction

- Beaker, 250 mL
- Centrifuge tubes, 50 mL
- Cutting board
- Erlenmeyer flask, 125 mL
- Graduated cylinder, 50 mL
- Filter paper, Whatman No. 1
- Fish, freshwater (e.g., catfish) and saltwater species (e.g., tilapia)
- Funnel
- Knife
- Pasteur pipettes and bulbs
- Test tube with cap
- Weighing boat

23.3.2 Protein Determination (BCA Method)

- Beaker, 50 mL
- Graduated cylinder, 25 mL
- Mechanical, adjustable volume pipettor, 1000 µL, with plastic tips
- Test tubes

23.3.3 Electrophoresis

- Beaker, 250 mL (for boiling samples)
- Two Erlenmeyer flasks, 2 L (for stain and destain solutions)
- Glass boiling beads (for boiling samples)
- Graduated cylinder, 100 mL
- Graduated cylinder, 500 mL
- Hamilton syringe (to load samples on gels)
- Mechanical, adjustable volume pipettors, 1000 µL, 100 µL, and 20 µL, with plastic tips
- Pasteur pipettes, with bulbs
- Rubber stopper (to fit 25-mL sidearm flasks)
- Two sidearm flasks, 25 mL
- Test tubes or culture tubes, small size, with caps
- Tubing (to attach to vacuum system to degas gel solution)
- Weighing paper/boats

23.3.4 Equipment

- Analytical balance
- Aspirator system (for degassing solutions)
- Blender
- Centrifuge
- Electrophoresis unit
- pH meter
- Power supply
- Spectrophotometer
- Top loading balance
- Vortex mixer
- Water bath

23.4 PROCEDURE

(Single sample extracted)

23.4.1 Sample Preparation

1. Coarsely cut up about 100-g fish muscle (representative sample) with a knife. Accurately weigh out 90 g on a top loading balance.
2. Blend one part fish with three parts extraction buffer (90-g fish and 270 mL extraction buffer) for 1.0 min in a blender. (Note: Smaller amounts of fish and buffer, but in the same 1:3 ratio, can be used for a small blender.)
3. Pour 30 mL of the muscle homogenate into a 50-mL centrifuge tube. Label tube with tape. Balance your tube against a classmate's sample. Use a spatula or Pasteur pipette to adjust tubes to an equal weight.
4. Centrifuge the samples at 2000× g for 15 min at room temperature. Collect the supernatant.
5. Filter a portion of the supernatant by setting a small funnel in a test tube. Place a piece of Whatman No. 1 filter paper in the funnel and moisten it with the extraction buffer. Filter the

supernatant from the centrifuged sample. Collect about 10 mL of filtrate in a test tube. Cap the tube.
6. Determine protein content of filtrate using the BCA method and prepare sample for electrophoresis (see below).

23.4.2 BCA Protein Assay

(Instructions are given for duplicate analysis of each concentration of standard and sample.)

1. Prepare the Working Reagent for the BCA assay by combining Pierce Reagent A with Pierce Reagent B, 50:1 (v/v), A:B. For example, use 50-mL Reagent A and 1.0-mL Reagent B to prepare 51.0-mL Working Reagent, which is enough for the BSA standard curve and testing the extract from one type of fish. (Note: This volume is adequate for assaying duplicates of five standard samples and two dilutions of each of two types of fish.)
2. Prepare the following dilutions of the supernatant (filtrate from Procedure, Sample Preparation, Step 5): dilutions of 1:5, 1:10, and 1:20 in *extraction buffer*. Mix well.
3. In test tubes, prepare duplicates of each reaction mixture of diluted extracts and BSA standards (using 2 mg BSA/mL solution) as indicated in the table that follows:

Tube identity	*dd water (µL)*	*BSA Std. (µL)*	*Fish extract (µL)*	*Working reagent (mL)*
Blank	100	0	–	2.0
Std. 1	80	20	–	2.0
Std. 2	60	40	–	2.0
Std. 3	40	60	–	2.0
Std. 4	20	80	–	2.0
Std. 5	0	100	–	2.0
Sample 1:5	50	–	50	2.0
Sample 1:10	50	–	50	2.0
Sample 1:20	50	–	50	2.0

4. Mix each reaction mixture with a vortex mixer and then incubate in a water bath at 37°C for 30 min.
5. Read the absorbance of each tube at 562 nm using a spectrophotometer.
6. Use the data from the BSA samples to create a standard curve of absorbance at 562 nm versus µg protein/tube. Determine the equation of the line for the standard curve. Calculate the protein concentration (µg/mL) of the extract from each fish species using the equation of the line from the BSA standard curve and the absorbance value for a dilution of the fish extract that had an absorbance near the middle point on the standard curve. Remember to correct for dilution used. (Note: Do not use the diluted samples for electrophoresis. Use the original extract prepared as described below.)

23.4.3 Electrophoresis

1. Assemble the electrophoresis unit according to the manufacturer's instructions.
2. Use the table that follows the list of electrophoresis reagents to combine appropriate amounts of all reagents for the *separating* gel, except APS, in a sidearm flask. Degas the solution and then add APS. Proceed immediately to pour the solution between the plates to create the separating (resolving) gel. Pour the gel to a height approximately 1 cm below the bottom of the sample well comb. Immediately, add a layer of butanol across the top of the separating gel, adding it carefully so as not to disturb the upper surface of the separating gel. This butanol layer will prevent a film from forming and help obtain an even surface. Allow the separating gel to polymerize for 30 min and then remove the butanol layer just before the stacking gel is ready to be poured.
3. Use the table that follows the list of electrophoresis reagents to combine appropriate amounts of all reagents for the *stacking* gel, except APS, in a sidearm flask. Degas the solution for 15 min (as per the manufacturer's instructions) and then add APS. Proceed immediately to pour the solution between the plates to create the stacking gel. Immediately, place the well comb between the plates and into the stacking gel. Allow the stacking gel to polymerize for 30 min before removing the well comb. Before loading the samples into the wells, wash the wells twice with dd water.
4. Mix the fish extract samples well (filtrate from Sect. 23.4.1, Step 5). For each sample, combine 0.1-mL sample with 0.9-mL electrophoresis sample buffer in screw cap culture tube. Apply cap.
5. Heat capped tubes for 3 min in boiling water.
6. Apply 10 and 20 µg protein of each fish extract to wells of the stacking gel using a syringe. Calculate the volume to apply based on the protein content of the extract and the dilution used when preparing the extract in electrophoresis sample buffer.
7. Apply 10 µl of molecular weight standards to one sample well.
8. Follow the manufacturer's instructions to assemble and run the electrophoresis unit. When the line of Bromophenol Blue tracking dye has reached the bottom of the separating gel, shut off the power supply. Disassemble the electrophoresis unit, and carefully remove the separating gel from between the plates. Place the gel in a flat dish with the Coomassie Brilliant Blue stain solution. Allow the gel to stain for at least 30 min. (If possible, place the dish with the gel

on a gentle shaker during staining and destaining.) Pour off the stain solution and then destain the gel for at least 2 h using the destain solution with at least two changes of the solution.

9. Measure the migration distance (cm) from the top of the gel to the center of the protein band for the molecular weight standards and for each of the major protein bands in the fish extract samples. Also measure the migration distance of the bromophenol blue tracking dye from the top of the gel.
10. Observe and record the relative intensity of the major protein bands for each fish extract.

23.5 DATA AND CALCULATIONS

23.5.1 Protein Determination

Tube identity	*Absorbance*	*µg protein/ tube*	*µg/mL sample*
Std. 1, 20 µL BSA			
Std. 1, 20 µL BSA			
Std. 2, 40 µL BSA			
Std. 2, 40 µL BSA			
Std. 3, 60 µL BSA			
Std. 3, 60 µL BSA			
Std. 4, 80 µL BSA			
Std. 4, 80 µL BSA			
Std. 5, 100 µL BSA			
Std. 5, 100 µL BSA			
Sample 1:5			
Sample 1:5			
			$\bar{X}$ =
Sample 1:10			
Sample 1:10			
			$\bar{X}$ =
Sample 1:20			
Sample 1:20			
			$\bar{X}$ =

Sample calculation for fish extract protein concentration:

For fish extract diluted 1:20 and 50 µL analyzed with absorbance of 0.677:

Equation of the line: $y = 0.0108x + 0.0022$

If $y = 0.677$, $x = 62.48$

$$C_i = C_f(V_2 / V_1)(V_4 / V_3)$$

(See Chap. 3 in this laboratory manual; C_i = initial concentration; C_f = final concentration)

C_i = (62.48-ug protein/tube) × (20 ml/1 mL) × (tube/50 uL)
= 24.99µg protein/uL fish extract

Sample calculation to determine the volume of prepared extract to apply 20 ug protein to each sample well:

How many µL are needed to get 20 µg protein? Remember the electrophoresis sample buffer dilution is 1:10:

$$24.99\,\mu g\,\text{protein}/\mu L \times Z\,\mu L \times (1\,mL/10\,mL) = 20\,\mu g$$

$$Z = 8.00\ uL$$

23.5.2 Electrophoresis

1. Calculate the relative mobility of three major protein bands and all the molecular weight standards. To determine the relative mobility (R_f) of a protein band, divide its migration distance from the top of the gel to the center of the protein band by the migration distance of the bromophenol blue tracking dye from the top of the gel:

$$R_f = \frac{\text{distance of protein migration}}{\text{distance of tracking dye migration}}$$

Sample identity	*Distance of protein migration*	*Distance of tracking dye migration*	*Relative mobility*	*Molecular weight*
Molecular weight standards				
1				
2				
3				
4				
5				
Fish species				
Freshwater				
Saltwater				

2. Prepare a standard curve by plotting relative mobility (*x*-axis) versus log molecular weight of standards (*y*-axis).
3. Using the standard curve, estimate the molecular weight of the major protein subunits in the freshwater and saltwater fish extracts.

23.6 QUESTIONS

1. Describe how you would prepare 1 L of the buffer used to extract the fish muscle proteins (0.15 *M* sodium chloride, 0.05 *M* sodium phosphate, pH 7.0). Show all calculations.

2. Discuss the differences between the fish species, regarding the presence or absence of major protein bands identified by the molecular mass and the relative amounts of these proteins.

RESOURCE MATERIALS

Bio-Rad (2013) A Guide to Polyacrylamide Gel Electrophoresis and Detection. Bulletin 6040 Rev B. Bio-Rad Laboratories, http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6040.pdf Accessed June 24, 2015

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