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The rAmylase Project by Ellyn Daugherty

Characterizing Proteins by PAGE

(Lab 5f)

(Cat. # BTNM-5F)

Developed in partnership with

The rAmylase Project by Ellyn Daugherty

Characterizing Proteins by PAGE (Lab 5f)

Teacher's Guide

The following laboratory activity is adapted from "Laboratory 5f: Characterizing Proteins by PAGE" from *Biotechnology: Laboratory Manua*l by Ellyn Daugherty. For more information about the program, please visit www.emcp.com/biotechnology.

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About Ellyn Daugherty: Ellyn Daugherty is a veteran biotechnology educator and recipient of the Biotechnology Institute's National Biotechnology Teacher-Leader Award. She is the founder of the San Mateo Biotechnology Career Pathway (SMBCP). Started in 1993, SMBCP has instructed more than 7,000 high school and adult students. Annually, 30-40 SMBCP students complete internships with mentors at local biotechnology facilities.

G BIOSCIENCES®

About G-Biosciences: In addition to the Biotechnology by Ellyn Daugherty laboratory kit line and recognizing the significance and challenges of life sciences education, G-Biosciences has initiated the BioScience Excellence™ program. The program features hands-on teaching kits based on inquiry and curiosity that explore the fundamentals of life sciences and relate the techniques to the real world around us. The BioScience Excellence™ teaching tools will capture the imagination of young minds and deepen their understanding of various principles and techniques in biotechnology and improve their understanding of various social and ethical issues.

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Upon receipt, store the materials as directed in the package literature.

MATERIALS INCLUDED

This kit has enough materials and reagents for 8 lab groups (32 students in groups of 4).

- 4 nUView™ 10-well 10% Tris-Glycine Protein Gels*, store at 4°C
- 8 tubes of 2X PAGE Sample Loading Dye Buffer (175µl), store at 4°C
- 1 bottle 10X PAGE Electrophoresis Running Buffer (250ml)
- 8 tubes of PAGEmark™ Protein Marker (sizing standards), store at -20°C
- 4 tubes of 0.1mg/ml α-Amylase (50µl), store at -20°C
- 4 tubes of 0.1mg/ml Cellulase (50µl), store at -20°C
- 1 bottle LabSafe™ GelBlue PAGE Stain (250ml)
- 50 Microcentrifuge tubes (1.5ml)
- 24 Lid Locks (keeps microcentrifuge tubes closed during heating)

ADDITIONAL EQUIPMENT & MATERIALS REQUIRED

- Micropipets and tips
- Protein Electrophoresis Equipment (vertical gel boxes and power supplies)
- Heat block (preferred) or water bath set at 90°C
- Large weigh boats or trays (about 10x10cm), for transferring gels to imaging system or for staining
- UV Light box
- Test Tubes
- Beakers
- Deionized water

SPECIAL HANDLING INSTRUCTIONS

- Store the PAGEmark™ Protein Marker, Amylase and Cellulase frozen at -20°C.
- Store the nUView™ Tris-Glycine Protein Gels and 2X PAGE Sample Loading Dye Buffer at 4°C.

*NOTE: This kit is sent with 4 "NG" nUView gels. These are considered universal gels and fit most 10X10cm gel box tanks. If requested at the time the order is placed, "NB" gels may be substituted for NG if the customer has Mini Protean gel tanks. If requested at the time the order is placed, "NN" gels may be substituted for NG if the customer has XCell SureLock gel tanks.

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GENERAL SAFETY PRECAUTIONS

- The reagents and components supplied in the *The rAmylase Project*™ kits are considered non-toxic and are safe to handle (unless otherwise noted), however good laboratory procedures should be used at all times. This includes wearing lab coats, gloves and safety goggles.
- The teacher should 1) be familiar with safety practices and regulations in his/her school (district and state) and 2) know what needs to be treated as hazardous waste and how to properly dispose of non-hazardous chemicals or biological material.
- Students should know where all emergency equipment (safety shower, eyewash station, fire extinguisher, fire blanket, first aid kit etc.) is located and be versed in general lab safety.
- Remind students to read all instructions including Safety Data Sheets (SDSs) before starting the lab activities. A link for SDSs for chemicals in this kit is posted at www.gbiosciences.com
- At the end of the lab**,** all laboratory bench tops should be wiped down with a 10% bleach solution or disinfectant to ensure cleanliness.
- Remind students to wash their hands thoroughly with soap and water before leaving the laboratory.

TEACHER'S PRE EXPERIMENT SET UP

1. In a large beaker (4L), pitcher, bucket or carboy, dilute 10X PAGE Electrophoresis Running Buffer to 1X with distilled water (mix 250ml buffer with 2250ml deionized water). The amount of Electrophoresis Running Buffer needed depends on the device used. Chill the buffer to 4°C to prevent overheating during electrophoresis.

2. Distribute the following items to each lab group:

    - 1 nUView™ 10-well 10% Tris-Glycine Protein Gel
    - 1 tube of 2X PAGE Sample Loading Dye Buffer (150µl)
    - 1 tube of PAGEmark™ Protein Marker (sizing standards)
    - 1 tube of 0.1mg/ml α-Amylase (50µl) or 1 tube of 0.1mg/ml Cellulase (50µl)
    - 4 Microcentrifuge tubes (1.5ml)
    - 1 large weigh boat or trays (about 10x10cm)

TIME REQUIRED

- 30 minutes pre-lab (preparation of buffer and distribution of reagents)
- 2- hour lab period (to prepare samples, load, run and analyze gel on IV imaging system, place gels in LabSafe™ GelBlue PAGE Stain)
- 1 hour post-lab analysis

NEXT GENERATION SCIENCE STANDARDS ADDRESSED

- HS-LS1: From Molecules to Organisms: Structures and Processes
- LS1.A: Structure and Function

For more information about Next Generation Science Standards, visit: http://www.nextgenscience.org/

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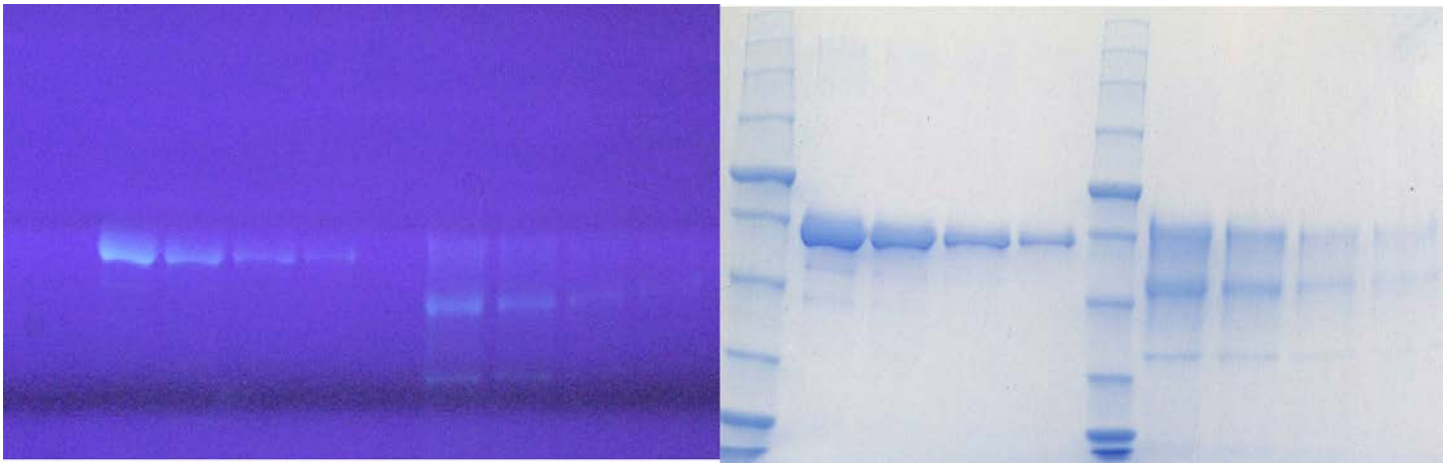
EXPECTED RESULTS



ANSWERS TO ADDITIONAL QUESTIONS

1. Explain how PAGE electrophoresis separates protein molecules?
   A. Proteins are charged molecules and when placed under an electrical field the protein molecules migrate through a matrix, either towards the cathode or anode depending on the net charge of the protein molecules. During migration, proteins separate into different species depending on the size or the net charge of the protein molecules

2. Briefly describe the role of SDS in protein electrophoresis.
   A. SDS binds and denatures protein molecules forming long rod like structures

3. A technician sets up and starts a PAGE. Current is flowing, and bubbling is visible at the electrodes. After 30 minutes, none of the samples have moved out of the wells. List three things the technician should check.
   Answers will vary, but if the sample has not moved out of the wells of a gel, some things to check include the following: Has the gel been placed in the gel box in the correct orientation— not backward? Does the buffer completely cover the wells? Are air bubbles blocking the flow of electricity at the bottom of the gel?

4. If a gel has a band that is significantly darker and fatter than all the other bands, suggest a few reasons for that result.
   Answers will vary. A fat band can mean a single, highly concentrated sample of a single polypeptide. It can also mean two or more bands of nearly the same molecular weight have not completely separated.

5. Every amino acid has a different molecular weight because of different R groups. Using amino-acid data from ProtScale at: http://biotech.emcp.net/molecularweight, one can determine that the average molecular weight of an amino acid is about 137 Da. Use the average molecular weight of an amino acid and the estimated molecular weight of the polypeptide chains in the protein you studied to determine the approximate number of amino acids in each protein.
   Answer will vary based on the protein studied and the estimates made, but the number of amino acids in a protein can usually be found at the NBCI Web site. For example, a good estimate for amylase is 61,000 Da/137 Da per amino acid = 445 amino acids.

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OBJECTIVES

What structural characteristics of amylase and cellulase can be determined from running samples on a SDS-PAGE gel? How is the resolution of the polypeptide bands on the gel affected by the concentration of the sample loaded?

BACKGROUND

One of the first things to learn about a protein is its size and structure. By running a denaturing sizing gel, one can determine the molecular weight and the number of different polypeptide chains of a protein. Molecular weights are reported in kilodaltons (kDa). A dalton (Da) is equal to the mass of one hydrogen atom. For example, 1kDa is equivalent to 1000Da. Proteins usually have molecular masses ranging from 10 to 300kDa.

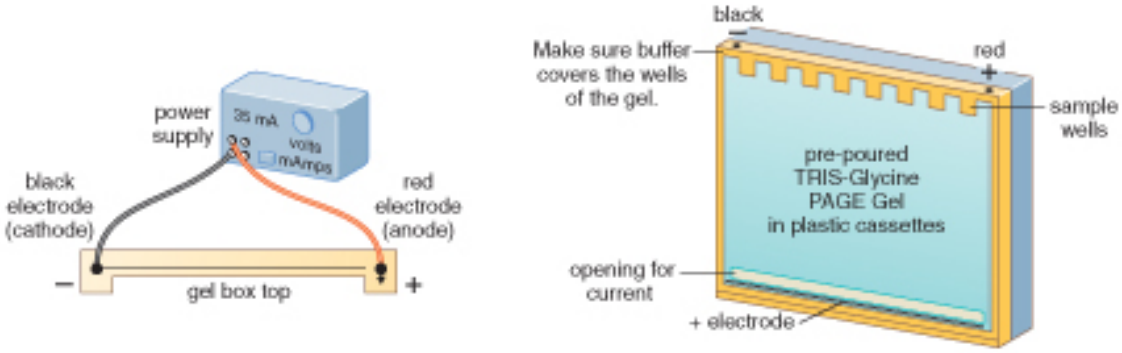

Figure: Vertical Gel Electrophoresis. *Although vertical gel boxes vary from one manufacturer to another, all are basically the same design. The gel cassettes are snapped or screwed in place (right). Running buffer is added behind the gel, covering the wells. Buffer is poured in front of the gel cassette to cover the front opening. When the top is placed on the box (left) and the power is turned on, electricity flows from top (negative charge) to bottom (positive charge). Negatively charged samples move down the gel towards the positive electrode.*

For size determination, a TRIS-glycine (TG) polyacrylamide gel at a given concentration is used. Premade gels can be purchased with a polyacrylamide concentration of anywhere from 4% to 20%. Samples of unknown molecular weight are loaded into wells. Standard proteins of known molecular weight are run in at least one lane.

The gel is run at a specific current measured in milliamps (mA) until the loading dye front is near the bottom of the gel. The SDS and other denaturing agents in the loading dye and buffer causes the peptide chains to unravel and linearize. If there is more than one type of polypeptide in the protein, it separates from the other peptides. Smaller peptides move through the gel faster than larger ones. The peptides "band out" based on size. After staining the peptides, you can size them by comparing the bands of unknown molecular weight to standards of known molecular weight.

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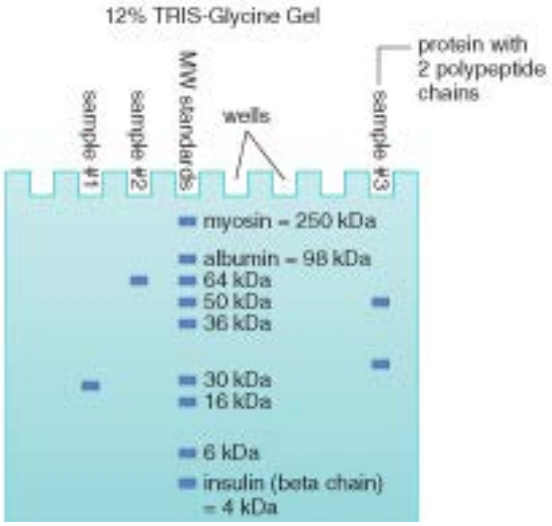

Figure: PAGE with standards. *The smaller the peptide chain, the faster it moves through the gel. Protein-sizing standards can be used to determine the size of unknown samples. Proteins sizes are in kilodaltons (kDa).*

The proteins studied in this experiment are the enzymes amylase and cellulase. Amylase breaks down starch to sugar. Cellulase breaks down the plant protein cellulose to simple sugars. By running pure samples of each protein on a SDS-PAGE gel, the number of polypeptide chains (one or more) in each protein may be determined and the size of each polypeptide chain may be estimated. Knowing that the average size of an amino acid is about 137kDa, the number of amino acids in each polypeptide chain may be estimated as well.

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MATERIALS FOR EACH GROUP

Supply each group with the following components.

- 1 nUView™ 10-well 10% Tris-Glycine Protein Gel (shared with another lab group)
- 1 tube of 2X PAGE Sample Loading Dye Buffer (150µl)
- 1 tube of PAGEmark™ Protein Marker (sizing standards)
- 1 tube of 0.1mg/ml α-Amylase (50µl) or 1 tube of 0.1mg/ml Cellulase (50µl)
- 4 Microcentrifuge tubes (1.5ml)
- Vertical Gel Box/Electrophoresis Apparatus (shared with another lab group)
- 300V Power Supply for use with gel box (shared with another lab group)

Some components will be shared by the whole class and should be kept on a communal table.

Caution: Never use latex gloves when using a heat block or water bath. A 90° heat block or water bath can cause severe burns. Use lid locks to keep tubes closed. Lid locks have handles to make for safer handling. If using a hot water bath, place tubes in a floating rack so the students do not come in contact with the near boiling water.

- Heat block (preferred) or water bath set at 90°C
- 24 Lid locks (at heat block or bath)
- 1X PAGE Electrophoresis Running Buffer (shared with whole class), 250-300ml per gel box
- Large weigh boats or trays (about 10x10cm), for transferring gels to imaging system or for staining
- LabSafe™ GelBlue PAGE Stain (shared with whole class)

ADDITIONAL MATERIALS FOR EACH GROUP

The following standard lab equipment should be available for each group.

- P-100 and P-20 Micropipets and tips
- Gloves and goggles
- Paper towels
- Glassware

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PROCEDURE

I. Prepare the Samples

NOTE: Each group will prepare dilutions of either amylase stock solution or Cellulase stock solution and will then load their samples on a protein gel shared with a group using the alternative protein. The teacher will assign the protein stock (amylase or Cellulase) to your lab group.

1. Set up 4 tubes and label them 1, 2, 3 and 4 plus the letter "A" or "C" to denote the protein being studied.
2. Add 30µl of 2X PAGE Sample Loading Dye Buffer to your "1", "2", "3", and "4" tubes.
3. Transfer 30µl of the 0.1mg/ml protein from your protein stock solution to your group's A1 or C1 tube and pipette up and down to mix. Now, this is a 0.05mg/ml protein solution.
4. Transfer 30µl of the 0.05mg/ml protein solution to Tube 2 and pipette up and down to mix. This is a 0.025mg/ml protein solution.
5. Transfer 30µl of the 0.025mg/ml protein solution to Tube 3 and pipette up and down to mix. This is a 0.0125mg/ml protein solution.
6. Transfer 30µl of the 0.0125mg/ml protein solution to Tube 4 and pipette up and down to mix. Discard 30µl of this solution. This is a 0.00625mg/ml protein solution.
7. Just prior to loading the gel (step 15), seal the 4 tubes with a lid lock and carefully place into a 90°C heat block (or water bath) for 3 minutes. Do not over boil.
8. Briefly vortex the sample tubes and centrifuge for 2-3 seconds to bring down the condensation.

II. Protein Electrophoresis

9. Set up a vertical electrophoresis gel box as directed in the following steps. Use a nuView 10% PAGE gel. In your notebook, record the gel concentration, the lot number, and the expiration date.
10. Cut open the packet and drain the preservative from the cassette and dry front of gel.
11. Notice how the wells of the gel are numbered and there is a line at the bottom of each well. Using a permanent marker to shade the well numbers makes the wells easier to see during loading.
12. Study the gel box to understand how it is put together and which side is the front. Put the gel(s) in the box with the high side facing out, so that the labeled side faces the front of the gel box. When running a gel, a tight seal must be formed between the gel cassette and the gasket of the running frame to prevent buffer and current leakage from the inner buffer chamber (where the gel wells are exposed to the buffer).
13. Pour sufficient 1X Electrophoresis Running Buffer into the inner buffer chamber of the gel running apparatus to completely cover the sample wells. Fill the outer tank with running buffer to ensure the bottom of the gel cassette is covered (3-4 cm). Make sure that buffer is being held in the inner chamber and that the chamber is not leaking.

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Make adjustments as necessary to prevent leakage. Gently rinse the wells of the gel thoroughly with running buffer (2 times the well volume) to remove air bubbles and to displace any storage buffer.

14. The amylase lab group should load 5µl PAGEmark™ Protein Marker to the first lane. The PAGEmark™ Protein Marker consists of a mix of twelve Prestained proteins of molecular weight 240, 180, 140, 100, 72, 60, 45, 35, 25, 20, 15 and 10kDa. The molecular weight markers are used to determine molecular weights of the amylase protein.

15. Load the 30µl amylase samples 1-4 to the remaining wells #2-5. Each student should load at least one well. Make sure to record the order samples were loaded.

16. The cellulase lab group should load 5µl PAGEmark™ Protein Marker to the lane #6.

17. Load 30µl cellulase samples 1-4 to the remaining wells #7-10. Each student should load at least one well. Make sure to record the order samples were loaded.

18. Run the gel at 250 volts (no more than 80mA) for about 30 minutes* until the blue dye front is 0.5-1cm from the bottom of the gel.

* The nUView™ Tris-Glycine Protein gels are designed to be run at 200-250 volts but no more than 80mA. Having the current above 80mA could melt the gel. At 80mA the gel run is over in about 30 minutes. The gels run that fast only when one gel is run on a power supply at a time. If 2 or more gels/gel boxes are run on the same power supply then the gel run time will be significantly longer.

19. Set up and label a dish, tray or large weigh boat with deionized water. This will be used to transfer the gel to the imaging system.

20. When the electrophoresis run is completed, remove the gel cassette(s) from the tank. Rinse the cassette in water. To open the cassette, pry apart from one of the cassette edges. Pull the top plate of the cassette away from the bottom plate. The two halves will snap apart completely, exposing the gel. Transfer the gel in the deionized water in the dish by inverting the plate with the gel on it over the water and touching the gel to the water. The gel will slip into the water. Carefully pour off the water leaving the gel in the tray. Do not soak the gel in water.

21. The supplied NuView™ gel has a special protein stain in the gel that allows visualization of high-resolution protein bands under UV light within 2 minutes of completing electrophoresis. Using UV safety glasses and gloves place the gel on a UV light box (250-300nm) and illuminate until bands are visible (approximately 2 minutes). UV bands will start to fade after approximately 7 minutes with cumulative exposure to UV. Capture (scan/photograph) the gel for a permanent record.

22. The gel can also be stained with the supplied Coomassie Protein Stain (LabSafe™ GelBlue). Wash the gel twice in distilled water, five minutes each.

23. Pour off all water from the gel.

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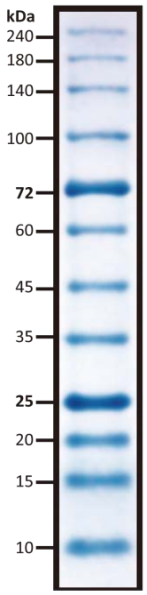
24. Add 50ml LabSafe™ GelBlue to cover the gel. Gently shake the gel for 60 minutes at room temperature.
    NOTE: Protein bands will begin to appear within 5 minutes and the gel can be removed from the stain when the desired bands are visible.

25. Decant the LabSafe™ GelBlue PAGE Stain and rinse the gel with distilled water. The gel can be stored in water. Longer de-staining in water will give a clearer view of the protein bands.

26. Following de-staining, place the gel on a glass plate and on top of a light box. A white light photograph of the gel may be taken for a permanent record.

III. Molecular Weight Determination:

Under appropriate conditions, one gram of a polypeptide binds 1.4 grams of SDS, resulting in the formation of a rod like particle with length proportional to the molecular weight of the polypeptide.



4-20% Tris-Glycine

The PAGEmark™ Protein Marker consists of a mix of eight proteins of molecular weight 240, 180, 140, 100, 72, 60, 45, 35, 25, 20, 15 and 10kDa.

The molecular weights of the sample polypeptides can be estimated by the following procedure.

a. Using the UV light gel photograph or a the white light photograph, measure the distance traveled by the bromophenol dye front and each standard polypeptide and several unknown protein bands from the top of the resolving gel.

b. Calculate R_f (relative mobility) for each polypeptide from the formula:

R_f = distance of protein migration / distance of dye migration

c. Plot R_f versus log M_r for the standard on semi-log paper or use a spreadsheet program such as Microsoft Excel.

d. Use the R_f value of the unknown sample polypeptide to estimate their molecular weight by interpolation.

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DATA ANALYSIS & CONCLUSION

In a written concluding statement (1-3 paragraphs), discuss the results of the experiment, including for each protein studied: the number of polypeptide chains present, the estimated molecular weight of each polypeptide chain, and the estimated total molecular weight of the protein. Determine the optimum concentration for visualizing the peptide chains of each protein studied. Discuss the sources of error in technique that could lead to fallacious data. What variables are hard to control in this experimental design? What might be done to improve the resolution of the peptide bands on the gel? Describe several ways in which the PAGE technique can be used in industry.

ADDITIONAL QUESTIONS

1. Explain how PAGE electrophoresis separates protein molecules?

2. Briefly describe the role of SDS in protein electrophoresis.

3. A technician sets up and starts a PAGE. Current is flowing, and bubbling is visible at the electrodes. After 30 minutes, none of the samples have moved out of the wells. List three things the technician should check.

4. If a gel has a band that is significantly darker and fatter than all the other bands, suggest a few reasons for that result.

5. Every amino acid has a different molecular weight because of different R groups. Using amino-acid data from ProtScale at: http://biotech.emcp.net/molecularweight, one can determine that the average molecular weight of an amino acid is about 137 Da. Use the average molecular weight of an amino acid and the estimated molecular weight of the polypeptide chains in the protein you studied to determine the approximate number of amino acids in each protein.

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