# Chapter 5

# Isolation of Myosin and Actin from Chicken Muscle

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## Contents



## Introduction

Obtaining enriched fractions of the major structural/contractile proteins from store-bought chicken muscle was an exercise we designed to expose first year students to the power of 'reductionist', analytical methods in biology. It is currently used in our Honors Introductory Biology classes. The lab spans two weeks: extraction and electrophoresis are accomplished during the first 3 hour lab period, and measurements from the dried gels are obtained during the first 20–30 minutes of the following week's lab. It is our plan to expand this exercise by incorporating elements that allow students to develop a more holistic impression of this sophisticated biological machinery, such as microscopic examination of muscle structure and demonstration of contractility (we are indebted in this regard to those ABLE participants who made us aware of the glycerin-stored muscle preps; e.g. from Carolina Biological).

The classic procedures for purifying myosin and actin from striated muscle are designed to use tissue from a freshly killed animal (rabbit muscle is a common choice), or from defatted, freeze-dried preparations (actin can be readily purified from an acetone-extracted powder of chicken muscle; i.e. Sigma chemical M7881) The relevant citations I've used in my work for myosin are Margossian and Lowey (1982) and for actin are Spudich and Watt (1971). It should be recognized that preparations derived from fresh muscle give decidedly cleaner purifications because the extraction begins with tissue in which the mechanisms that regulate the many interactions (myosin and actin being the most prominent) are mostly intact. Thus, one's knowledge of the roles of ATP, protein phosphorylation, and various ion fluxes and binding can be used in designing an effective strategy for gently dissociating the relevant proteins. Store-bought muscle, on the other hand, contains muscle in which the regulatory machinery expired with the organism, and has thus been rendered in a state of permanent rigor. Depending on how rapidly the meat was chilled, there is probably also some enzymatic degradation, and disruption due to freezing. What is more, our modifications of the classic procedures, to compress the exercise into a 3-hour student lab, eliminate many of the meticulous steps (spread over several days) that ensure high purity and yield of intact, functional molecules. The result of these trade-offs is that the protein fractions that are collected contain significant cross-contamination and some enzymatic degradation into protein sub-fragments.

While not being purified in the strictest biochemical sense, one could say that the actin and myosin fractions are 'comparatively' enriched with respect to each other. Besides the element of time, it turns out that these transgressions against sound biochemistry have some benefits for the novice learner. The electrophoretic gels of the protein fractions have many bands, and, once students have determined their molecular masses, they can undertake the useful detective work to deduce what the fragments might be.

For instance, the irreversible formation of actomyosin complexes that accompany rigor, means that even the myosin fraction which presumably has been separated from most actin by the high salt buffers, will exhibit a prominent actin band in the gel lane (the actin's tenacious grip in rigor having been finally broken by the SDS detergent in the gel buffers). The long myosin molecule tends to be clipped at specific location by enzymes released from cells; with a model of an intact myosin molecule (I like the depictions in Lehninger's Biochemistry text, 2nd ed., Worth Publishers, 1975, pp. 754–755) students can make informed guesses about the identity of the primary myosin sub-fragments.

The underlying principle which guides the extraction and purification of muscle proteins involves differential solubility. The highly asymmetric myosin molecule, with its globular heads and long-intertwined tails (it spontaneously forms dimers, and can be induced to assemble further into macroscopic filaments) is soluble in high ionic strength. Globular actin is more soluble in low salt buffers, especially if low levels of divalent ions and ATP are present. When a myosin fraction that has been solubilized in high salt, is diluted into low salt conditions, the myosin molecules quickly come out of solution (occasionally forming visible filaments) - an event which is instructive for students. The solubilization/precipitation of actin during changes in buffer composition is far more subtle (requiring an ultracentrifuge to produce a pellet of filamentous [f-] actin), and we simply eliminated those steps from the short protocol.

## Materials (one lab section of 20 students working in pairs)

High speed microcentrifuge (>10,000 rpm) (1)
Polyacrylamide gel electrophoresis apparatuses (e.g., Biorad) (3–5)
Power supplies (one per electrophoresis apparatus) (3–5)
Gel dryer or gel drying film and frames (e.g., Promega) (1)
Boiling water bath or hot plate with beaker of water (1)
Rack for immersing microcentrifuge tubes in water bath (1)
P-200 micropipetors or equivalent (5)
P-20 micropipetors or equivalent (5)
Micropipetor tips (100)
Insulated ice buckets filled with crushed ice (10)
Mortars and pestles
Glass plates (approximately 9" × 9") (10)
Single-edged razors (10)
Small plastic containers with lids (e.g., Rubbermaid sandwich containers) (3–5)
Plastic metric rulers (10)
Glass beakers, 100 ml (20)
Glass Erlenmeyer flasks, 25 ml (10)
Polypropylene microcentrifuge tubes, 2.0 ml, with attached flat cap (160)
Cheesecloth squares, 5" × 5" (100)
Disposable gloves (1 pair per student) (box)
Frozen chicken breast (100 g)
High salt buffer for myosin extraction (4.5 g KCl, 4.0g $KH_2PO_4$, pH to 6.5, and bring to final volume of 200 ml in distilled, deionized water) (200 ml)
Low salt buffer for actin extraction (0.12 g Tris, 4 ml of 10 mM solution of $CaCl_2$, 400 ml of 50 mM stock of ATP [sodium salt], pH to 8.0 and bring to 200 ml volume with distilled, deionized water) NOTE: This buffer must be made fresh each day and stored on ice due to the rapid degradation of ATP at room temperature. (200 ml)
Solubilizing buffer (5 ml)

Vial pre-stained high range SDS-PAGE protein molecular weight standards (e.g., Sigma #C3312). NOTE: One vial is sufficient for 5–6 laboratory sections (1)
Bottle 30% acrylamide/bis solution, 29:1 (purchased pre-mixed) (1)
10% ammonium persulfate (100 mg ap in 1 ml distilled, deionized water) (1 ml)
TEMED
Coomassie Brilliant Blue R250 solution (0.25 g CBB in 90 ml methanol [1:1 v/v] and 10 ml glacial acetic acid) (1 liter)
Destain solution (30% methanol and 10% acetic acid in distilled, deionized water) (4 liter)

## Notes for the Instructor

### Sources of chemicals and equipment

Virtually everything we use in our labs can be obtained from Hoefer (now Pharmacia/Hoefer), Sigma Chemical, and VWR (or Fisher). BioRad also has a complete line of electrophoresis/molecular biology supplies.

### Extraction

*Chicken*

We use chicken breast because 1) it is not pigmented, and 2) students are familiar with it as something to eat. Pigmented meat contains all sorts of circulation proteins (especially hemoglobin), and the color is imparted to the extraction buffers making unaided visualization of centrifugation pellets and precipitating filaments impossible. Because the chicken breast has essentially been genetically engineered to have high white meat content, one can, for the purposes of this lab, envision the life of the chicken as part of the myosin/actin purification process. We tend to keep the muscle frozen then present it for use in the slightly thawed (but still crystalline) state. This makes it easier to slice and chop the meat before the grinding steps.

*Extraction Buffers*

The recipes we use are as follows:

High salt buffer for myosin:
4.5 g KCl (gives final concentration of 0.3M KCl)
4.0 g $KH_2PO_4$
0.44 g KOH (for initial pH adjustment)
bring final volume to 200 ml with $H_2O$
adjust final pH to 6.5

Low salt buffer for actin:
0.12 g Tris (gives 5 mM concentration)
4 ml of 10 mM stock of $CaCl_2$ (0.2 mM final concentration)
400 µl of 50 mM stock of (Na)ATP (keep stock frozen until use; final concentration 0.1 mM)
bring final volume to 200 ml with $H_2O$
adjust final pH to 8.0

*Centrifuge*

Ideally one would like to keep protein samples as cold as possible throughout the isolation, and the original procedures call for refrigerated centrifugation steps. Refrigerated centrifuges however take time and care in loading, and their run-times create the dreaded lab dead-time (for which there is occasionally no recovery). We thus divide up the extracts into 1.5 ml (conical sides) or 2.0 ml (cylindrical sides) Eppendorf tubes and spin them as quickly as possible at room temp. in the typical (12,000 rpm) lab microfuge. One can put these machines in a cold room or a refrigerator, but we have found it isn't worth the bother of added congestion and progressive damage (from humidity) to the centrifuge. Tell students to keep the samples on ice as much as possible and to handle the tubes near the top rim of the Eppendorfs (even though they nestle so naturally in the 98.6° F hand).

Centrifuge tubes should be marked with the name of the group and the contents of the tube before being placed in the centrifuge. When loading the microcentrifuge, it's helpful to put the microcentrifuge tube hinges such that they all face in the same direction (e.g., toward the outside of the microcentrifuge). This way, even if the pellet is very small it will be in a predictable location (i.e., the bottom of the tube, on the same side as the hinge) and is less likely to wash out of the tube as the supernatant is poured off.

**Electrophoresis**

*Sample Preparation*

The blue 'solubilizing' or 'sample' buffer that is added to the protein samples contains SDS (which, in addition to the heat of the boiling water bath, denatures the proteins), bromophenol blue (the tracking dye), β-mercaptoethanol (which breaks protein sulfhydral bridges, removing protein secondary structure), and 4 M urea (which increases the density of the samples so that they drop through the buffer when loaded into the gel wells. The actual recipe for a 4∞ solution (one adds 1 part sample buffer to 3 parts protein sample) is:

| | Final Concentration |
|---|---|
| 6 g SDS | 6% |
| 24 g urea | 4 M |
| 1.5 g Tris | 125 mM |
| 0.15 g EDTA | 4 ml |

Add water to 50 ml, adjust pH to 6.9.
For 10 ml working solution, add 750 µl β-mercaptoethanol and 650 µl of 2% (w/v) bromophenol blue.

*Gels*

We use the Hoefer mini-gel system (Hoefer mighty small, SE200). The size of these gels is 8 cm ∞ 10 cm, and they are 0.75 mm thick. With a 10% final concentration of acrylamide/bis mixture, these gels have reasonable strength (given their thickness) and run to completion (they can be stopped earlier, of course) in 45 to 60 min., without frying the unit. We use the very popular 2 gel-layer system (originally called discontinuous electrophoresis) for obtaining high resolution protein separations. The samples are loaded into wells formed from a narrow "stacking" gel that is 4% acrylamide (soft and porous to proteins) and has a pH of about 7. Under voltage the proteins and move rather quickly towards the main, "running" gel layer This medium is 10% acrylamide (firmer and less porous) and has a

pH of about 9. The sudden change in surface charge density (due to the pH shift), coupled with the decreased porosity, causes the proteins to sort themselves according to their masses (small proteins run closer to the front) and to concentrate into tight bands that are visible after staining. With 0.75 mm thickness and a ten-well comb, the sample wells each hold a max. of 15 µl; we find that 1–5 µl of the muscle extracts is sufficient to easily visualize bands on gels stained with Coomassie blue. One can get spacers and combs to make thicker gels (and larger well volumes), but thicker gels take longer to stain, destain, and dry. Of course the sample can be run on other gel systems (including the older tube gels), and it shouldn't take but a few trial runs to learn what it will take to substitute another system to this basic outline.

Our gel recipes are as follows:

30% Acrylamide/2% Bis acrylamide - ONE MUST USE GLOVES AND WEAR A FILTER MASK TO WEIGH OUT THE ACRYLAMIDE POWDER. ONCE IT IS IN SOLUTION THE DUSK FACTOR IS REMOVED, BUT ONE MUST STILL WEAR GLOVES TO WORK WITH THESE SOLUTIONS IN THE UNPOLYMERIZED FORM.

We generally make a liter volume of the acrylamide stock, and this will keep in the refrigerator for up to a year:
300 g Acrylamide
8 g bis acrylamide
bring to 1 liter with water

Buffer A (for running gel) - 3 M tris-HCL
363 g tris
add 1 N HCl to bring volume to 800 ml
adjust pH to 8.9 with concentrated HCl
bring final volume to 1 liter with water

Buffer B (for stacking gel) - 1 M tris-HCl
36.3 g tris
add 200 ml 1 N HCl
adjust pH to 6.7 with concentrated HCl
bring volume to 300 ml with water

10% SDS - 1 g SDS/bring to 10 ml with water

5% (w/v) ammonium persulfate (catalyst)
(This component is the most problematic of all the reagents used in casting gels. Many people make a fresh solution for each batch of gels; I've had the solution keep in the refrigerator for months and still effect polymerization. On the other hand I've had the dry crystals "die" right on the shelf (i.e. solutions made fresh fail to cause the gel to harden). If you experience any problems in polymerization, my experience suggests that it's time to get a new bottle of the ammonium persulfate)

5% (v/v) TEMED (catalyst)

*Procedure for Casting 12, 10% Minigels*

I use the very efficient Hoefer gel caster (SE215) to cast 12 gels at a time. These can be stored in a snap-top box (Rubbermaid) with a little water to provide humidity for a month or more. I leave about 1 cm of headspace at the top of the gels for a stacking gel (into which the comb is inserted, forming the sample wells) to be applied near the time of the lab. The recipe for the running gels (in casting unit) is as follows:

| | |
|---|---|
| Water | 30 ml |
| Acrylamide/bis solution | 20 ml |
| Buffer A | 10 ml |
| 10% SDS | 1.25 ml |
| 5% AmPS | 120 µl |
| TEMED | 75 µl (add last and pour gels) |

Applying the stacking gel

The stacking gel (to top 4 running gel slabs) is made as follows:

| | |
|---|---|
| Water | 4 ml |
| Acrylamide/bis solution | 750 µl |
| Buffer B | 750 µl |
| 10% SDS | 120 µl |
| 5% AmPS | |
| TEMED | |

I set up 4 gel sandwiches (i.e. using the running gels poured in the caster, above) at a time and fill the remaining volume above the running gel with a Pasteur pipet. I then gently insert the 10 well teflon comb (which displaces some of the stacking gel mix), and watch to be sure that there are no leaks or air bubbles.

*Loading and Running Gels*

The gel tanks are filled with SDS/tris/glycine buffer. We make up a 10∞ concentrate which sits out at lab temp. (the SDS will precipitate in the cold), and then make up 1∞ as needed. The 10∞ recipe is: 143 g glycine, 30 g tris, 20 g SDS, bring to 1 liter with water.

Once both chambers (including the sample wells) are filled (one wants no air bubbles at the bottom of the gel, which would create an open circuit - I dutifully check, but have never had this problem with the Hoefer cast gels), the cooked, blue protein samples are loaded into the wells. Because the yield is somewhat unpredictable, we usually load 1 µl and 5 µl of each extract into the gel, with an open lane between them in case the higher volume lane overflows. Because of the urea-enhanced density, they drop right through the buffer into the well. The protein standards we use, that actually separate during the run as colored bands (liked by students), are from Sigma (C3312).

Wires are connected—the lower end of the gel is [+] (red wire), and the sample end is [-] (black wire)—and the power turned on. I prefer a power supply with volt and current (amperage) meters, but many student models (including ours) lack these features. The meters are a great aid in trouble-shooting: if the voltage is high (over 100v) with very low current (< 2 ma), then there is an open circuit (or very high resistance) somewhere. The problem could be due to unconnected wires (don't laugh, I do this), air bubbles, insufficient buffer in the tank (must cover the platinum wires), buffer with incorrect composition (too low) of tris/glycine. If the amperage is high (> 50 ma)—DON'T LEAVE THE SUPPLY ON FOR EVEN A FEW SECONDS IF THIS IS TRUE—and the voltage is low (< 10 v), then there is a current leak somewhere (and this might be dangerous). This problem is almost always traceable to a leaking buffer chamber, or (less likely) to an incorrectly-made (tris/glycine too high) tank buffer (i.e. we've had students fail to dilute the 10∞ concentrate to 1∞ ). With the 0.75 mm thick, 10% acrylamide, Hoefer-cast gels, I consistently see 70 to 100 v and 15 to 25 ma on the meters (I should note that the voltage is lower and the current higher when one first turns on the power supply, and, as the resistance of the gel

rises during the 1st 15 min of the run, one must increase the voltage to maintain a current of about 20 ma).

When the tracking dye nears the bottom of the gel, the TA turns off the power supply, disconnects the wires, and dumps out the tank buffer. Because it usually takes most of the lab period to extract the proteins and load the gels, this is usually done after the students have left the lab. The glass plates are separated, and the gel is carefully stripped from the plate and dropped into a staining dish (Rubbermaid again). Don't worry if the gel is folded or wrinkled, it will straighten out as soon as the stain is added.

*Staining, Destaining and Drying the Gels*

The staining solution is:
400 ml methanol
70 ml glacial acetic acid
0.25 g Coomassie blue R-250
add water to 1 liter

Staining will be faster (complete in 1 hr) with constant agitation on a slow speed shaker. But you can just let them sit overnight with occasional hand-swirling. Stain is dumped off (we reuse it) and destain is added (recipe: 375 ml methanol, 250 ml Acetic acid, bring to 2.5 liters with water). Again swirling speeds the destaining process - it also helps to add a wadded-up Kimwi—for some unknown reason the cellulose preferentially binds the dye and one has to exchange the destain solution only once or twice.

The gels can be examined wet (you can lay them out and keep them moist on a sheet of glass), or, as we do, you can dry them. We use a commercial dryer (all molecular biology labs have them, and there are air drying methods also). The dried gels have the advantage that they can be handled and photocopied (and enlarged) for measurements of the migration distances of the protein bands. We have the students plot the relative mobility (distance traveled by protein band/distance traveled by tracking dye) of each known band (MW standards) as a semilog plot. The classic reference for this procedure is Weber and Osborn (1969). Similarly, the mobilities of the unknown muscle proteins (and fragments) are determined. The standard curve plotted from the MW standards is then used to determine the approximate molecular masses of the unknown protein bands.

If a commercial dryer is available, this is the fastest method of drying gels. When using a commercial dryer, be sure to put a cold trap between the vacuum pump and the dryer, since acid from the destain solution can destroy the seals in the vacuum pump. In the absence of a dryer, gels can be air dried. For air drying, wet gels should be placed between a sheet of wet paper and a sheet of wet cellulose. The top cellulose layer should be smoothed against the gel so that no air bubbles remain. It is helpful to leave a margin of paper around the gel so that it may be labeled. Alternatively, the gel can be sandwiched between two layers of cellulose. The gel "sandwich" is then placed on a sheet of glass (the glass plate from the electrophoresis apparatus works well) and plastic strips are affixed to all four edges. Binder clips are then placed around all sides to affix the gel to the glass and to prevent it from curling as it dries. Glass plates should stand vertically for 2 days to dry.

## Lab Prep and in Class Procedures

Glass plates and mortars and pestles should be chilled (e.g., in a refrigerator) prior to use. The chicken breast should be thawed slightly, so that it is not frozen solid when students begin to chop.

We place labeled beakers on each bench top for liquid waste and used pipet tips. A labeled plastic "Biohazard" bag is used for disposal of used cheesecloth and leftover chicken pieces. Ice buckets filled

with ice are placed on the lab benches. We use one bucket for every pair of students, but one bucket per group of 4 students would suffice if buckets are in short supply. Stock flasks of the buffers are labeled and kept on ice at the front of the classroom. Students dispense required amounts as necessary.

Prior to beginning the experiment, the instructor should review the structure of muscle fiber and provide some basic information on the principles behind electrophoresis. We draw a diagram of a gel on the black board to indicate where each sample should be loaded. Students should wear latex gloves for all procedures requiring the handling of muscle matter and should wash their hands thoroughly afterward. Students work in groups of four. Within each group, one pair prepares the myosin crude extract while the other pair prepares the actin crude extract. Each pair should periodically observe what the other pair is doing so that differences between myosin and actin at various stages of the procedure can be observed.

## Student Laboratory Handout

## Isolation of Myosin and Actin from Chicken Muscle

### Background

The muscles of your body perform various essential functions such as moving parts of the body, helping circulate blood and pushing food through the digestive system. They accomplish these by contracting. For example, skeletal muscles are composed of muscle fibers that run the length of the muscle. Within these fibers are smaller elements that are composed of parallel, partially overlapping **thick** and **thin filaments**. Muscle contraction occurs when these filaments slide past each other and mesh more closely. These filaments are made up of several proteins. Thin filaments are composed primarily of **actin**, a globular protein, while thick filaments are made up of **myosin**, which is a long, stringy protein with paddle-like protrusions at both ends. During muscle contraction, the myosin paddles (called **heads**) attach to the actin in the thin filaments and then swivel, which causes the thin filament to be pushed alongside the thick filament. The heads detach and attach again farther down the thin filament and pull the thin filament farther along (kind of like pulling a rope in towards you, hand over hand). This ultimately results in shortening of the muscle and movement of the body part to which the muscle is attached. See your text for a more detailed explanation of this process.

In this lab you will learn basic **extraction** procedures for separating the proteins actin and myosin from other molecules that make up muscle tissue, and you will determine some of their physical properties using a technique called **protein electrophoresis**.

During electrophoresis, the proteins are exposed to an electrical field. Because the proteins themselves are electrically charged, the electrical field causes them to move. Smaller proteins are able to move faster than larger ones. After allowing the apparatus to run for a certain amount of time, the protein samples will have spread themselves out based on their relative sizes. By running a set of standards (molecules of known molecular weight) along with our actin and myosin samples, we can determine the relationship between molecule size and distance moved, and use this to estimate the sizes of our two proteins.

What kind of substrate should we use? One problem we would encounter if we just had the proteins moving in a solution would be convection currents. Also if someone bumped the solution, the different substances might be mixed together and we wouldn't be able to distinguish which was which. To avoid

mixing of the proteins in solution, we will put them into a gelatin-like substance called **polyacrylimide gel**.

Another problem that we have is that proteins are often not visible. How will we know how far they have moved? We will add a small tracking dye called **bromophenol blue**. Since the bromophenol blue is small and negatively charged, it will move faster than proteins and its blue color allows us to know when to stop running the gel. It does not stain the proteins, however. After the gel has run we will have to stain the proteins using a different dye to see how much they have moved. This step takes a day or two, so your TA will do it for you, and we will see the final results and take our measurements at the beginning of next week's lab.

**Procedure**

*Muscle Tissue Preparation*

1) Obtain a chunk of partially frozen chicken breast (~5 g), 20 ml high salt buffer, and 20 ml low salt buffer.
2) Chop up the chicken breast with a single-edged razor on a cold glass plate. Mince until it looks like a chickenburger.
3) Scrape the chopped material into a cold mortar and grind with the pestle briefly to crush the tissue.
4) Split into to two equal portions, one for the myosin extraction and one for the actin extraction.

*Crude Extraction of Myosin*

1) To one portion (myosin extraction), add approximately 5 ml of the high salt buffer and grind with the pestle. Keep adding buffer a little at a time until the muscle is thoroughly crushed.
2) Filter through 4–6 layers of cheesecloth that has been moistened and wrung out with cold water. Make a little "cup" over a beaker with the wet cloth and pour in the extract. How can you make sure that you've separated the buffer (containing the dissolved protein) from the rest of the muscle matter? Discard the cheescloth in a marked waste container.
3) Centrifuge approximately 8 ml of this crude extract at the highest speed (10,000 rpm) for 3 minutes. You will have to split each extract up into about 4 microcentrifuge tubes. If there is a large **pellet** remaining in the tube after centrifugation, pour off the liquid portion (the **supernatant**) into another microcentrifuge tube and discard the tube with the pellet into a marked waste container. Centrifuge the supernatant portion again for 3 minutes to get a clearer supernatant. Keep the supernatants <u>on ice</u> as much as possible while you do this.
4) Pour off supernatants into a 25 ml glass flask and store on ice. Discard the microcentrifuge tubes and the pellets they contain in the marked waste container.

*Crude Extraction of Actin*

1) To the second portion of the ground muscle (the portion that you saved for the actin extraction), add approximately 5 ml of the low salt buffer and continue as in steps 1–4 above. Each of these steps must be done <u>separately</u> for the myosin and the actin preparations.

Can you tell a difference in consistency between the two extracts (especially during step 2)?

*Further Purification Steps for Myosin*

1) Remove 200 µl of the myosin crude extract from step 4 of the myosin extraction procedure (above) and save in a plastic microcentrifuge tube, on ice.
2) Estimate the volume of supernatant in the remaining myosin crude extract and dilute 1:6 with cold deionized water (e.g., if you have 5 ml of extractant, add 25 ml of water). Do this in a clear beaker so you can watch the myosin filaments form.
3) Let this sit on ice for 10–15 minutes, swirling periodically. Make notes in your lab notebook describing what you see.
4) Remove 10 ml of this diluted myosin into 6 microcentrifuge tubes and spin at 10,000 rpm for 3 minutes. The filaments will form pellets. Pour off the supernatant into a liquid waste beaker. Store the tubes containing pellets on ice.

*Preparation for Electrophoresis*

1) In a microcentrifuge tube, add 75 µl of protein solubilizing buffer (with blue tracking dye) to 200 µl of the actin crude preparation.
2) In a second microcentrifuge tube, add 75 µl of protein solubilizing buffer to 200 µl of the myosin crude preparation.
3) Add 75 µl of protein solubilizing buffer to the first of the 6 tubes with the myosin filament pellets, then remove the contents of the tube with a pipettor and transfer it to the second tube. Sequentially transfer each pellet to the next tube, ultimately combining them all in the last tube.
4) Place the tubes in a tube boiling rack and boil in a water bath for 1 minute.
5) Load lanes in your gel with prestained standards, actin preparation, crude myosin preparation and the purified myosin preparation. Your TA will tell you the exact amounts to load and the order in which they need to be loaded.
6) Your TA will connect the electrodes and run the gel.

The gel will take an hour or so to run, so you may not be able to see the complete process, but you will be able to see some migration of the tracking dye and the pre-stained standards (proteins of known size, each stained with a dye of a different color). Tomorrow, your TA will apply a stain to the actin and myosin (called **coomassie brilliant blue**) that will enable you to visualize the locations of your preparations.

The following steps will be carried out next week:

*General Observations*

1) How do the crude preparations compare to the purified myosin preparation? Do they share similar bands, or are specific bands present in one preparation and absent from others?
2) What is the significance of all the extra bands?

*Determining the Molecular Weights of Actin and Myosin*

1) With a ruler, measure the distance moved by each of the prestained standards. Convert the raw distances to **mobility** scores. These are calculated as the distance moved by the molecule of interest

divided by the distance moved by the dye front. This calculation standardizes the mobilities so that they range from 0 to 1, enabling you to compare your results with those of other groups.

2) Plot the molecular weight of the standard (y-axis) versus mobility (x-axis) on semi-log graph paper.
3) Label each of the major bands in each of your preparations and measure the distance moved.
4) Using the plot you obtained in step 2, estimate the molecular weights of actin and myosin.
5) Estimate the molecular weights of the two bright bands that lie below the myosin band on the purified myosin prep. What might these be?

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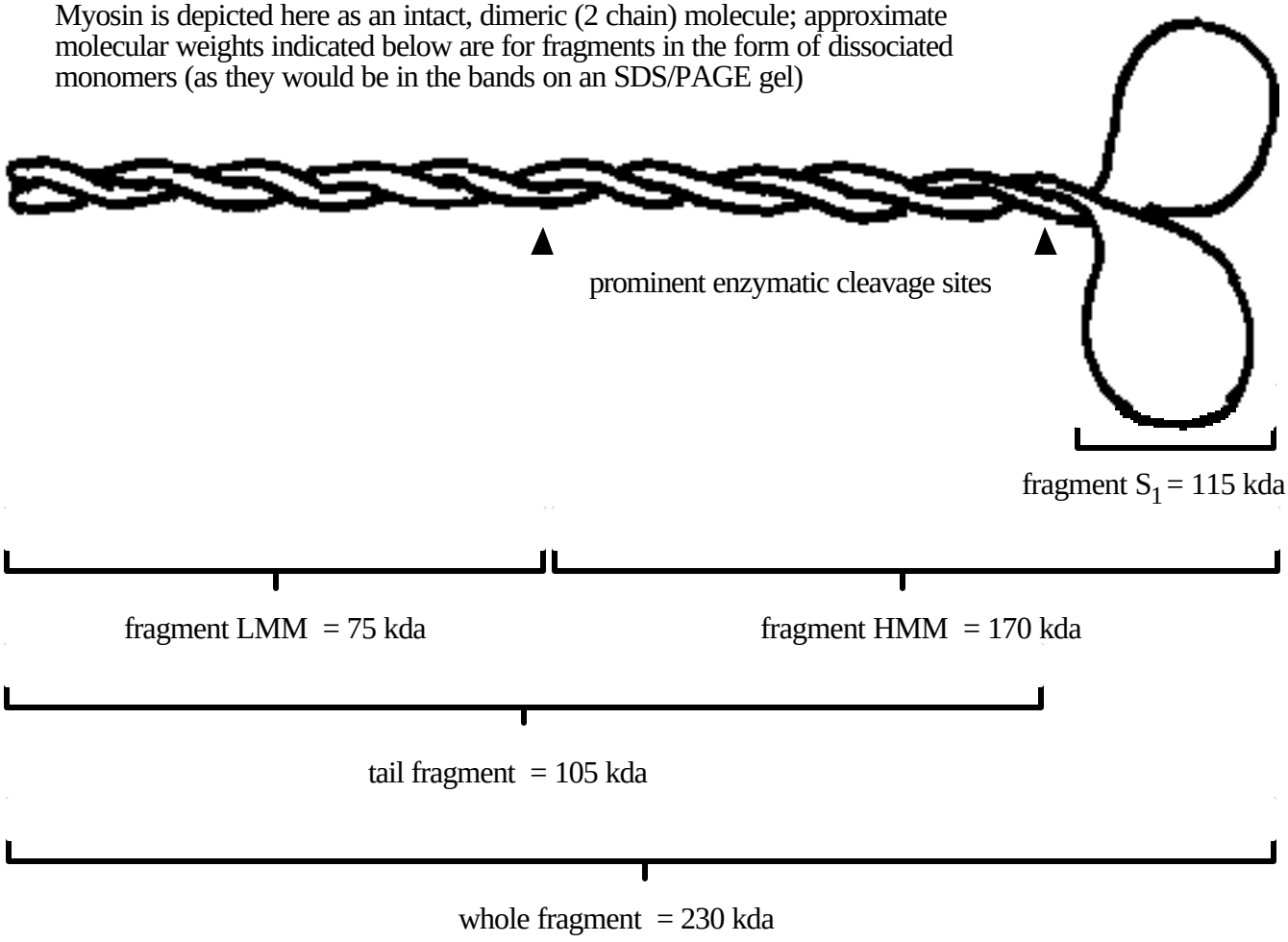
APPENDIX A

*Structure of Myosin and Actin*

**Sketches of Proteins in Skeletal Muscle**

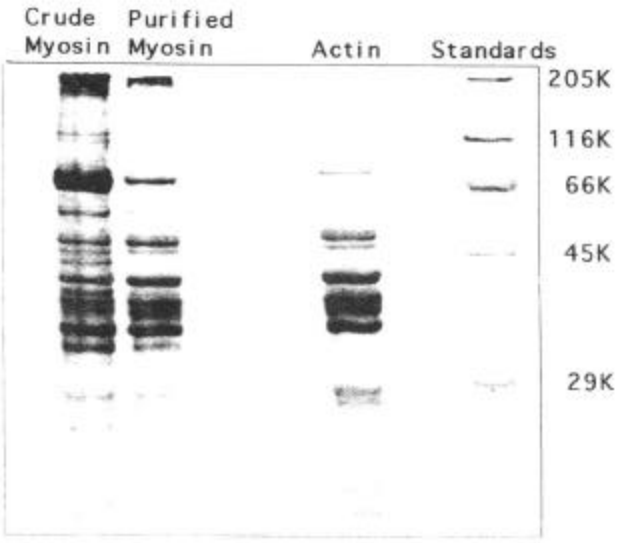
Myosin is depicted here as an intact, dimeric (2 chain) molecule; approximate molecular weights indicated below are for fragments in the form of dissociated monomers (as they would be in the bands on an SDS/PAGE gel)




Actin is depicted as polymerized f(filamentous)-actin, which is comprised of repeating, g(globular)-subunits, each with a molecular weight of about 46 kda. Both actin and myosin molecules are drawn at approximately the same scale.

APPENDIX. B

*Sample Protein Electrophoresis Gel*



## APPENDIX. C
*Addresses of Sources*

Pharmacia Biotech/Hoefer
800 Cenntenial Ave
P.O. Box 1327
Piscataway, NJ 08855-1327
1-800-526-3593
http://www.biotech.pharmacia.se

Sigma Chemical Company
P.O. Box 14508
St. Louis, MO 63178-9916
1-800-325-3010
http://www.sigma.sial.com

VWR Scientific Products
7211 Hanover Parkway Suite D
Greenbelt, MD 20770-9888
1-800-932-5000
http://www.vwrsp.com