Chapter 3

Purification of Maltose-Binding Protein from *E. coli* Periplasm

Judith R. Levin

Department of Biological Sciences
Goucher College
1021 Dulaney Valley Rd.
Baltimore, MD 21204
jlevin@goucher.edu

Judy Levin is an Assistant Professor of Biological Sciences and Chemistry at Goucher College. She received her A.B. in Biochemical Sciences from Harvard University, and her Ph.D. in Biochemistry from U. C. Berkeley. She did postdoctoral work at U. C. San Francisco and at Johns Hopkins University before joining the faculty at Goucher in 1992. Judy's teaching responsibilities include cell biology, molecular biology, and biochemistry lecture and lab courses. Her research focuses on protein-nucleic acid interactions during transcription by *E. coli* RNA polymerase.

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Introduction

This laboratory exercise, used in the sophomore cell biology lab at Goucher College, was designed to give students with little or no biochemistry background an understanding of the fundamental concepts involved in protein purification. In the first of two 3-hour lab periods, a one-step purification using affinity chromatography (Riggs, 1990) is used to isolate maltose-binding protein (MBP) from the periplasmic space of *Escherichia coli*. During the second 3-hour lab period, fractions from the purification are visualized by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The students observe that the overall amount of protein as well as the number of bands on the gel declines as the protein is purified. Using the method of Bradford (1976), they measure the protein content of various fractions to correlate protein concentration with the appearance of the samples on the gel, and to get a feel for the type of "bookkeeping" involved in protein purification. Also, they use molecular weight standards on the gel to estimate the subunit molecular weight of their purified protein. Students are able to see what it means to purify a protein without getting bogged down in the minutia of enzyme assays and calculations of specific activities and yields. Further, they are exposed to concepts and techniques of biochemical fractionation and the use of standard curves to analyze properties of an unknown sample.

Because my goal was to teach the concepts of protein purification with a minimum of student intimidation, I have intentionally made the procedure somewhat "cookbook" in nature. The idea is to make it as easy as possible for students to collect useful data, so that they can focus on understanding what their results *mean*. Many participants at the workshop felt that this was a valuable approach, while some felt it would be desirable to incorporate an aspect of scientific "inquiry", as opposed to simply reproducing an established purification scheme. Further discussion of this issue can be found in the "Notes for the Instructor" section.

I find that this exercise is reliable, easy to prepare and carry out, and relatively inexpensive as biochemistry labs go. While some of the reagents are expensive if obtained commercially, they can be prepared inexpensively if you are willing to invest the time and effort. Ideally, the exercise requires the use of a high-speed preparative centrifuge, but an alternate protocol using a table-top clinical centrifuge is provided.

Materials

Day One

E. coli cells: 10 ml/pair (see Appendix A for preparation)
0.5 M EDTA - 0.1 ml per pair
pipets, 10-ml - 2 per pair, plus appropriate pipet aids
5 mM $MgSO_4$, ice cold - 10 ml per pair
Laemmli protein sample buffer (2× concentration, to mix with equal volume of sample) (0.125 M TrisCl pH 6.8, 4% SDS, 20% glycerol, 2% [v/v] 2-mercaptoethanol, 0.01% [w/v] bromophenol blue) - 0.5 ml per pair
1M TrisCl, pH 7.4 - 2 ml per pair
Column buffer (20 mM TrisCl, pH 7.4, 0.2 M NaCl, 10 mM 2-mercaptoethanol [optional]) - 50 ml per pair
Amylose resin (New England Biolabs [NEB] #800-21S, 15 ml, $100 US) - 1 ml suspension per pair. Mix well in bottle supplied. Divide suspension evenly among tubes for 1 tube/pair. Beads can be resuspended in a little additional buffer if necessary to make them "go around" for all students. Beads can be regenerated (follow manufacturer's instructions), but it may be a nuisance to collect small amounts from many columns. Alternatively, resin can be prepared inexpensively according to the procedure of Kellerman and Ferenci (1982).
Elution buffer (column buffer + 10 mM maltose) - 3 ml per pair
Micropipettors (such as Gilson P20, P200, P1000 [optional]) - at least one set per 2 pairs of students, plus appropriate tips
Microcentrifuge tubes (1.5 ml size) - 9 per pair, plus rack
Sorvall RC-5B or comparable preparative centrifuge (standard protocol) or tabletop clinical centrifuge (alternate protocol), and tubes (2 per pair) suitable for 10-ml sample volume
Top-loading balances for balancing centrifuge tubes
Ice buckets (1 per pair) and ice
plastic conical screw-cap tubes, 15-ml (or other tubes suitable for sample storage) - 5 per pair
Column setups - 1 per pair (see Appendix A for preparation)
Beakers (for catching column outflow, and for weighing and carrying centrifuge tubes) - 2 per pair
Markers for labeling tubes
Pasteur pipets - several per pair, plus bulbs

Day Two

Student samples from day one
Boiling water bath and floats for holding 4 samples per pair plus markers and controls
E. coli cell sample for gels (see Appendix A for preparation)
MBP positive control - from instructor's trial run or previous year's class samples. (*Note:* the wild-type MBP protein provided in the NEB kit is not the same size as the fusion protein purified in this exercise.)
Protein molecular weight markers (Bio-Rad "low range", catalog #161-0305, enough for 100 gels, $70 US; or any comparable product encompassing the 40–50 kilodalton range)
Protein mini-gel apparatus and power supply (such as Bio-Rad MiniProtean II; catalog 165-2940, $475 US with power supply; catalog 165-2991, $785 US without power supply)
SDS-polyacrylamide gels and running buffer, staining, and destaining solutions - see Appendix A
Gel loading tips (these are longer and narrower at the tip than standard tips, and are available from most suppliers of standard tips)
Micropipettors (such as Gilson P20, P200, P1000 [optional]) - at least one set per 2 pairs of students, plus appropriate tips
Spectrophotometers (such as Sequoia-Turner Model 340 or Fisher "SpectroMaster" Model 415) - at least one per 2 pairs of students
Disposable glass culture tubes, 13×100 mm - 10 per pair for use in Bradford assay; these also serve as cuvettes for the above-mentioned spectrophotometers
Protein Assay Dye Reagent Concentrate (Bio-Rad catalog # 500-0006, $65 US for 450 ml) - 10 ml per pair (*Note:* in lieu of purchasing the commercial product, the reagent may be prepared according to the method of Bradford [1976], but keep in mind that the protocol used in this exercise uses the reagent at 5× concentration relative to the standard assay described by Bradford; the Bio-Rad "concentrated" product is used *undiluted*.)
Bovine serum albumin (BSA) (0.1 mg/ml) - 2 ml per pair, for use as protein standard in Bradford assays; make up in water or 10 mM TrisCl, pH 7.4
Vortexers - at least one per 2 pairs of students
Rulers for measuring mobilities of bands on gels

Notes for the Instructor

General instructional comments:

This exercise is based on the Protein Fusion and Purification System utilizing maltose-binding protein marketed by New England Biolabs (Kit Catalog #800, $400 US; individual components also available separately). The protein purified is actually a plasmid-encoded fusion of MBP (product of the *E. coli malE* gene) and the α-fragment of β-galactosidase (product of the *E. coli lacZα* gene), and so has a higher molecular weight (approximately 51 kilodaltons) than that of wild-type MBP (approximately 43 kilodaltons). I do not mention this to the students, nor the fact that the protein is encoded on a plasmid rather than the chromosome, as I feel that this information would unnecessarily complicate their thinking about the experiment.

Two versions of the plasmid-encoded fusion are available: pMAL-p2, which includes the signal peptide region of *malE*, causing the protein to be secreted to the periplasmic space, and pMAL-c2, which lacks the

signal peptide and therefore produces a cytoplasmic protein. I have employed pMAL-p2 for two reasons: 1) purifying a periplasmic protein teaches the students the advantage of having the cell essentially do some of the purification work for you; 2) preparation of the whole-cell extract involves a sonication step not required for preparation of the periplasmic fraction, which is obtained by osmotic shock. At the ABLE workshop, it was suggested that some instructors might want to use the cytoplasmic protein for some students, and challenge the students to determine whether their particular sample is cytoplasmic or periplasmic. It should not be overly difficult to adjust the protocol to include this modification. However, introducing this level of scientific inquiry will undoubtedly sacrifice some of the simplicity that makes this exercise so valuable for beginning students.

Another way to decrease the "cookbook" nature of this exercise would be to omit the detailed protocol provided for the Bradford assay, leaving it to the students to design their own experiment. If your students are already familiar with the Bradford assay and are comfortable with the concept of a standard curve, you may well want to try this. I have found that first semester sophomores at Goucher are still struggling with this concept, and in the interest of ensuring that they collect useful data with a minimum of hassle so that they can focus on interpreting the data, I have opted to spell out the protocol for them in this exercise.

While for this exercise you need only to obtain plasmid pMAL-p2 (or pMAL-c2) and the amylose resin, other components of the New England Biolabs kit, such as the anti-MBP antiserum and sequencing/PCR primers, may be useful for expanding this exercise for other courses involving more advanced students.

For detailed discussion of the maltose-binding protein fusion and purification system refer to the original journal article by Maina *et al*. (1988) and a review by Riggs (1995). Preparation of an *E. coli* periplasmic fraction by osmotic shock was first described by Neu and Heppel (1965).

Notes on Centrifugation

In our course at Goucher, we typically perform the centrifugations in Part I of this exercise in a Sorvall refrigerated high-speed centrifuge (such as model RC-5B) capable of attaining much higher g-forces (ca. 10,000× g) than the clinical centrifuges that were available for this workshop. At the workshop we found that the presence of unpelleted cells in the periplasmic fraction clogged the amylose column and caused it to run excruciatingly slowly. If you do not have access to a high-speed centrifuge, you may wish to substitute the provided alternate procedure for binding MBP to the amylose resin in "batch" style rather than on a column. This alternate procedure has the students transfer the resin to a column for the elution step, providing the dual advantages of exposing the students to the column procedure, and ensuring that the final eluted sample is not contaminated with resin.

Even with periplasmic fractions prepared by high-speed centrifugation, the flowrate of the column typically slows down during the course of sample loading. If students become frustrated, they can gently resuspend the resin with a Pasteur pipet; this will usually redistribute any material clogging the top surface of the column, and allow flow to resume. This approach can also be used during the wash (step B3, standard procedure), but is not recommended during the elution step (step B4). If you find that flowrate is consistently a problem, try substituting Part II of the alternate procedure for Part II of the standard procedure.

at low speed. It is possible that this problem can be avoided by using freshly grown cells rather than ones that have been stored frozen. In carrying out the alternate procedure, I have noticed that there is usually a layer of loosely packed material above the cell pellet at the first centrifugation step. Removing this layer seems to give cleaner results in subsequent steps. My guess is that this layer contains cells that have

been damaged in the freeze-thaw process (cells are prepared in advance and stored at –20° C) and do not pellet well

Note that centrifugation speeds are given in g-force here. You will need to determine the speed in rpm that is appropriate for your particular rotor. If using a clinical centrifuge, use the highest possible setting, which is usually not more than about 1500× g.

Student Outline

Day One

Introduction

In order to elucidate the role of a particular protein in cellular processes, it is often desirable to isolate the protein and study its physical and biochemical properties in vitro. Protein purification is a skill that encompasses the many techniques involved in extracting molecules from living cells and separating a particular protein of interest from the multitude of other molecules (protein and nonprotein) in the cell.

During the next two lab periods we will be purifying a protein called maltose-binding protein (MBP) from the bacterium *Escherichia coli*. MBP resides in the periplasmic space of the bacterial cell (the space between the plasma membrane and the outer membrane) and is involved in transport of the disaccharide maltose into the cell. The maltose-carrying site of this protein recognizes the α-1,4 glycosidic linkage of maltose but is rather nonspecific about the size of molecule it can bind to. While its highest affinity is for maltose, it is capable of binding to α-1,4 linked polysaccharides such as glycogen, amylose and amylopectin. We will take advantage of this property in our purification scheme.

Our goal of purifying this protein is abetted by its periplasmic location: there are many fewer types of molecules in the periplasm than there are in an entire bacterial cell. Therefore, if we can somehow separate the periplasmic space from the rest of the cell, we will have already achieved a substantial purification of MBP. We will do this by putting the bacteria in hypotonic buffer; the resulting osmotic shock causes the outer membrane to burst while the plasma membrane remains intact because it is protected in these bacteria by the presence of a peptidoglycan cell wall. The contents of the periplasmic space (mostly proteins) are therefore released into the medium and can be separated from the remaining shocked cells by centrifugation.

The remaining task is to separate MBP from the other proteins in the periplasmic fraction. To do this, we will use a technique known as *affinity chromatography*. In this technique, the MBP-containing sample is mixed with beads that are coated with the polysaccharide amylose. MBP can bind to amylose, but few, if any, of the other molecules in the periplasmic sample can. Therefore, when the beads are subsequently washed with buffer, MBP remains attached to the beads while virtually everything else is flushed away. In order to retrieve the MBP from the beads, we will immerse them in a buffer containing maltose. The MBP will release from the amylose coating on the beads, bind to the maltose, and diffuse into the buffer surrounding the beads. MBP is said to have *eluted* from the beads. The resulting *eluate* should contain only buffer, maltose and MBP. When a particular protein is the only macromolecule present in a mixture, it is said to have been purified to *homogeneity*.

How will we assess the success of our purification? If we were purifying a protein with enzymatic activity, we could use an assay to measure the amount of enzyme activity at each stage of purification, and compare this to the total amount of protein present. The ratio of enzyme activity to total protein, or *specific*

activity (units/mg), increases as the protein is purified away from other proteins that do not have the enzymatic activity being measured.

Since MBP has no enzymatic activity that we can measure, we need to resort to another strategy to assess its purification. We will use a technique known as SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to visualize the variety of proteins present in our samples at each stage of the purification. In this technique, proteins are sorted according to their molecular weight and appear as a series of "bands" after staining with a protein-binding dye. When the mixture of proteins in an entire bacterial cell is analyzed by SDS-PAGE, thousands of bands, each corresponding to a different protein, are seen. In contrast, our periplasmic sample will reveal a substantially decreased number of bands (one of which corresponds to MBP), and the eluate from the amylose column will likely reveal only one prominent band (presumably corresponding to MBP). This will provide a dramatic, though qualitative, visualization of the success of our purification.

To help quantify these results, we will save a sample from each stage of the purification and measure the total amount of protein. Even though we can't measure a specific activity because there is no enzymatic activity, we still expect the amount of total protein in our sample to decline as we remove all the cellular proteins that are not of interest, leaving only MBP behind. Our measurements will help us determine what percentage of the total cellular protein was eliminated from the MBP sample by the osmotic shock procedure, what percentage of the periplasmic proteins were removed by the affinity chromatography procedure, and finally how much MBP (assuming homogeneity was achieved) remained at the end of the purification.

This week in lab we will perform the osmotic shock and affinity chromatography techniques to purify MBP. We will save samples from each stage of our purification to use next week for SDS-PAGE analysis and measurement of protein.

Prelab Assignment

Today's experiment consists of many steps and you will be saving samples at many points for later analysis. To make sure you have a clear picture of the procedure, draw yourself a flowchart showing the progression from starting material (*E. coli* cells) to final sample (column eluate), indicating the approximate total volume of sample expected at each stage, and indicating the amounts saved for later analysis. Make sure to identify where samples "A", "B", "C", and "D" come from.

Standard Procedure

I. Osmotic Shock to Isolate Periplasmic Fraction

1. To 10 ml of *E. coli* cells in 30 mM TrisCl pH 8.0 + 20% sucrose, add 20 µl 0.5 M EDTA. (EDTA, or ethylenediaminetetraacetic acid, binds to divalent cations, such as magnesium, that are required for stability of the bacterial outer membrane.) Cover the tube and shake gently for 5 minutes. Pour the contents into a centrifuge tube and balance with another group.
2. Centrifuge the cells for 10 minutes at 10,000×g. Pour off the supernatant fluid, being careful not to disturb the cell pellet.
3. Resuspend the cell pellet with vigorous pipetting in 10 ml of ice-cold 5 mM $MgSO_4$. Keep the tube on ice for 10 minutes, swirling often.

Remove 150 µl from the tube into a microfuge ("Eppendorf") tube labeled with your initials and the letters "TP" (for "total protein"). This sample will be used next week for measurement of protein. Keep this, and all other samples in the italicized sections below, on ice.

4. Centrifuge the cells again for 10 minutes at 10,000× g. (Note: During this spin, you can begin preparing your column, as described in section II.A below.)
5. Pour the supernatant fluid to a fresh tube (*do not discard!*) labeled "periplasm". Resuspend the cell pellet in 10 ml of column buffer and label the tube "shocked cells".
   Put 20 µl of shocked cells plus 20 µl of sample buffer into a microfuge tube labeled with your initials and the letter "A". Put 150 µl of shocked cells into a microfuge tube labeled with your initials and the letters "SC".
   Put 20 µl of periplasm plus 20 µl of sample buffer into a microfuge tube labeled with your initials and the letter "B". Put 150 µl of periplasm into a microfuge tube labeled with your initials and the letter "P".
   These samples will be used next week for SDS-PAGE and measurement of protein.
6. Add 0.2 ml 1 M TrisCl pH 7.4 to the "periplasm" tube (the 10-ml sample, *not* the microfuge tube).

II. Purification of MBP by Affinity Chromatography

A. Pouring the column
   1. Mount the column and close off the outlet at the bottom.
   2. Add a Pasteur pipet full of column buffer. Open the column outlet briefly so that the glass wool is wetted and the outlet tubing fills with buffer. Reclose the column outlet.
   3. Add 1 ml of amylose resin suspension. As the beads begin to settle at the bottom of the column, open the outlet at the bottom of the column to let the buffer flow through. DO NOT LET THE BUFFER LEVEL GET DOWN TO THE TOP SURFACE OF THE PACKED BEADS. Keep adding buffer to the top of the column until about 10 ml of buffer have flowed through. Then close off the bottom outlet until you are ready to load your sample.

B. Loading, washing and eluting sample
   1. Open the bottom outlet and allow the buffer to drain through until the level is just barely above the top of the packed column resin. Close the outlet.
   2. Using a Pasteur pipet, load your "periplasm" sample onto the top of the column. Open the outlet enough to allow about one drop per second to flow from the column, and collect the flowthrough into a tube labeled "flowthrough". Keep adding sample to the top of the column, being careful not to let the column run dry, until all the sample has been loaded and collected into this same tube. When the sample fluid level gets down to just barely above the top of the packed resin, close the outlet.
      Put 20 µl flowthrough plus 20 µl sample buffer into a microfuge tube labeled with your initials and the letter "C". Put 150 µl of flowthrough into a microfuge tube labeled with your initials and the letters "FT". These samples will be used next week for SDS-PAGE and protein measurement.
   3. Add some column buffer to the top of the column, and open the outlet, collecting into a fresh tube labeled "wash". Keep adding column buffer to the column until at least 10 ml has washed through the column. Allow the buffer level to come down to just above the column resin, and close the outlet.
   4. Add 2–3 ml of elution buffer (column buffer + 10 mM maltose) to the column. Squeeze the liquid out of the outlet tubing before opening the outlet (this is important so that your eluted sample is not diluted by buffer that was already in the tubing). Collect 1.5 ml into a graduated microfuge tube labeled "eluate".
      Put 20 µl eluate plus 20 µl sample buffer into a microfuge tube labeled with your initials and with the letter "D". ***Save all nine microfuge tube samples (including the final "eluate") to be frozen for use in next week's experiment.***

Alternate Procedure

I. Osmotic Shock to Isolate Periplasmic Fraction

1. To 10 ml of *E. coli* cells in 30 mM TrisCl pH 8.0 + 20% sucrose, add 20 µl 0.5 M EDTA. (EDTA, or ethylenediaminetetraacetic acid, binds to divalent cations, such as magnesium, that are required for stability of the bacterial outer membrane.) Cover the tube and shake gently for 5 minutes. Pour the contents into a centrifuge tube and balance with another group.
2. Centrifuge the cells for 10 minutes at high speed in a table-top clinical centrifuge. Using a Pasteur pipet, remove the supernatant fluid, being careful not to disturb the cell pellet. (Note: There may be a layer of loosely packed, damaged cells above the more tightly packed intact cell pellet. Remove this layer along with the supernatant, and discard.)
3. Resuspend the cell pellet with vigorous pipetting in 10 ml of ice-cold 5 mM $MgSO_4$. Keep the tube on ice for 10 minutes, swirling often.
   Remove 150 µl from the tube into a microfuge ("Eppendorf") tube labeled with your initials and the letters "TP" (for "total protein"). This sample will be used next week for protein measurement. Keep this, and all other samples in the italicized sections below, on ice.
4. Centrifuge the cells again for 10 minutes at high speed.
5. Using a Pasteur pipet, remove the supernatant fluid to a fresh tube (*do not discard!*) labeled "periplasm". Resuspend the cell pellet in 10 ml of column buffer and label the tube "shocked cells".
   Put 20 µl of shocked cells plus 20 µl of sample buffer into a microfuge tube labeled with your initials and the letter "A". Put 150 µl of shocked cells into a microfuge tube labeled with your initials and the letters "SC".
   Put 20 µl of periplasm plus 20 µl of sample buffer into a microfuge tube labeled with your initials and the letter "B". Put 150 µl of periplasm into a microfuge tube labeled with your initials and the letter "P".
   These samples will be used next week for SDS-PAGE and measurement of protein.
6. Add 0.2 ml 1 M TrisCl pH 7.4 to the "periplasm" tube (the 10-ml sample, *not* the microfuge tube).

II. Purification of MBP by Affinity Chromatography

A. "Batch" binding of MBP to amylose resin
   1. Add 1 ml of amylose resin suspension to the periplasmic fraction (you will now have approximately 11 ml). Keep on ice for 10 minutes, inverting frequently to keep resin suspended.
   2. Allow the resin to settle (this may be aided by a low-speed centrifugation), and carefully transfer the supernatant liquid to a fresh tube labeled "flowthrough".
      Put 20 µl flowthrough plus 20 µl sample buffer into a microfuge tube labeled with your initials and the letter "C". Put 150 µl of flowthrough into a microfuge tube labeled with your initials and the letters "FT". These samples will be used next week for SDS-PAGE and measurement of protein.
   3. Resuspend resin in 5 ml column buffer. Keep on ice for 5 minutes, inverting frequently.
   4. Allow the resin to settle, and carefully remove the supernatant liquid.
   5. Resuspend resin in another 5 ml of column buffer.

B. Eluting MBP from the amylose resin
   1. Mount the column and close off the outlet at the bottom.
   2. Add a Pasteur pipet full of column buffer. Open the column outlet briefly so that the glass wool is wetted and the outlet tubing fills with buffer. Reclose the column outlet.

3. Add the resuspended resin. As the beads begin to settle at the bottom of the column, open the outlet at the bottom of the column to let the buffer flow through. Close off the column outlet when the buffer level is even with the surface of the packed beads.
4. Add 2–3 ml of elution buffer (column buffer + 10 mM maltose) to the column. Squeeze the liquid out of the outlet tubing before opening the outlet (this is important so that your eluted sample is not diluted by buffer that was already in the tubing). Collect 1.5 ml into a graduated microfuge tube labeled "eluate".
   Put 20 µl eluate plus 20 µl sample buffer into a microfuge tube labeled with your initials and with the letter "D". ***Save all nine microfuge tube samples (including the final "eluate") to be frozen for use in next week's experiment.***

Day Two

Introduction

In today's lab we will complete the analysis of our purification of MBP by performing a qualitative assessment of protein content using SDS-PAGE (SDS-polyacrylamide gel electrophoresis) and by quantifying the amount of protein using the Bradford assay.

Electrophoresis is the movement of charged molecules under the influence of an electric field. In the most common form of electrophoresis, the sample is applied to a crosslinked matrix (called a "gel") of agarose or polyacrylamide that is soaked in buffer. When an electric current is applied to the buffer, charged molecules begin to migrate in the buffer, through the pores in the gel matrix, at a rate proportional to their charge/mass ratio. Small molecules move through the gel unimpeded, but larger molecules tend to collide with the gel matrix, and are thus slowed down. This "molecular sieving" property of gels causes molecules to be sorted according to size and shape. If all of the molecules in a mixture have the same charge/mass ratio and the same shape, then gel electrophoresis will cause them to be sorted solely according to their size, or molecular weight.

While native proteins come in an assortment of charge/mass ratios and shapes, this variation can be eliminated by denaturing the proteins with the detergent sodium dodecyl sulfate (SDS). SDS is an anionic detergent with the following chemical structure:

$CH_3–CH_2–CH_2–CH_2–CH_2–CH_2–CH_2–CH_2–CH_2–CH_2–CH_2–CH_2–O–SO_3–Na^+$

SDS binds noncovalently to all proteins, saturating their surfaces to produce protein-SDS complexes with about one SDS molecule for every two to three amino acid residues. In doing this it coats the surface of each protein with many negatively charged sulfate groups, swamping out the charge on the native protein. The charge repulsion caused by SDS causes denaturation of the protein. As a result of the detergent binding and unfolding of the protein, many different SDS-protein complexes can be approximated as rigid rods of uniform charge density, with lengths proportional to the number of amino acid residues in the polypeptide chain. They will therefore experience a uniform force in an electric field in a polyacrylamide gel. Since different size protein-SDS complexes will be differentially retarded by the pores of the gel matrix, the separation will be based solely on the size of the protein. The molecular weight of an unknown protein can be determined by comparing its mobility in an SDS-PAGE experiment to that of a set of protein standards of known molecular weight under identical experimental conditions.

In practice, the protein sample is mixed with a buffer containing SDS, β-mercaptoethanol (to reduce disulfide bonds, allowing complete denaturation) and a small molecule *tracking dye*, such as bromophenol blue, which migrates faster than all of the proteins and allows us to monitor the progress of the sample through the gel. After heating the sample in boiling water to promote denaturation, the sample is carefully

layered into a rectangular shaped indentation (called a sample well) at the top of the gel, and the electric current is turned on. The negatively charged SDS-protein complexes (and the tracking dye) move away from the negative electrode at the top of the gel toward the positive electrode at the bottom of the gel. The current is turned off when the tracking dye approaches the bottom edge of the gel. Following electophoresis, the protein bands in the gel are typically visualized through the use of a general protein stain called Coomassie Blue (the same dye used in the Bradford assay, described below), and the *relative mobility* (R_f) of the band(s) of interest is determined. This refers to the mobility of the protein band relative to that of the tracking dye:

$$R_f = \frac{\text{distance migrated by protein}}{\text{distance migrated by dye}}$$

For a given gel composition, there will be a size range of proteins for which the R_f is inversely proportional to the log of the molecular weight (mw). Protein standards with known molecular weights in that range are chosen, and a standard curve is generated by plotting R_f *vs*. log mw. The molecular weights of the unknown bands are then determined from the standard curve.

Procedure

I. SDS-Polyacrylamide Gel Electrophoresis

1. Heat your gel samples (A through D, blue-colored) for 5 minutes in a boiling water bath.
2. When everyone is ready, carefully layer 20 µl of each of your four samples into a sample well on the assembled polyacrylamide gel apparatus. Also loaded onto the gel will be one sample of protein molecular weight standards and one sample of the whole *E. coli* cells used in the purification.
3. Once the electrophoresis has started, it will take approximately an hour for the tracking dye to migrate down the gel. While this is occurring, perform the protein assays on the remaining samples saved from the purification (part II below).
4. When electrophoresis is complete, the gel apparatus will be disassembled. Measure the distances migrated by the tracking dye and the protein molecular weight standards. Use these measurements to calculate the R_f of each of the proteins. Construct a standard curve plotting R_f *vs*. log mw for the protein standards (the mw's of the standards will be provided in class).
5. The gel will be placed in staining solution. After staining, the gel will need to soak in destaining solution overnight to wash away dye from everywhere on the gel that did not contain protein. Only after this destaining step will the protein bands in our experimental samples be visible. Sometime during the week following your lab period, come into lab to view your gel. Write down a rough estimate of the number of bands in each of your samples. What does this tell you (qualitatively) about each stage of the purification? Measure the distance migrated by your purified MBP band (sample D). Use this measurement, along with the distance migrated by the tracking dye, to calculate the R_f of MBP. Using the standard curve constructed in step 4, calculate the molecular weight of MBP. If there is more than one band in your sample D, calculate the mw of the two most prominent bands. Can you think of any explanation for this result? If no bands are visible in your sample D, note this in your notebook and use someone else's lane to measure an R_f and calculate a mw. Can you think of an explanation for this result?

II. Measurement of Protein by the Bradford Assay

How can we quantify the amount of total protein in our samples? Most proteins do not have a visible color, so direct colorimetry is not useful. To measure the amount of protein, we can mix the protein with a compound that turns a color when it reacts with protein. There are a few known colorimetric reactions that allow measurement of protein. One such assay (the Bradford reaction) uses a dye, Coomassie Blue, that turns from red to blue upon binding proteins. If a protein solution of known concentration is used in this assay, the relationship between protein concentration and blue color can be determined. Spectrophotometric principles (i.e., Beer's Law) can then be applied to determine the protein concentration in an unknown sample.

We will use a total assay volume of 3 ml. The dye reagent solution is 5 times as concentrated as it should be in the final assay mixture, and is therefore referred to as a "5×" stock. To plan your experiment, complete Table 3.1 below showing what volumes of reagents to add to each tube. Note that the amount of 5× dye reagent added to each tube will be constant. (A dash in the table below indicates none of that reagent is added to that tube). Tables like this are commonly used when many tubes are to be set up similarly in an experiment.

Table 3.1. Protocol for Bradford Assay.

Tube:	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
BSA (0.1 mg/ml)	–	0.1 ml	0.3 ml	0.5 ml	0.7 ml	–	–	–	–	–
total protein ("TP")	–	–	–	–	–	0.1 ml	–	–	–	–
shocked cells ("SC")	–	–	–	–	–	–	0.1 ml	–	–	–
periplasm ("P")	–	–	–	–	–	–	–	0.1 ml	–	–
flowthrough ("FT")	–	–	–	–	–	–	–	–	0.1 ml	–
eluate	–	–	–	–	–	–	–	–	–	0.5 ml
H_2O										
5× dye reagent										
Total volume	3.0 ml	3.0 ml	3.0 ml	3.0 ml	3.0 ml	3.0 ml	3.0 ml	3.0 ml	3.0 ml	3.0 ml

1. Using the completed protocol in Table 3.1, set up your protein assay. Vortex the samples and allow to sit for 5 minutes.
2. Blank the spectrophotometer at 595 nm using tube 1, then read the absorbances of the remaining nine samples.
3. Construct a standard curve using the data from the BSA samples (tubes 1–5; tube 1 serves as the 0 mg standard, with an absorbance value of 0). Use units of milligrams (mg) for the *x*-axis. Use this curve to calculate the *amount* of protein in each of the unknown assay tubes. Taking into account the volume of

each sample placed in the Bradford assay tube, calculate the *concentration* of protein in each unknown sample (tubes 6–10).

4. Using the known *total* volume of each sample (not just the volume saved for analysis), calculate the *total* amount of protein in the samples at each stage of the purification.
   a) What percentage of the protein found in intact cells was found in the periplasm?
   b) What percentage of protein in the periplasm flowed through the column? Is this consistent with the appearance of the samples on the gel?
   c) How much protein is in your final eluted sample? What percentage of total cellular protein does this represent? What percentage of the periplasmic protein does it represent?
   d) Using the mw for MBP calculated in part I, and assuming all of the protein in your final sample is MBP, calculate the number of moles of MBP in your final sample. How many molecules of MBP is this? Assuming you started with 10^{10} *E. coli* cells, how many molecules of MBP are there in a typical *E. coli* cell?

Further Reading for Students

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72:248–254. *(Protein measurement using Coomassie Blue.)*

Deutscher, M. P., ed. 1990. Guide to Protein Purification. Vol. 182, Methods in Enzymology. Academic Press, San Diego, 894 pages. *(Protein purification.)*

Duplay, P., Bedouelle, H., Fowler, A. V., Zabin, I., Saurin, W., and M. Hofnung. 1984. Journal of Biological Chemistry, 259:10606–10613. *(Background on MBP.)*

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227:680–685. *(SDS-polyacrylamide gel electrophoresis.)*

Neu, H. C., and L. A. Heppel. 1965. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. Journal of Biological Chemistry, 240:3685–3692. *(Isolation of a periplasmic fraction.)*

Literature Cited

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72:248–254.

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Kellerman, O. K. and T. Ferenci. 1982. Maltose-binding protein from *E. coli*. Methods in Enzymology, 90:459–463.

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Neu, H. C., and L. A. Heppel. 1965. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. Journal of Biological Chemistry, 240:3685–3692.

Riggs, P. D. 1990. Expression and purification of maltose-binding protein fusions. Unit 16.6, Supplement 19, *in* Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and K. Struhl, eds.), Wiley and Sons, New York.

Sambrook, J., Fritsch, E. F., and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Second Edition. Cold Spring Harbor Press, New York, 1659 pages.

APPENDIX A
Lab Preparation

Commercial Suppliers

The specialized equipment and reagents used in this exercise can be obtained from the following two suppliers:

New England Biolabs	Bio-Rad Laboratories
32 Tozer Road	2000 Alfred Nobel Drive
Beverly, MA 01915-5599	Hercules, CA 94547
1-800-632-5227	1-800-424-6723

Preparation of Cells

Note: This preparation requires several days, and should preferably be done at least a week in advance.

Bacterial strain and plasmid:

New England Biolabs suggests strain TB1 (NEB catalog #801-A, available free with any order), but any transformable strain of *E. coli* can be substituted. Transform with plasmid pMAL-p2 (NEB #800-65S, $80 US for 5 µg) by standard procedure (Sambrook *et al.*, 1989). Plasmid-bearing strains can be stored long-term at –70° C in the presence of glycerol or DMSO (Sambrook *et al.*, 1989).

Materials:

(The amounts specified here are based on a lab with 16 pairs of students.)
40% (w/v) glucose (autoclaved or filter sterilized) - 15 ml
30 mg/ml ampicillin - 10 ml
LB medium - 1.5 liters. Per liter: 10 g tryptone, 5 g yeast extract, 10 g NaCl, 0.5 ml 4 M NaOH. Autoclave 20 minutes at 121° C. Can store at room temperature. Add glucose (from 40% stock) to final concentration of 0.2% (w/v) and ampicillin (from 30 mg/ml stock) to final concentration of 100 µg/ml just before cells.
0.1 M IPTG (isopropyl β-D-thiogalactoside; for induction of plasmid-encoded MBP-lacZα fusion gene; available from many commercial sources) - 1.5 ml
1M TrisCl, pH 8.0 - 100 ml stock
30 mM TrisCl, pH 8.0/20% (w/v) sucrose - 200 ml for cell resuspension (or minimally 10 ml per pair)
Autoclave (or filter sterilization apparatus)
Erlenmyer or Fernbach flasks for growth of 1.5 liters of *E. coli* liquid culture
37° C shaker for bacterial growth
Preparative centrifuge and appropriate tubes or bottles for harvesting 1.5 liters of bacterial culture

Cell growth:

a. Grow 20 ml overnight culture of *E. coli* cells containing pMAL-p2 in LB containing 0.2% glucose and 100 µg/ml ampicillin.
b. Inoculate 3 × 500 ml cultures (prewarmed LB/glucose/amp) each with 5 ml of cells. Grow at 37° C with vigorous shaking to an OD_{600} of 0.4–0.6.
c. Add 0.5 ml 0.1 M IPTG to each culture.
d. Continue growth 2 hours.
e. Harvest cells by centrifugation for 20 minutes at 4,000µg at 4° C.
f. Resuspend cells in a total of 175 ml 30 mM TrisCl/20% sucrose.
g. Take out 100 µl and mix with 100 µl Laemmli sample buffer and save as "whole cells" sample for gels.
h. Aliquot remainder in 10-ml portions for students. Store at –20° C.

Column Setups

For each pair of students, set up a column using a 3-cc plastic syringe as follows:

a. Remove the plunger from the syringe and insert a small wad of glass wool into the bottom of the syringe. Use enough glass wool to prevent amylose resin from leaking through, but don't compress it so tightly that the flow of liquid is impeded. A Pasteur pipet is useful in pushing the glass wool to the bottom of the syringe.
b. Attach a short (about 2 inches) piece of latex tubing to the syringe outlet (no needle is necessary), and close it off with a pinch clamp.
c. Clamp the "column" to a ring support so that it is held firmly in a vertical position.
d. If students have trouble getting buffer to flow through column initially, have them push some buffer through with the syringe plunger. (*Note:* Once the amylose resin has been poured into the column, do *not* use the syringe plunger, as it will suck the resin up with it when it is pulled back out! If flow slows once the column is poured, it can sometimes be improved by stirring up the resin with a Pasteur pipet. See instructor's notes.)
e. Be sure to have students squeeze buffer out of outlet tube prior to collecting eluate, as instructed in the student outline. Otherwise the eluted protein will be substantially diluted, and may not be detectable in the protein assay or on the gel.

SDS-Polyacrylamide Gel Electrophoresis

Note: This technique has been presented in detail in a previous volume of this series (Karcher *et al.*, 1996). Materials are listed here for convenience; for specifics on pouring, running, and staining of gels refer to the above reference or the BioRad MiniProtean II instruction manual.

Materials:

30% (w/v) acrylamide (BioRad 161-0158) - 100 ml of premixed solution containing a 37.5:1 ratio of acrylamide:bisacrylamide. This is a carcinogen and neurotoxin, so handle with care. Mix gels according to package directions.
0.5 M TrisHCl pH 6.8 - 50 ml
1.5 M TrisHCl pH 8.8 - 50 ml

SDS (sodium dodecyl sulfate) - 10 g - Do not refrigerate solutions as they will precipitate.

TEMED (N,N,N', N'-tetramethylethyldiamine) - 1 ml

10% (w/v) ammonium persulfate - must be made fresh - 1 ml

10% acrylamide running gels - can be prepared the day before lab according to manufacturer instructions. The MiniProtean II apparatus comes with combs that have 15 wells. This is enough for 3 pairs of students (4 samples each) plus markers, whole cell control, and MBP control. Plan accordingly for the number of students in your lab. After running gels have polymerized (at least 1 h), overlay with 0.375 M TrisHCl, pH 8.8. Seal entire assembly in a plastic bag for overnight storage, if necessary

4% acrylamide stacking gel - 1 per gel - have solution ready according to package instructions to demonstrate gel pouring to students immediately when lab begins. Insert 15 well combs.

Running buffer (5× stock) - 500 ml - (per liter: 15 g Tris base, 72 g glycine, 5 g SDS)

Coomassie blue protein stain (0.1% [w/v] Coomassie Brilliant Blue R in 50% [v/v] methanol/10% [v/v] acetic acid) - 500 ml (can be reused)

Destain solution (5% [v/v] methanol/10% [v/v] acetic acid in distilled water) - 2 liters

APPENDIX B
Sample Data

Sample SDS-polyacrylamide gel

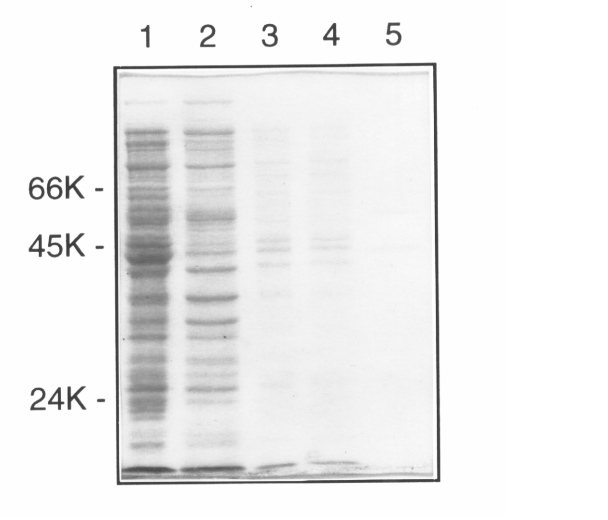

Figure 3.1. Photograph of typical stained SDS-polyacrylamide gel of MBP purification. Lane 1, whole cells; lane 2, shocked cells; lane 3, periplasm; lane 4, amylose resin flowthrough; lane 5, amylose resin eluate. Samples were prepared as described in student outline. 20 µl of each sample, corresponding to 10 µl of fraction plus 10 µl of 2× Laemmli sample buffer, were loaded on a 10% polyacrylamide minigel. Sizes of protein molecular weight markers (not shown) are indicated at the left.

Figure 3.1 shows a typical gel obtained in this exercise. Sample volumes corresponding to equal proportions of all fractions were loaded, except for the final eluate, which has a total volume of 1.5 ml compared to the approximately 10-ml volumes of the preceding samples. Some important features to notice are the following:

1) The shocked cell sample (lane 2) looks similar to the whole cell sample (lane 1), except for appearing depleted for some bands. In at least some cases, it is clear that these bands are enriched in the periplasmic fraction.
2) The periplasmic fraction (lane 3) clearly accounts for less of the total cellular protein than the shocked cells (lane 2). Results of the Bradford assays should help quantify this observation.
3) The flowthrough sample (lane 4) appears essentially *identical* to the periplasm sample (lane 3). This is consistent with results of the Bradford assay, which normally reveal that the protein content of the eluate is neglible compared to the onput (periplasm) and flowthrough samples.
4) Consistent with the extremely low protein content of the eluate, barely any protein is visible in the eluate lane (lane 5). On this particular gel, two bands are visible, with apparent molecular weights of ~53,000 kilodaltons and ~43,000 kilodaltons. The larger of these two proteins is about the expected molecular weight for the plasmid-encoded MBP-lacZα fusion, while the smaller is roughly the size of the endogenous wild-type maltose-binding protein. It may be that induction of the fusion gene was not optimal in this particular batch of cells, so that the fusion protein was not obtained in substantially greater yield than the endogenous protein.

Sample Bradford Assay Data

Table 3.2. Typical results of Bradford assay standard curve and MBP purification samples

Standard Curve:				
vol BSA (0.1 mg/ml) (ml)	**mg protein**	**Absorbance (595 nm)**		
0.000	0.00	0.000		
0.100	0.01	0.234		
0.300	0.03	0.569		
0.500	0.05	0.961		
0.700	0.07	1.048		
Experimental Data:				
Fraction	**vol. in assay (ml)**	**Absorbance (595 nm)**	**mg protein**	**conc. protein (mg/ml)**
Total protein (TP)	0.100	0.468	0.0239	0.239
Shocked cells (SC)	0.100	0.676	0.0350	0.350
Periplasm (P)	0.100	0.169	0.0081	0.081
Column Flowthrough (FT)	0.100	0.120	0.0054	0.054
Column Eluate (E)	0.500	0.018	0.0000	0.000

Table 3.2 shows typical student data obtained in this exercise. Standard curve data are plotted in Figure 3.2. The equation for the standard curve is applied to the absorbances of experimental samples to calculate the *amount* of experimental sample in each assay tube. This amount of protein is then divided by the volume of sample that was placed in the assay tube to determine the *concentration* of protein for any given experimental fraction. To account for the partitioning of protein among fractions at various stages of purification, the total amount of protein in each fraction at each stage of purification must be calculated. To do this, the total volume of each fraction is multiplied by the concentration of protein in that fraction. The results of such calculations are shown in the purification flowchart in Table 3.3. Note that in the experiment from which the data set of Table 3.3 was taken, the shocked cells were resuspended in 5 ml rather than the 10 ml instructed in the student outline. Having total volumes that differ from fraction to fraction emphasizes to students the necessity of accounting for fraction volumes when comparing protein content. If you decide to instruct the students to resuspend the shocked cells in a smaller volume, you may wish to instruct them to load less of this sample on the SDS-polyacrylamide gel, so that the protein contents of the fractions will be directly comparable on the gel.

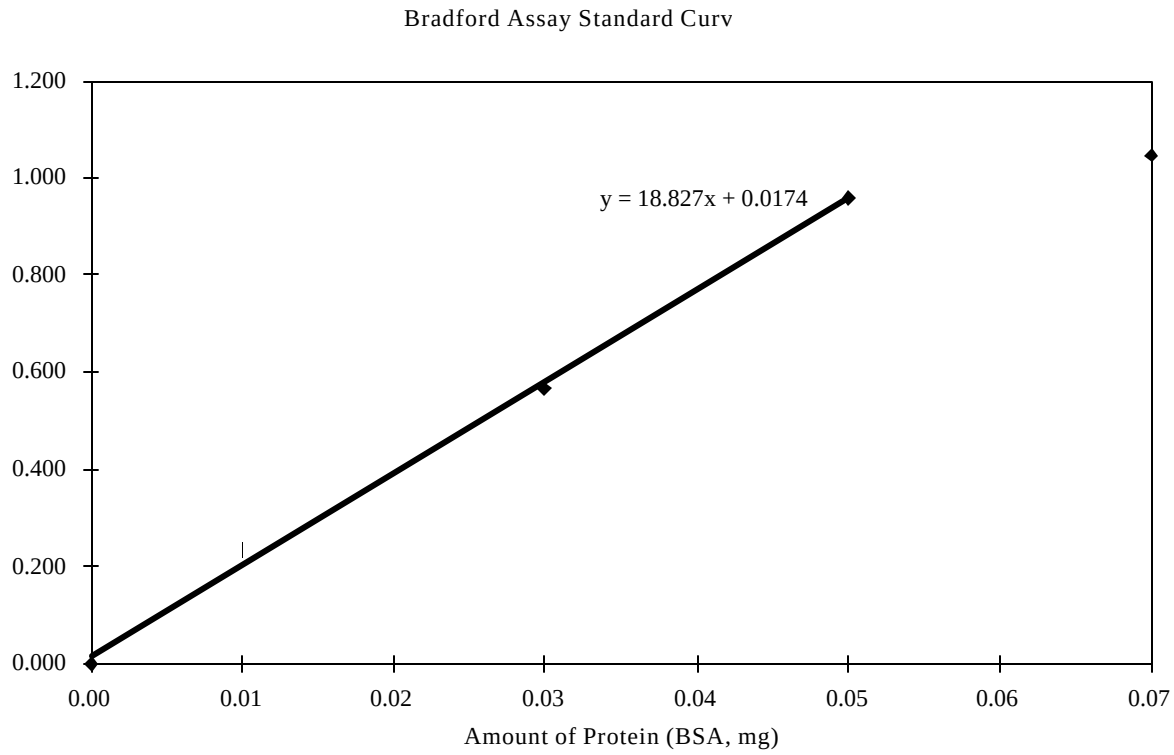

Figure 3.2. Bradford assay standard curve. Data points shown in Table 3.2 are plotted. The linear region of the data was fitted to a line (equation shown) using the "trend" command in Microsoft Excel.

Table 3.3. Purification table for MBP isolation

Fraction	Total Volume (ml)	Protein Conc. (mg/ml)	Amt. protein (mg)	% Original Protein
Total protein (TP)	10.0	0.239	2.39	100%
Shocked cells (SC)	5.0	0.350	1.75	73%
Periplasm (P)	10.0	0.081	0.81	34%
Column Flowthrough (FT)	10.0	0.054	0.54	23%
Column Eluate (E)	1.5	0.000	0.00	0%

According to the data in Table 3.3, roughly 30% of the protein in *E. coli* cells is periplasmic, while the remainder is cytoplasmic. This result seems consistent with the result visualized in the gel of Figure 3.1. Note that no protein was detectable in the eluted sample, yet faint bands are visible by SDS-PAGE. This will be disturbing to many students, but I think teaches them an important lesson about assay sensitivity: just because you don't detect it, doesn't mean it isn't there!