PROTEIN PURIFICATION MANUAL
Purification of ß-galactosidase from E. Coli

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Chapter 1

Introduction: Overview of Protein Purification

This laboratory manual outlines the procedures for the purification of an enzyme, ß-galactosidase, from a strain of E. coli cells. Extracting and purifying this protein from E. coli will be a multi-step process, starting from cell paste of E. Coli, progressing through several separation methods, and ending with analysis and verification of the purified protein by polyacrylamide gel electrophoresis (PAGE) and Immunoblotting procedures. Unlike many classroom laboratory activities, purification of ß-galactosidase is a project which will span the entire semester. The project is designed, as much as possible, to simulate a "real world" biotechnology experience in order to give you the skills you need to do similar projects on the job. Laboratory notebook documentation is always important, but becomes even more important in a project environment. Written and oral communication skills will be emphasized in several ways – by laboratory notebook documentation, individual progress reports and by an oral presentation summarizing the project. Teamwork skills will be emphasized by working with the same lab partner all semester and by collaboration with other students to give the oral presentation.

What is protein purification?

Protein purification is the separation of a specific protein from contaminants in a manner that produces a useful end product.

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What is a protein?

Proteins are the major components of living organisms and perform a wide variety of essential functions in cells. Many of the products that are produced in modern biotechnology companies are proteins. These proteins may be drugs, such as insulin or tissue plasminogen activator (TPA), or they may be the molecular tools that enable biotechnology research such as the enzymes that are used to cut and paste DNA. In biochemical terms, each protein is composed of varying numbers and kinds of 20 different amino acid building blocks. The sequence of amino acids determines the relative positioning of individual chemical groups of each amino acid which in turn determines the final structure and function of the molecule. Protein structure and function will be explored in more depth in Chapter 2.

Why purify proteins?

Technicians and scientists in biotechnology must face the challenge of protein purification regularly. In a research environment, proteins must be purified in order to determine their structure and study their biochemical properties. In industrial settings, proteins are purified on a larger scale in order to be sold as products such as drugs, vaccines, diagnostic tools or food additives.

Protein purification is a challenge.

Protein purification is a challenge because, in addition to the target protein that you want to purify, the cell contains several thousand other proteins along with nucleic acids

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(DNA and RNA), polysaccharides, lipids, and small molecules. A target protein for purification may constitute as little as 0.001% of the total protein in the cell. At most, it may be 10%, unless over-expression is induced by genetic engineering methods- in that case, it may be expressed at higher levels, up to 60% of the total cell protein. In any case, the challenge is to separate the target protein from all the other components in the cell with reasonable efficiency, yield and purity. The two most important things required to make a purification possible are a specific assay for the target protein and a means of stabilizing it during the purification.

Separation methods are based on protein properties.

Because of the differences in amino acid composition and sequence, and the possible presence of non-protein groups, each protein has different chemical characteristics that make it unique. These characteristics include size (molecular weight), charge, solubility, hydrophobicity and biological affinity. Differences in these characteristics are the basis for separation methods such as filtration, salt precipitation, and chromatographic procedures. A wide variety of separation methods are available.

Strategies for Purification.

A purification strategy includes a series of separation methods or steps. Each purification strategy is unique to the individual protein. This situation is in marked contrast to that for purification of DNA and RNA where only a few accepted purification protocols exist. This laboratory manual outlines the procedures involved in **ONE** strategy for protein purification. The choice of separation procedures for a particular

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protein purification strategy is partially based on the characteristics of the target protein that you want to purify. The choice of methods is also affected by the characteristics of the source material (microorganism, animal or plant tissue) from which the protein is being purified as well as the scale necessary (µg to kg amounts). The ideal purification strategy has the following goals: maximum recovery of the target protein; minimal loss of biological activity; and maximum removal of contaminating proteins, as well as low cost, as diagrammed in Figure 1.1. Like most protein purification strategies, purification of ß-galactosidase involves several protein separation steps along with procedures for analysis of purity and yield. The separation methods for this purification of ß-galactosidase will be outlined in Chapter 4.

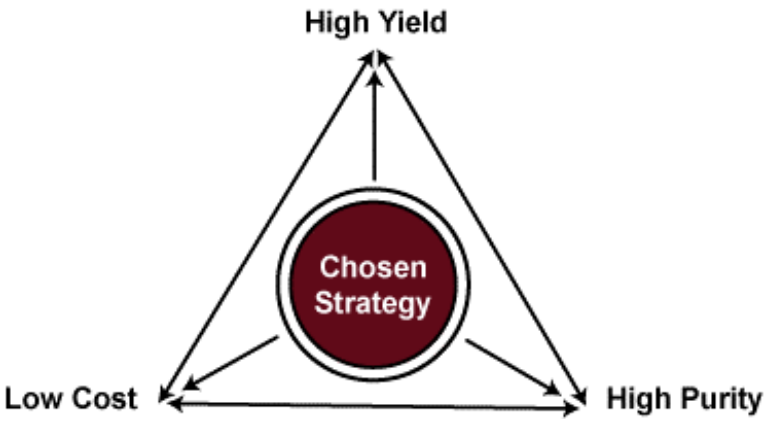

Figure 1.1: A particular strategy for protein purification is a compromise between the goals of high purity, high yield and low cost.

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Analysis of Yield and Purity.

In order to purify a protein, it is essential to have a specific assay, a method for detecting and measuring the target protein. The assay makes it possible to determine the yield, which is how much of the target protein has been purified. In rare cases, the target protein can be detected easily because it is colored (myoglobin) or fluorescent (Green Fluorescent Protein) but most proteins are colorless. If the protein is an enzyme, such as ß-galactosidase, it may be assayed by its ability to catalyze a reaction, provided that there is a way to monitor the formation of product, or the disappearance of substrate.

$$\text{Substrate(s)} \xrightarrow{\text{Enzyme}} \text{Product(s)}$$

These assays are often colorimetric assays based on a component of the reaction that absorbs light at a given wavelength. This light absorbance is measured by a spectrophotometer (See Seidman and Moore, Chapter 19 and 20 for more detailed treatment of spectrophotometry.) If the protein is not an enzyme, the assay is usually based on the biological activity of the protein. For example, if the target protein causes contraction of cultured muscle cells, an assay can be designed to measure this property.

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Since this specific assay for target protein is a measure only of the amount of target protein present and says nothing of the presence of contaminating proteins, an additional assay is needed to determine the amount of all proteins, called the total protein assay. The specific activity is expressed in units/mg and is a ratio of the amount of target protein (units) to the total mass of all proteins (mg).

After each separation step is performed, an aliquot of the product mixture is set aside for analysis of purity and yield. As the purification process proceeds, the specific activity should increase because contaminants are being removed, thereby decreasing the total weight of all proteins in the sample. This indicates greater purity due to fewer contaminating proteins. Maximum specific activity will be obtained when the protein is purified to homogeneity; in other words, there are no other proteins present other than the target protein. Losses of yield are inevitable as the product mixture is manipulated - the goal is to minimize this loss. The assays used for measuring the activity of ß-galactosidase and monitoring the specific activity of this purification strategy will be described in more detail in Chapter 3.

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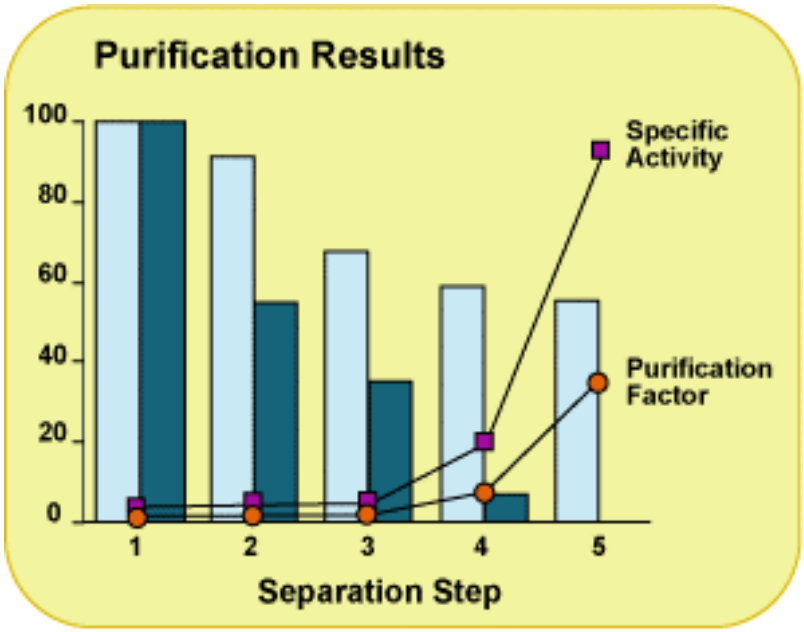

Light Blue: Activity of target
Dark Blue: Total protein

Figure 1.2: Typical results from a 5-step protein purification scheme are presented. The graph demonstrates that good recovery of biological activity, together with reduced protein mass, results in increasing specific activity and protein purity at each step.

But how do we know when we have removed ALL contaminants? Gel electrophoresis is one of the most powerful methods for analysis of protein purity. Protein samples are applied to a gel matrix and separated by size (SDS-PAGE), then stained to allow visualization of all proteins so the number of different proteins in the mixture can be determined. Two-dimensional PAGE offers even better resolving power since it separates by charge in the first dimension and size in the second dimension. Western

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blotting (immunoblotting) using specific antibodies to the target protein may also be used to verify that the target protein has been purified. Other methods, such as High Performance Liquid Chromatography (HPLC), may also be used to monitor purity but the instrumentation required is generally too expensive for teaching laboratories. Analysis and verification will be discussed in Chapter 5.

Purification goals will vary according to application.

The required purity of the end product will vary according to the intended use of the product. If complete removal of all contaminating proteins is not necessary, then it would not make good economic sense to spend time and money removing them. For example, a drug for human injection would obviously demand the highest standard of purity, even at high cost. But if the product is an enzyme for cutting DNA, a restriction endonuclease, then it might only be necessary to remove contaminating nucleases and other enzymes. The presence of some contaminating proteins would not affect product performance –in other words, the enzyme would be "functionally pure".

The scale of the purification process will affect the design of the purification strategy.

Different purification strategies may be required in order to purify the same protein for two different applications. For example, if a researcher is trying to purify a very small amount of a protein in order to determine the sequence of amino acids in a particular protein, the purification strategy may be quite different from one required for the industrial production of the same protein for use as a drug. In both cases the protein

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must be very pure; however, the amounts needed vary from µg to kg so that a different purification strategy will probably be required.

For these reasons, the exact approach that you will learn for ß-galactosidase in this lab manual will probably not work for another protein. Furthermore, the method which is presented in this lab manual is not the only method for purifying ß-galactosidase -- there are other methods which are equally effective. However, the concepts and techniques you will learn in this course will help you to understand and implement other strategies for protein purification that you will encounter. Throughout our purification project, we will discuss our approach for purifying and analyzing ß-galactosidase and compare it to alternative strategies of protein purification that could be used. Keep in mind, however, that it will not be possible to cover every possible strategy for protein purification in this course.

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Characteristics Of The Target Protein, ß-galactosidase.

ß-galactosidase is an enzyme that is produced in *E. coli.* In the cell, ß-galactosidase hydrolyzes (splits) the ß 1,6 bond in lactose, a disaccharide, producing two monosaccharides, glucose and galactose, which can be used as energy sources. (See Figure 3.1) If lactose is unavailable, or the organism already has plenty of glucose as an energy source, then it would be inefficient for the cell to make ß-galactosidase. But if its substrate, lactose, is present, and glucose is low, then the enzyme is made ("induced") because the cell needs it. Scientists use the term "inducible" to refer to enzymes that are only made when the inducing agent, usually the substrate of the enzyme reaction, is present. If an enzyme is made all the time, it is called "constitutive". As a source material for purification of the enzyme, a mutant strain of *E. coli* that is constitutive for ß-galactosidase was chosen because this strain is producing the maximum amount of the target protein.

Assignment: ß-galactosidase has been extensively studied and characterized. Commercial sources are available as we are certainly not the first to purify it.! Find ß-galactosidase in the Sigma catalog. How many different products are there? Look at the reported specific activity for each one. What accounts for these differences? Try typing ß-galactosidase into a search engine or a biomedical library database. (You will

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get thousands of hits.) From your research and discussion in lecture, what are some of the characteristics of ß-galactosidase?

Overview of the Project

We will begin the purification with cell paste from a strain of E. Coli that is a constitutive producer of ß-galactosidase and use the following series of procedures to purify and characterize this enzyme:

- Extraction
  Ultrasonic disruption OR Novagen Bugbuster™ (Enzymatic method)

- Purification Steps
  Ammonium sulfate precipitation (salting out the ß-galactosidase protein)
  Dialysis (desalting and buffer exchange)
  Ion exchange chromatography

- Analysis and verification steps
  Assays for specific activity
  Polyacrylamide gel electrophoresis

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Concentrating the sample from the column fractions

Western blotting

This manual is not designed to provide a complete body of theory for protein purification, although we do try to provide enough background information to understand the scientific basis of the methods in **THIS** purification strategy. Consult Appendix C for additional resources which present a more complete treatment of all separation methods.

Classroom compromises: In the "real world", the entire purification process outlined in this manual would routinely be performed in a cold room at 4°C, working quickly to minimize the loss of enzyme activity. Most classroom laboratories do not have access to cold rooms and working "quickly" is not possible in a class with 6 hours of laboratory time each week for 16 weeks. Fortunately, the activity of ß-galactosidase stands up admirably under these adverse conditions and most student groups still have activity to assay at the end of the semester.

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Important Tips for Protein Purification

Keep your protein cold.

Throughout this project, the enzyme extract must be kept cold, in an ice bath or in a cold room or refrigerator. There are enzymes called proteases in the cell extract that become more active at temperatures above 4° C. These enzymes will hydrolyze other proteins including ß-galactosidase. So to keep the proteases inactive, and ß-galactosidase active, keep the extract cold. Use refrigerated buffers, refrigerated centrifuges (if possible) and keep your samples on ice at all times.

Save materials.

Never throw away any supernatant, pellet, fraction, or sample until you have completed analysis of that purification step.

Use fume hoods for buffers.

ß-mercaptoethanol (ß-ME) and dithiothreitol (DTT) are reducing agents that you will be adding to your buffers to preserve the activity of ß-galactosidase. DTT and ß-ME are volatile and gradually evaporate from solutions so you will be adding these compounds to your solutions just before you use them. They are extremely foul smelling and toxic at high concentrations. Use fume hoods to add the concentrated DTT to your working solutions and minimize the amount of time you have uncovered solutions at the bench.

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Keep Good Records.

As always, it is extremely important that you keep extensive and accurate notes in your lab notebook. Follow all rules for keeping a notebook, such as using only indelible ink, never erasing anything, dating each entry and initialing your entries when appropriate. Keep track of the volumes you have at each step and other details such as where things are stored (which refrigerator or freezer, etc). Finally, for every chemical or solution, find and record the chemical name, concentration, supplier and catalog number and any other information you may need to reproduce your work. Remember, you - or another person unfamiliar with the work - should be able to repeat each step based on your laboratory notebook!

Course Organization

The set of procedures in this manual is a project that will take most of the semester. Unlike many other classroom laboratory projects and experiments, the organization of this course is designed to model "the real world" where laboratory work is generally project based. The course is intentionally organized to give you an opportunity to work in partners or teams, fairly independently of the instructors.

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Presentation of Results.

One of the most important aspects of biotechnology research is presentation of the results. Regardless of where you work, you will need to present your results orally and in written form to your supervisor and to others in the company or institution in which you are employed. It is also possible that your work will be presented at scientific meetings and, ultimately, be published in journals where other scientists will evaluate them critically.

Each student group will present a final oral report to the class and each student will submit a written formal lab report on the purification project. Although this report is due when the project is completed, take the time now and several times during the first month of the semester to read over the guidelines for your report that are contained in Section 5 of this lab manual. Familiarity with this format will help you to collect and analyze your data in ways that will save you time and much grief when you write your report.

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Chapter 2
What is a Protein?
Structure and Function

The word protein was first coined in 1838 to emphasize the importance of this class of molecules. The word is derived from the Greek word *proteios* which means "of the first rank".

This chapter will provide a brief background into the structure of proteins and how this structure can determine the function and activity of proteins. It is not intended to substitute for the more detailed information provided in a biochemistry or cell biology course.

Proteins are the major components of living organisms and perform a wide range of essential functions in cells. While DNA is the information molecule, it is proteins that do the work of all cells - microbial, plant, animal. Proteins regulate metabolic activity, catalyze biochemical reactions and maintain structural integrity of cells and organisms. Proteins can be classified in a variety of ways, including their biological function (Table 2.1).

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Table 2.1 Classification of Proteins According to biological function.	
Type:	**Example:**
Enzymes- Catalyze biological reactions	ß-galactosidase
Transport and Storage	Hemoglobin
Movement	Actin Myosin
Immune Protection	Immunoglobulins (antibodies)
Regulatory Function within cells	Transcription factors
Hormones	Insulin Estrogen
Structural	Collagen

How does one group of molecules perform such a diverse set of functions? The answer is found in the wide variety of possible structures for proteins.

In the English language, there are an enormous number of words with varied meaning that can be formed using only 26 letters as building blocks. A similar situation exists for proteins where an incredible variety of proteins can be formed using 20 different

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building blocks called amino acids. Each of these amino acid building blocks has a different chemical structure and different properties (Figure 2.2). Each protein has a unique amino acid sequence that is genetically determined by the order of nucleotide bases in the DNA, the genetic code. Since each protein has different numbers and kinds of the twenty available amino acids, each protein has a unique chemical composition and structure. For example, two proteins may each have 37 amino acids but if the sequence of the amino acids is different, then the protein will be different. How many different proteins can be formed from the twenty different amino acids? Consider a protein containing 100 different amino acids linked into one chain. Since each of the 100 positions of this chain could be filled with any one of the 20 amino acids, there are 20^{100} possible combinations, more than enough to account for the 90-100 million different proteins that may be found in higher organisms.

A change in just one amino acid can change the structure and function of a protein. For example, sickle cell anemia is a disease that results from an altered structure of the protein hemoglobin, resulting from a change of the sixth amino acid from glutamic acid to valine. (This is the result of a single base pair change at the DNA level.) This single amino acid change is enough to change the conformation of hemoglobin so that this protein clumps at lower oxygen concentrations and causes the characteristic sickle shaped red blood cells of the disease.

The unique structure and chemical composition of each protein is important for its function; it is also important for separating proteins in a protein purification strategy.

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Each of these differences in properties can be used as a basis for the separation methods that are used to purify proteins. Because these differences in protein properties originate from differences in the chemical structure of the amino acids that make up the protein, we need to explore the structure of amino acids and their contribution to protein properties in more detail.

Chemical Composition of Proteins: (Protein Structure)

Amino Acid Structure:

Amino acids are composed of carbon, hydrogen, oxygen, and nitrogen. Two amino acids, cysteine and methionine, also contain sulfur. The generic form of an amino acid is shown in Figure 2.1. Atoms of these elements are arranged into 20 kinds of amino acids that are commonly found in proteins. All proteins in all species, from bacteria to humans, are constructed from the same set of twenty amino acids. All amino acids have an amino group (NH_2) and a carboxyl group (COOH) bonded to the same carbon atom, known as the alpha carbon. Amino acids differ in the side chain or R group that is bonded to the alpha carbon. (Figure 2.2) Glycine, the simplest amino acid has a single hydrogen atom as its R group - Alanine has a methyl ($-CH_3$) group.

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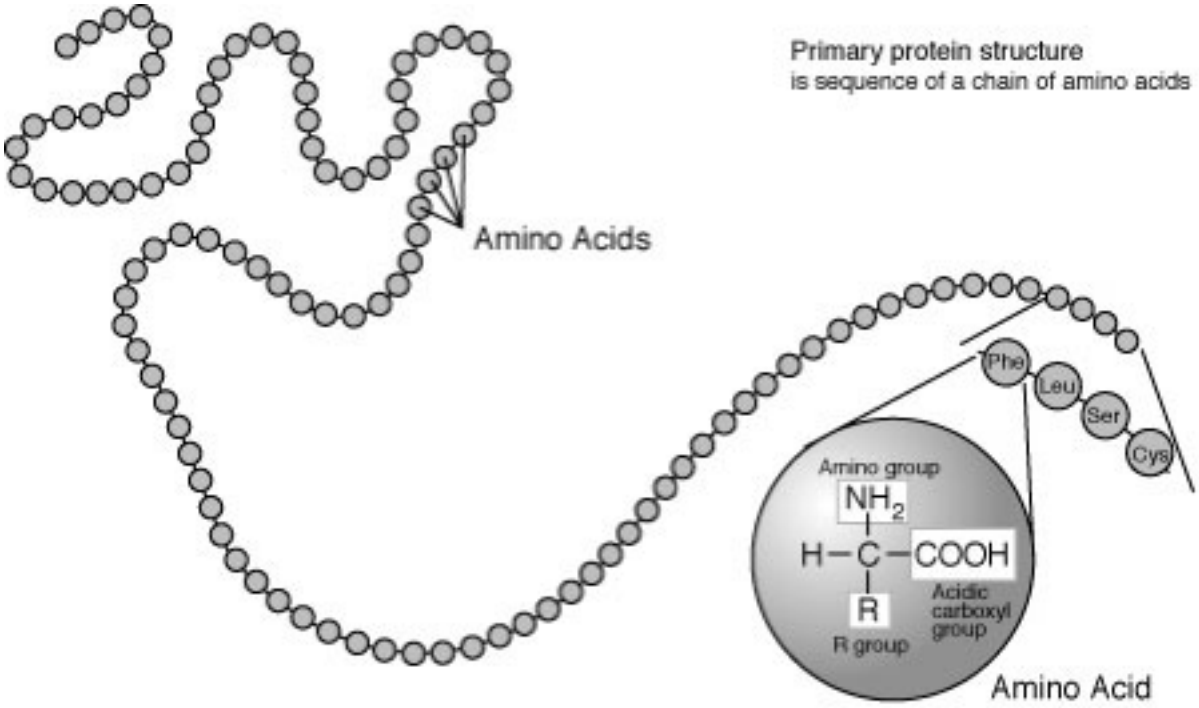

Figure 2.1: A diagram showing a generic amino acid structure. All amino acids have an alpha carbon, an amino group, and a carboxyl group, but each of the 20 amino acids has a different side chain or R group. Amino acids are linked together to form a polypeptide chain, like beads on a string, the primary level of protein structure. Figure used with permission from National Human Genome Research Institute (NHGRI), artist Darryl Leja, http://www.genome.gov/page.cfm?pageID=10000552

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The chemical composition of the unique R groups is responsible for the important characteristics of amino acids such as chemical reactivity, ionic charge and relative hydrophobicity. In Figure 2.2, the amino acids are grouped according to their polarity and charge. They are divided into four categories, those with polar uncharged R groups, those with apolar (nonpolar) R groups, acidic (charged) and basic (charged) groups.

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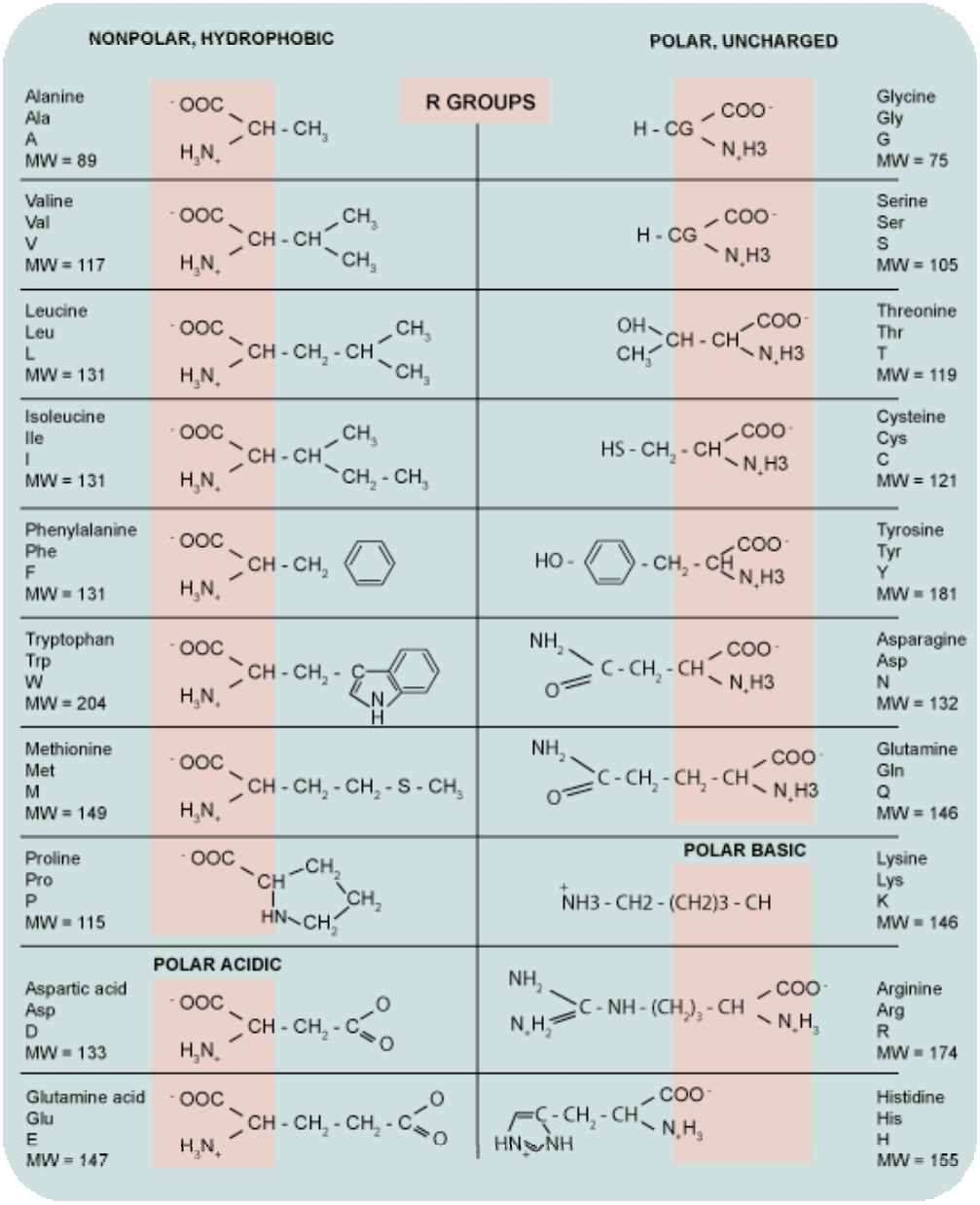

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Figure 2.2. The twenty amino acids, grouped according to the character of their side chain or R group. From *Guide to Protein Separations*, Rainin Instrument Co, Inc.

The polar amino acids are soluble in water because their R groups can form hydrogen bonds with water. For example, serine, threonine and tyrosine all have hydroxyl groups (OH). Amino acids that carry a net negative charge at neutral pH contain a second carboxyl group. These are the acidic amino acids, aspartic acid and glutamic acid, also called aspartate and glutamate, respectively. The basic amino acids have R groups with a net positive charge at pH 7.0. These include lysine, arginine and histidine. There are eight amino acids with nonpolar R groups. As a group, these amino acids are less soluble in water than the polar amino acids. If a protein has a greater percentage of nonpolar R groups, the protein will be more hydrophobic (water hating) in character.

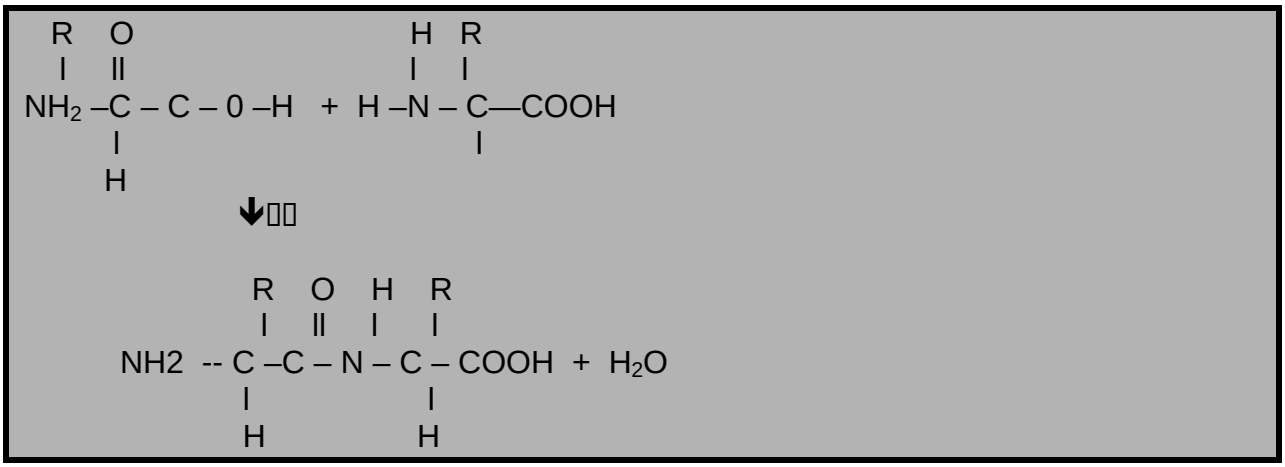

Figure 2.3. A diagram showing the formation of a Peptide bond between two Amino acids.

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A protein is formed by amino acid subunits linked together in a chain. The bond between two amino acids is formed by the removal of a H_20 molecule from two different amino acids, forming a dipeptide (Figure 2.3). The bond between two amino acids is called a peptide bond and the chain of amino acids is called a peptide (20 amino acids or smaller) or a polypeptide.

Each protein consists of one or more unique polypeptide chains. Most proteins do not remain as linear sequences of amino acids; rather, the polypeptide chain undergoes a folding process. The process of protein folding is driven by thermodynamic considerations. This means that each protein folds into a configuration that is the most stable for its particular chemical structure and its particular environment. The final shape will vary but the majority of proteins assume a globular configuration. Many proteins such as myoglobin consist of a single polypeptide chain; others contain two or more chains. For example, hemoglobin is made up of two chains of one type (amino acid sequence) and two of another type.

Although the primary amino acid sequence determines how the protein folds, this process is not completely understood. Although certain amino acid sequences can be identified as more likely to form a particular conformation, it is still not possible to completely predict how a protein will fold based on its amino acid sequence alone, and this is an active area of biochemical research.

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The final folded 3-D arrangement of the protein is referred to as its conformation. In order to maintain their function, proteins must maintain this conformation. To describe this complex conformation, scientists describe four levels of organization: primary, secondary, tertiary, and quaternary (Figure 2.4). The overall conformation of a protein is the combination of its primary, secondary, tertiary and quaternary elements.

Four Levels Of Organization Of Protein Structure:

- Primary Structure refers to the linear sequence of amino acids that make up the polypeptide chain. This sequence is determined by the genetic code, the sequence of nucleotide bases in the DNA. The bond between two amino acids is a peptide bond. This bond is formed by the removal of a H_20 molecule from two different amino acids, forming a dipeptide. The sequence of amino acids determines the positioning of the different R groups relative to each other. This positioning therefore determines the way that protein folds and the final structure of the molecule.

- The secondary structure of protein molecules refers to the formation of a regular pattern of twists or kinks of the polypeptide chain. The regularity is due to

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hydrogen bonds forming between the atoms of the amino acid backbone of the polypeptide chain. The two most common types of secondary structure are called the alpha helix and ß pleated sheet. (Figure 2.4)

- Tertiary structure refers to the three dimensional globular structure formed by bending and twisting of the polypeptide chain. This process often means that the linear sequence of amino acids is folded into a compact globular structure. The folding of the polypeptide chain is stabilized by multiple weak, noncovalent interactions. These interactions include:

    - Hydrogen bonds that form when a Hydrogen atom is shared by two other atoms.

    - Electrostatic interactions that occur between charged amino acid side chains. Electrostatic interactions are attractions between positive and negative sites on macromolecules.

    - Hydrophobic interactions: During folding of the polypeptide chain, amino acids with a polar (water soluble) side chain are often found on the surface of the molecule while amino acids with non polar (water insoluble) side chain are buried in the interior. This means that the folded protein is soluble in water or aqueous solutions.

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Covalent bonds may also contribute to tertiary structure. The amino acid, cysteine, has an SH group as part of its R group and therefore, the disulfide bond (S-S) can form with an adjacent cysteine. For example, insulin has two polypeptide chains that are joined by two disulfide bonds.

Quaternary structure refers to the fact that some proteins contain more than one polypeptide chain, adding an additional level of structural organization: the association of the polypeptide chains. Each polypeptide chain in the protein is called a subunit. The subunits can be the same polypeptide chain or different ones. For example, the enzyme ß-galactosidase is a tetramer, meaning that it is composed of four subunits, and, in this case, the subunits are identical - each polypeptide chain has the same sequence of amino acids. Hemoglobin, the oxygen carrying protein in the blood, is also a tetramer but it is composed of two polypeptide chains of one type (141 amino acids) and two of a different type (146 amino acids). In chemical shorthand, this is referred to as $\alpha_2ß_2$. For some proteins, quaternary structure is required for full activity (function) of the protein.

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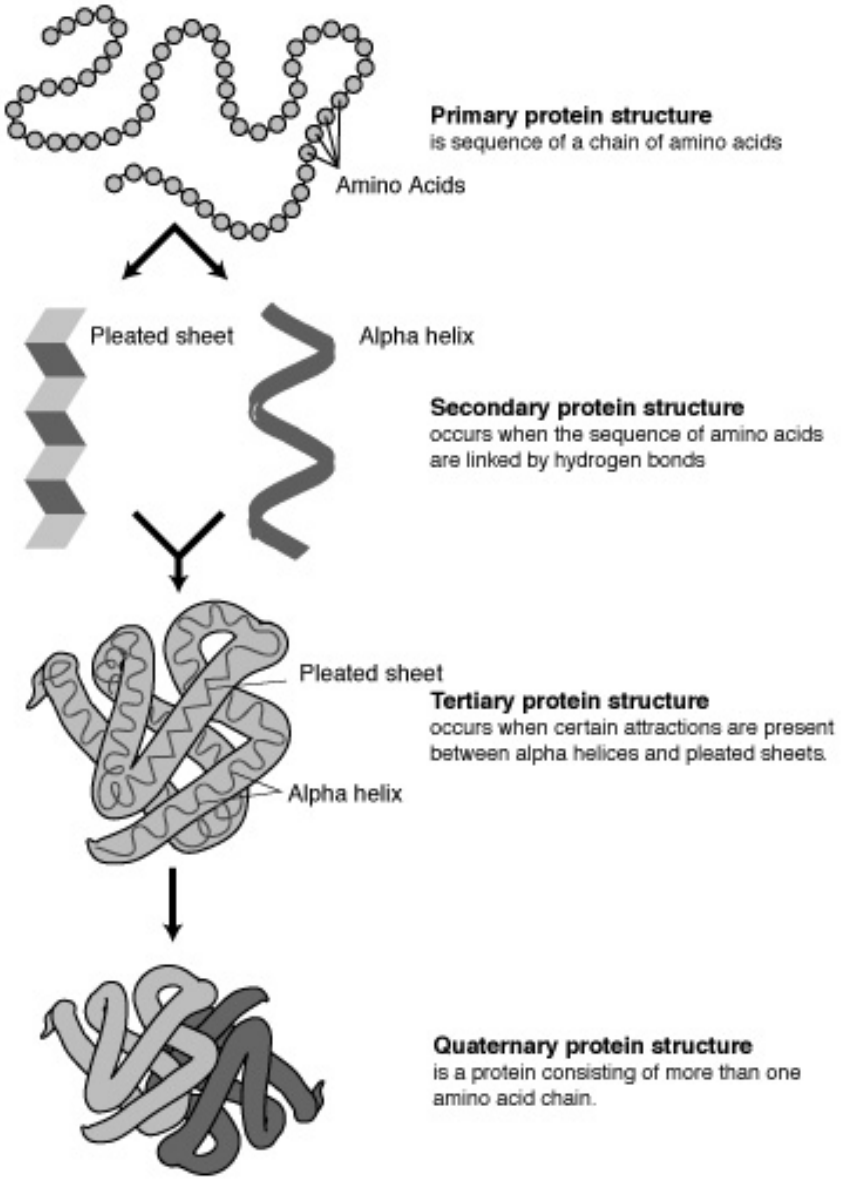

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Figure 2.4: The four levels of protein structure are illustrated in this diagram. Figure used with permission from National Human Genome Research Institute (NHGRI) , artist Darryl Leja: http://www.genome.gove/page.cfm?pageID=10000552

Conjugated Proteins:

Some proteins combine with other kinds of molecules such as carbohydrates, lipids, iron and other metals, or nucleic acids, to form glycoproteins, lipoproteins, hemoproteins, metalloproteins, and nucleoproteins respectively. The presence of these other biomolecules affects the protein properties. For example, a protein that is conjugated to carbohydrate, called a glycoprotein, would be more hydrophilic in character while a protein conjugated to a lipid would be more hydrophobic in character.

Protein Properties And Separation:

Proteins are typically characterized by their size (molecular weight) and shape, amino acid composition and sequence, isolelectric point (pI), hydrophobicity, and biological affinity. Differences in these properties can be used as the basis for separation methods in a purification strategy (Chapter 4). The chemical composition of the unique

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R groups is responsible for the important characteristics of amino acids, chemical reactivity, ionic charge and relative hydrophobicity. Therefore protein properties relate back to number and type of amino acids that make up the protein.

SIZE: Size of proteins is usually measured in molecular weight (mass) although occasionally the length or diameter of a protein is given in Angstroms. The molecular weight of a protein is the mass of one mole of protein, usually measured in units called daltons. One dalton is the atomic mass of one proton or neutron. The molecular weight can be estimated by a number of different methods including electrophoresis, gel filtration, and more recently by mass spectrometry. The molecular weight of proteins varies over a wide range. For example, insulin is 5,700 daltons while snail hemocyanin is 6,700,000 daltons. The average molecular weight of a protein is between 40,000 to 50,000 daltons. Molecular weights are commonly reported in kilodaltons or (kD), a unit of mass equal to 1000 daltons. Most proteins have a mass between 10 and 100 kD. A small protein consists of about 50 amino acids while larger proteins may contain 3,000 amino acids or more. One of the larger amino acid chains is myosin, found in muscles, which has 1,750 amino acids.

Separation methods that are based on size and shape include gel filtration chromatography (size exclusion chromatography) and polyacrylamide gel electrophoresis.

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Amino Acid Composition and Sequence:

The amino acid composition is the percentage of the constituent amino acids in a particular protein while the sequence is the order in which the amino acids are arranged.

CHARGE: Each protein has an amino group at one end and a carboxyl group at the other end as well as numerous amino acid side chains, some of which are charged. Therefore each protein carries a net charge. The net protein charge is strongly influenced by the pH of the solution. To explain this phenomenon, consider the hypothetical protein in Figure 2.5. At pH 6.8, this protein has an equal number of positive and negative charges and so there is no net charge on the protein. As the pH drops, more H+ ions are available in the solution. These hydrogen ions bind to negative sites on the amino acids. Therefore, as the pH drops, the protein as a whole becomes positively charged. Conversely, at a basic pH, the protein becomes negatively charged. pH 6.8 is called the pI, or isoelectric point, for this protein; that is, the pH at which there are an equal number of positive and negative charges. Different proteins have different numbers of each of the amino acid side chains and therefore have different isoelectric points. So, in a buffer solution at a particular pH, some proteins will be positively charged, some proteins will be negatively charged and some will have no charge.

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Separation techniques that are based on charge include ion exchange chromatography, isoelectric focusing and chromatofocusing.

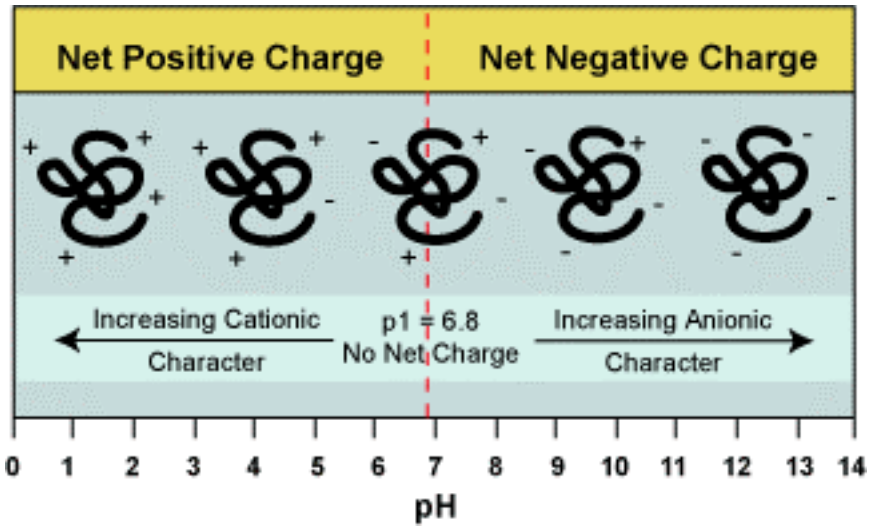

Figure 2.5. The pI is the pH at which there is no net charge on the protein. At lower pH readings, there are more positive charges in the environment and therefore, the protein has an increased cationic character. The reverse is true at pH readings above the pI.

HYDROPHOBICITY: Literally, hydrophobic means fear of water. In aqueous solutions, proteins tend to fold so that areas of the protein with hydrophobic regions are located in internal surfaces next to each other and away from the polar water molecules of the solution. Polar groups on the amino acid are called hydrophilic (water loving) because they will form hydrogen bonds with water molecules. The number, type and distribution

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of nonpolar amino acid residues within the protein determines its hydrophobic character. (Chart of hydrophobicity or hydropathy)

A separation method that is based on the hydrophobic character of proteins is hydrophobic interaction chromatography.

SOLUBILITY: As the name implies, solubility is the amount of a solute that can be dissolved in a solvent. The 3-D structure of a protein affects its solubility properties. Cytoplasmic proteins have mostly hydrophilic (polar) amino acids on their surface and are therefore water soluble, with more hydrophobic groups located on the interior of the protein, sheltered from the aqueous environment. In contrast, proteins that reside in the lipid environment of the cell membrane have mostly hydrophobic amino acids (non polar) on their exterior surface and are not readily soluble in aqueous solutions.

Each protein has a distinct and characteristic solubility in a defined environment and any changes to those conditions (buffer or solvent type, pH, ionic strength, temperature, etc.) can cause proteins to lose the property of solubility and precipitate out of solution. The environment can be manipulated to bring about a separation of proteins- for example, the ionic strength of the solution can be increased or decreased, which will change the solubility of some proteins.

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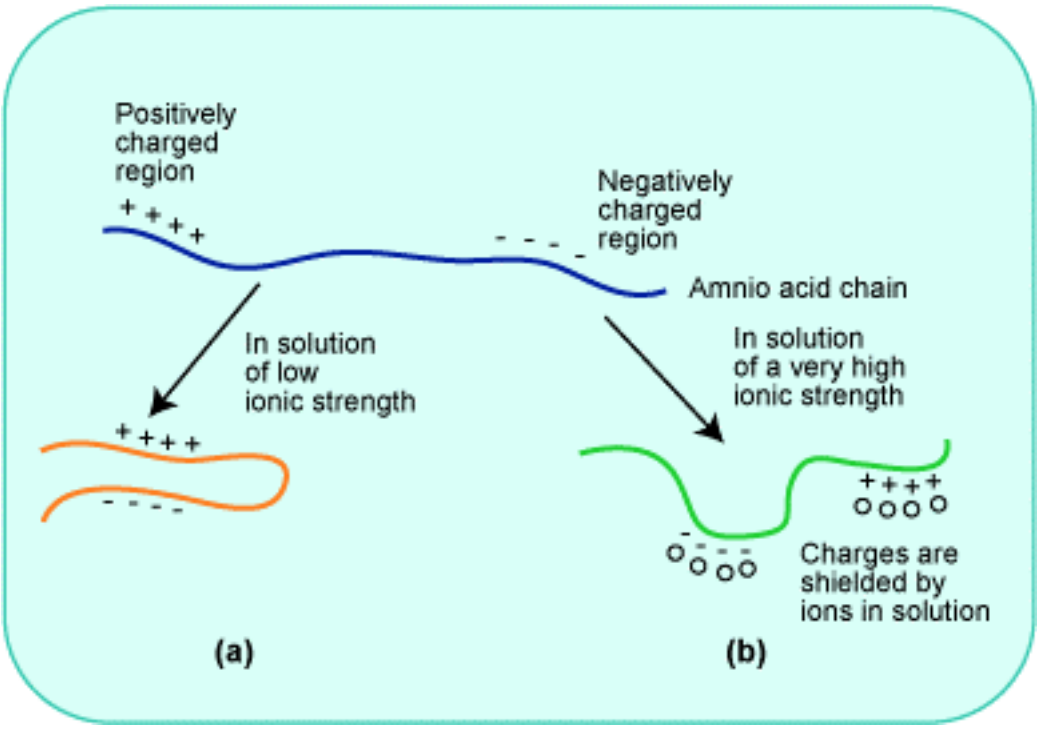

Figure 2.6: Ionic Strength and Protein Folding. This figure shows the effect of ion concentration on protein folding.

BIOLOGICAL AFFINITY (FUNCTION): Proteins often interact with other molecules *in vivo* in a specific way- in other words, they have a biological affinity for that molecule. These molecular counterparts, termed ligands, can be used as "bait" to "fish" out the target protein that you want to purify. For example, one such molecular pair is insulin and the insulin receptor. If you want to purify (or catch) the insulin receptor, you could couple many insulin molecules to a solid support and then run an extract (containing the

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receptor) over that column. The receptor would be “caught” by the insulin bait. These specific interactions are often exploited in protein purification procedures. Affinity chromatography is a very common method for purifying recombinant proteins (proteins produced by genetic engineering). Several histidine residues can be engineered at the end of a polypeptide chain. Since repeated histidines have an affinity for metals, a column of the metal can be used as bait to “catch” the recombinant protein.

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Table 2.2: Methods Used for Protein Separation and Analysis

Technique	Protein Property Exploited
Bulk Methods	
Ammonium sulfate precipitation	Solubility
Filtration	Size
Chromatography Methods	
Ion-Exchange	Charge
Gel Filtration (Gel Permeation)	Size or molecular wt.
Hydrophobic Interaction	Hydrophobicity
Affinity	Biological Activity
Reversed Phase	Hydrophobicitiy
Chromatofocusing	pI (Charge)
Electrophoresis	
Native Gel	Mass/charge
Denaturing Gel (SDS-PAGE)	Mass (Molecular weight)
IEF	pI or charge
2D gels	Molecular weight and pI (charge)

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Working With Proteins:

How proteins lose their structure and function.

Although DNA can be isolated and amplified from thousand year old mummies, most proteins are more fragile biomolecules. Therefore, laboratory reagents and storage solutions must provide suitable conditions so that the normal structure and function of the protein is maintained. To understand how the structure of proteins is protected in laboratory solutions, it is necessary to understand how that structure can be destroyed.

- Proteins can **denature**, or unfold so that their three dimensional structure is altered but their primary structure remains intact.(Figure 2.7) Many of the interactions that stabilize the 3-D conformation of the protein are relatively weak and are sensitive to various environmental factors including high temperature, low or high pH and high ionic strength. Protein vary greatly in the degree of their sensitivity to these factors. Sometimes proteins can be renatured but often the denaturation is irreversible.

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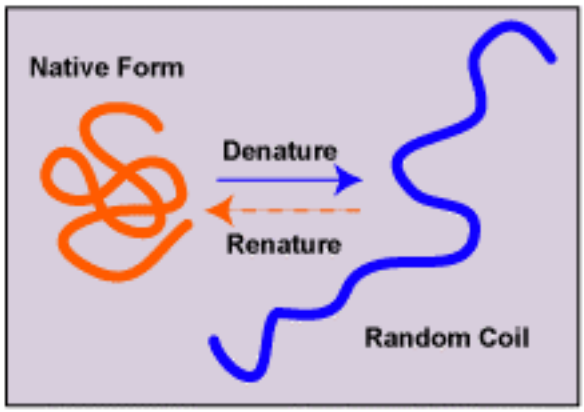

Figure 2.7. A figure showing the process of denaturation. The polypeptide chain has lost its higher order structure and is now a random coil.

- Proteins can also be broken apart by enzymes, called proteases, that digest the covalent peptide bonds between amino acids that are responsible for the primary structure. This process is called **proteolysis** and is irreversible. Cells contain proteases that are found in lysosomes, membrane bound organelles inside the cell. When cells are disrupted, lysosomes break and release these proteases, which can damage the other proteins in the cell. In the laboratory, it is therefore necessary to minimize the activities of cellular proteases to protect proteins from proteolysis. Methods used to minimize proteolysis include working at lower temperatures (4°C), adding chemicals that inhibit protease activity.

- Sulfur groups on cysteines may undergo oxidation to form disulfide bonds that are not normally present. Extra disulfide bonds can form when proteins are

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removed from their normal environment. Reducing agents such as dithiothritol or ß-mercaptoethanol are often added to prevent disulfide bond formation.

- Proteins readily adsorb (stick to) surfaces, thereby reducing their available activity. To prevent significant loss, do not store dilute solutions of proteins for prolonged periods of time. Always dilute them right before use.

The composition of the extraction buffer is important for maintaining structure and function of the protein. To prevent denaturation, the buffer pH is based on the pH stability range of the protein. Other components such as ionic strength, divalent cations (Ca++ and Mg++), or reducing agents (dithiothreitol or ß-mercaptoethanol) may be needed to maintain activity. In making the extract, cells are lysed and proteases (enzymes that degrade proteins) are released from their intracellular compartments. To prevent proteases from digesting the target protein, two strategies are commonly followed:

- The extract is kept cold. The activity of protein purification process is often conducted in cold rooms. At the very least, an effort is made to keep the extract at 4°C. 2) protease inhibitors are sometimes added to the mixture to prevent degradation by proteases. The drawback

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to this strategy is that the inhibitors must eventually be removed, along with other contaminant proteins.

Chapter 3
Assays and Calculations

Note: In this chapter, two assays are described that are necessary in order to follow and document the purification of ß-galactosidase. Both of these assays are colorimetric assays that rely on a spectrophotometer, an instrument that measures the absorbance of light. If a review of the principles of spectrophotometry and colorimetric assays is necessary, see Seidman and Moore, Basic Lab Methods for Biotechnology, chapters 19-20.

During the purification process for ß-galactosidase, two different assays will be used. The first is the Bradford assay (Bradford 1976), which measures the total amount of all proteins present in a sample. The second is an assay that measures the enzymatic activity of the target protein, ß-galactosidase. The values obtained from these two assays can be used to calculate the amount of enzymatic activity in each milligram of protein. This latter value is known as the specific activity and will allow you to assess the purity of the target protein, ß-galactosidase. Using the assay for ß-galactosidase, you will also be able to calculate the yield of your target protein, which is the total

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amount of ß-galactosidase that you purified. Yield can be expressed in several ways; as the total units of enzyme; as a percentage of the number of units present in the starting material; or as a weight of ß-galactosidase.

The protein assay. The total protein concentration, which includes the target protein along with all other contaminating proteins, must be known in order to determine specific activity. Total protein concentration can be determined by several different colorimetric assays, such as the Biuret, the Lowry or the (Bradford 1976) assay. In this purification project, a commercially prepared dye from Bio-Rad will be used. The Bio-Rad assay is based on the Bradford assay (Bradford 1976) (Reference). Like other colorimetric assays, a dye is added to the solution that changes color depending on how much protein there is in the sample. The amount of color change is quantitated by a spectrophotometer which measures the change in light absorbance at a particular wavelength. This assay is not specific for ß-galactosidase; rather, it measures the total amount of all proteins.

Colorimetric assays such as those described here are destructive to the sample. The total protein concentration can be reliably estimated using ultraviolet light absorption at 280 nm. Although not as accurate for solutions containing unknown proteins or mixtures of proteins, this method is not destructive to the sample and will be used to estimate the protein concentration of column fractions in Chapter 4.

The ß-galactosidase assay. The assay we will use for ß-galactosidase activity measures the biological activity of the enzyme; in this case, cleavage of a specific bond

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found in a type of sugar known as a ß-galactoside. Like the Bradford assay, the ß-galactosidase assay is a colorimetric assay. In vivo, the substrate is lactose which is cleaved into glucose and galactose, both of which are colorless. For the assay, we use a substrate, ONPG, that produces a colored product when cleaved. (Figure 3.1) The amount of enzyme activity is measured by the appearance of a yellow product that is produced when the substrate is cleaved. If there is activity in the sample, the solution will turn from clear to yellow as the enzyme does its job.

A specific assay for the target protein is essential in order to follow the progress of the purification. Without a specific assay, there would be no point in trying to purify the target protein.

Important Tips For These Assays

Accurate and reproducible assays are critical to the project. These assays are the basis for all the work this semester since you are using them to track your protein and monitor its purity and activity. Therefore it is extremely important that you work out your methods and develop proper technique **NOW**, before we make the crude extract.

- Write out your own procedure in your own words.

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- Pay careful attention to all the steps.

- Document all dilutions and volumes. Record the units for each measurement and calculation.
- Develop strategies for staying organized, such as moving your test tubes over in the rack after the addition of reagents.

- Repeat assays as needed to ensure reliable data.

Dilute your protein appropriately. The assays we will be using are designed to measure a fairly narrow range of protein and enzyme concentrations so you will need to dilute your protein in order to be in the range of the assay. The proper dilutions for each assay will change as you proceed with the purification project. You will need to determine the correct dilutions largely through trial and error.

Procedure 3.1 The Bradford Microassay

Background

The basis of the Bradford assay is the observation that certain amino acids found in most proteins selectively bind the dye, Coomassie Brilliant Blue, also called Bradford's

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reagent. Under acidic conditions when the dye binds these amino acids, it changes color from reddish-brown to blue. The more of these amino acids there are, the more color change in the solution. This change can be quantitated using a spectrophotometer at 595 nm. We will be using the "Bio-Rad Microassay," a version of this assay designed to measure 25 µg or less of protein.

For a standard curve, it is necessary to measure the absorbance of "standards" of known concentration, plot the absorbance of the standards, and derive the values for our unknown samples graphically from the values of our standards. The absorbance values of the standards may be graphed in two different ways, either relative to their protein concentration (µg/mL) or to the amount of protein (µg or mg). For these assays, the protein standard will be bovine serum albumin (BSA), a stable, inexpensive protein used for a variety of purposes.

Procedure

These assays are routinely performed using disposable glass tubes. After standards and unknown samples have been prepared and/or diluted, 0.2 ml of dye is added to 0.8 ml of each of the standards and samples. After a short wait to make sure all the dye has bound the protein, the absorbance of the sample is read using a spectrophotometer

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1. **MAKE A FLOW CHART.** Read through the entire procedure here, and consult the technical literature provided by the manufacturer of the dye (Bio-Rad). Save the flow chart you have prepared because you will be doing this assay many times throughout the purification project.

2. **TURN ON THE SPEC.** Turn on the spectrophotometer and set the wavelength to 595 nm.

3. **PREPARE THE STANDARDS AND BLANK.** Starting with a BSA stock solution of 1 mg/ml, make a series of dilutions so that the final concentrations range from 1 to 25 µg/ml. (You will need 2 mls of each solution in order to have duplicates.) These are your standards. Table 3.1 is designed to help you with your dilutions. The first line of the table has been filled out for you. Use this as a guide to complete the table. Remember that pipetting 1 µl or less is not very accurate.

4. Pipette 0.8 ml of each standard into a separate tube for assay.

5. In addition, prepare a control tube with 0.8 ml of buffer or water (depending on the diluent for the standards) that will serve as your blank for the

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spectrophotometer. (If your protein sample of unknown concentration is in a buffer, that buffer will be your blank.)

Table 3.1 BSA DILUTION TABLE FOR STANDARD CURVE

Tube	Final BSA Concentration	Volume of Stock Solution needed In µl for	Volume of H_20 µl
1	1.0 ug/ml	2	1998
2	5.0 ug/ml		
3	10.0 ug/ml		
4	20.0 ug/ml		
5	25.0 ug/ml		
6 (Blk)	0	0	1000

* To determine the amount of stock you will need to make the desired final protein concentrations, use the $C_1V_1 = C_2V_2$ equation, where C_1 and C_2 are the concentrations of the stock and the diluted sample respectively and V_1 and V_2 are the volumes of the stock and the diluted sample.

Sample Calculation:

$C_1V_1 = C_2V_2$

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C_1 = stock concentration of 1 mg/mL or 1000 µg/mL

V_1 = the unknown (volume of stock needed)

C_2 = concentration of BSA standard we are trying to make, 1 µg/mL

V_2 = volume of the standard we are trying to make, 2 mLs

1000 µg/ml x V_1 = 1 µg/mL x 2 mLs

Solving for V_1 we get an answer of 0.002 mLs or 2 µl

6. **PREPARE THE UNKNOWN SAMPLES.** In order for the assay to be valid, the unknown sample must be in the concentration range of the standards you have prepared, 1-25 µg/mL. Since you have no idea how much protein there is in the unknown sample, you should prepare several dilutions. What should you use as your diluent? Bear in mind that if none of those dilutions lie within the linear range of the assay, you will need to repeat the entire assay, standards and all. (Why do you have to repeat the ENTIRE assay?)

7. **CONDUCT A CHECK.** At this point you should have 5 standards, a blank (negative control) and several different dilutions of your ß-galactosidase sample. All standards, samples and the blank should have a volume of 0.8 ml. **Hold the rack of tubes up to your eye.** If the volumes do not appear to be the same, **do not proceed.** If the volumes are not accurate, the assay will not be accurate and you will be wasting reagents and time if you continue.

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8. **ADD DYE.** Add 0.2 ml of Bio-Rad dye to each of the assay tubes. Mix the tubes gently but thoroughly after the addition of the dye: either vortex **GENTLY** on a low power setting or mix gently by flicking. **Avoid foaming!**

9. **CHECK FOR COLOR.** After 5-15 minutes, check to see that color is developing. Solutions without protein should still be reddish brown. Solutions with protein should turn blue: the more concentrated the protein, the more blue the solution should be. You should be able to see the progression of the color in the standard tubes. If not, something is wrong.

10. **READ AND RECORD THE SAMPLES.** After a period of 10 minutes to 1 hour, read the absorbance of each tube at 595 nm on the DU64 Spectrophotometer using plastic cuvettes. The spectrophotometer should be calibrated using the blank (negative control stap 5). Then start with the most dilute solution and progress to the most concentrated. Be careful to thoroughly remove the solution from the cuvette each time after reading its absorbance. If you leave some solution in the cuvette, it will dilute the next standard and your curve will not be accurate. Save each solution by putting it back into its tube in case you need to read it again. Record your sample absorbance values in Table 3.1.

11. **PREPARE THE STANDARD CURVE.** Generate your standard curve by graphing the absorbance readings of your diluted BSA standards as a function of the protein concentration. The x-axis should be labeled "BSA (µg/µl)" and the y-axis "A_{595}". See Figure 3.1 below.

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12. **DETERMINE THE SAMPLE VALUES.** Figure out the concentration of protein in your unknown(s) by using your standard curve . Record the results in your data table.
13. **DO CALCULATIONS.** Figure out the concentration of protein in the original undiluted sample. (See the sample calculation below.)

Table 3.2. Bradford Assay Data Table.

Sample	Tube	Protein Conc µg/ml	Volume Added Volume of standard µl	Dye µl	OD 595 nm
Std	1	1.0	800	200	
Std	2	5.0	800	200	
Std	3	10.0	800	200	
Std	4	20.0	800	200	
Std	5	25.0	800	200	
Blank	6				
Unknown	9				
Unknown	10				

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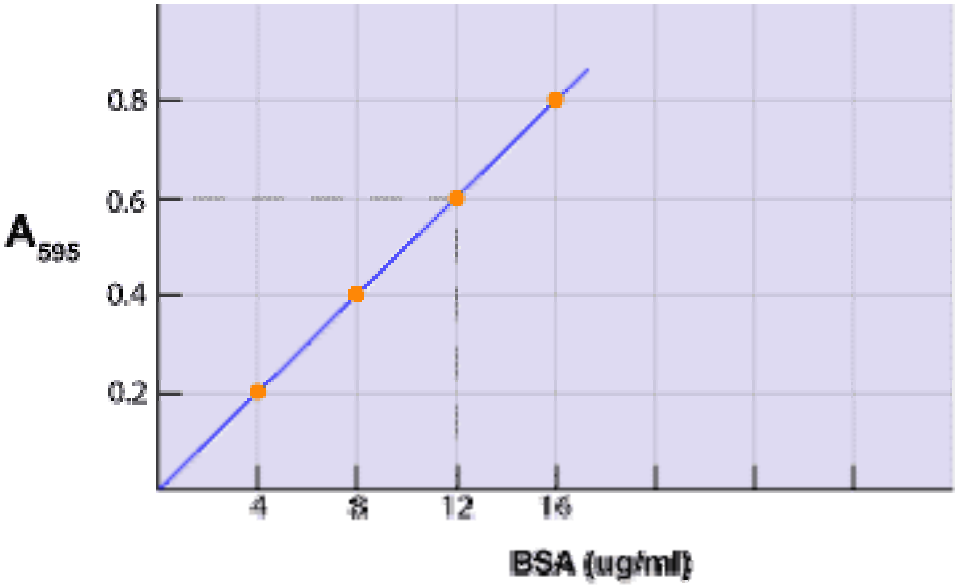

Figure 3.1. Sample Bradford Assay Standard Curve.

http://matcmadison.edu/biotech/

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Sample Calculation - Bradford Assay

- After performing the Bradford assay, make a graph by plotting the spectrophotometer absorbance readings vs. the concentration of each of the BSA standards. Draw a straight line through the points. If all the points do not lie on a line, draw a line of best fit. A sample standard curve is shown in figure 3.1 above.

- Using the spectrophotometer readings(s) for your unknown(s), determine the concentration (in µg/ml) for your unknown sample(s) from the standard curve. Suppose that your sample had an absorbance of 0.61. If you look at the graph above, this absorbance value corresponds to a protein concentration of 15 µg/ml.

- The concentration that you read from the graph in step 2 is the protein concentration of your diluted sample.

- Now you need to account for any dilutions that you made. If you took 20 µl of undiluted sample and 780 µl of water to make the 800 µl, that dilution is 20/800 or 1/40. You will therefore multiply the answer by 40. (If you made a 1/100 dilution, you will need to multiply by 100, etc., etc.)

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- If your dilution factor was 40, your original undiluted sample contains 600 µg/ml or 0.6mg/ml of total protein. (If your dilution factor was 100, then your undiluted sample contains 1500 µg/ml or 1.5 mg/ml

Procedure 3.2 The ß-Galactosidase Assay

Background

The basis of this assay is the biological activity of the enzyme: in this case, cleavage of the bond between the two sugars found in a family of disaccharides known as ß-galactosides. Because it has ß-galactosidase, *E. coli* is able to utilize lactose, the sugar found in milk, as an energy source. As a result of cleaving lactose, two monosaccharides are produced that can then be metabolized to produce energy through glycolysis and the citric acid cycle. When ß-galactosidase cleaves lactose, its natural substrate in *E. coli*, the reaction looks like this:

$$\text{lactose} \xrightarrow{\text{ß-galactosidase}} \text{glucose} + \text{galactose}$$

The natural products of this reaction are colorless and not readily detectable. Therefore, an assay system has been developed in which a synthetic sugar is cleaved,

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producing a colored product that absorbs light in the visible range. The artificial sugar is o-nitrophenyl-ß-galactoside (ONPG). The product, o-nitrophenol (ONP), is yellow and can be quantitated spectrophotometrically at 420 nm.

$$\text{o-nitrophenyl-ß-galactoside} \xrightarrow{\text{ß-galactosidase}} \text{o-nitrophenol (ONP)} + \text{galactose}$$

When more enzyme is present, more product will be formed and more yellow color will be seen. Enzyme assays must be performed at temperatures at which the enzyme is active and, for the sake of comparison, must always be performed at the same temperature; in this case, 37°C. Since measuring the activity of the enzyme is dependent on having an ample supply of substrate, you want the substrate to always be present in excess so that it is not limiting your ability to measure the enzyme. Therefore, if the assay tubes turns bright yellow immediately, the substrate is being used too quickly and the assay will not be accurate. Because the reaction continues as long there is substrate available, you will need to stop the reaction after a designated time in order to measure the absorbance consistently. This is done by adding $NaCO_3$, a strong base (pH 10-11) that denatures the enzyme and renders it inactive.

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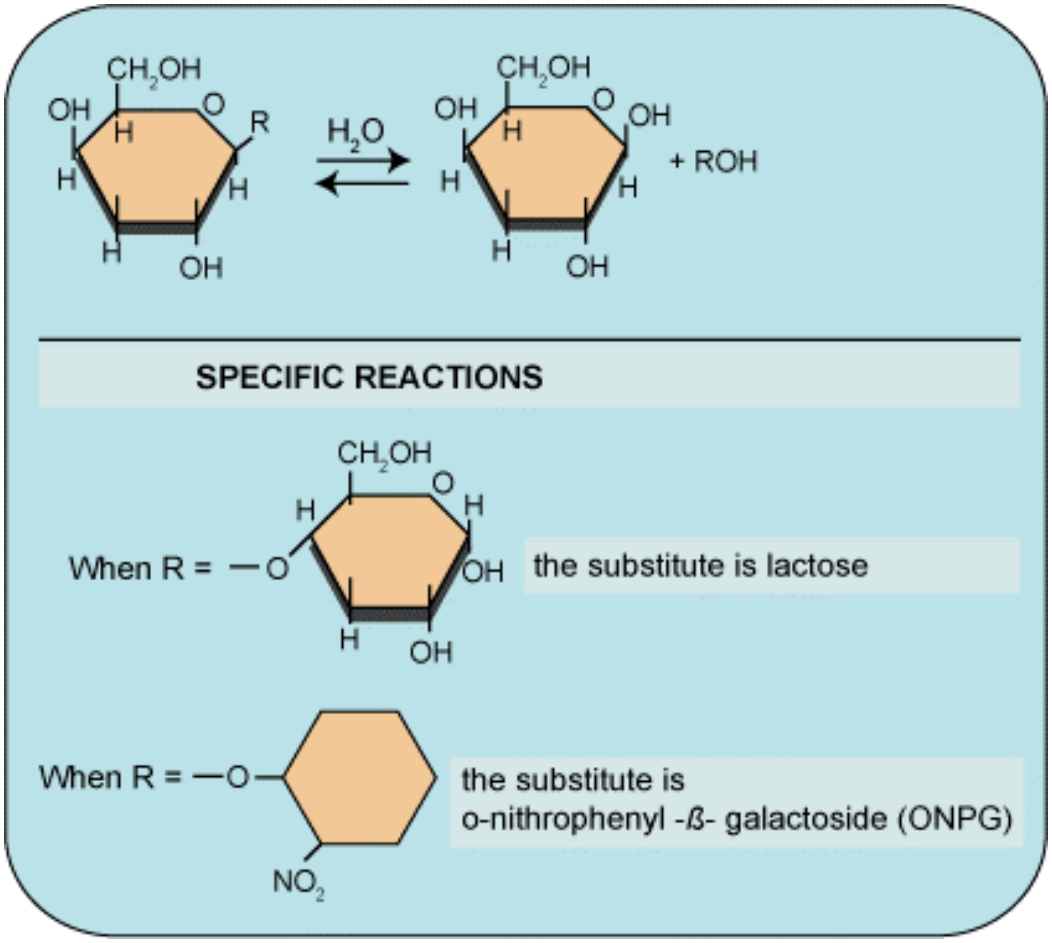

Figure 3.2: Reactions catalyzed by ß-galactosidase

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Overview of Assay

Using disposable glass tubes for these assays, you will add 10-50 µl of sample to 1 ml of Z buffer, a buffer in which ß-galactosidase has full activity. The Z buffer containing the sample and the ONPG substrate will then be separately pre-incubated to 37°C. The enzymatic reaction will begin as soon as you add the substrate to the samples. After you incubate the tubes for a sufficient length of time, you will terminate the reaction in all the tubes, using the STOP solution ($NaCO_3$). Read the sample absorbance on the spectrophotometer after transferring the contents of the tube to a plastic cuvette.

1. **MAKE A FLOW CHART.** Begin your preparations by making a flow chart or outline that details exactly what you are going to do in this assay. Save this flow chart as you did for the Bradford Assay and modify it as you refine and streamline your technique.

2. **TURN ON THE SPEC.** Turn on the spectrophotometer and set the wavelength to 420 nm.

3. **DILUTE THE SAMPLE(S).** In your first assay you will be using a sample of ß-galactosidase provided by your instructor. It will be your job to determine the activity of this sample. (After starting the purification, you will be using your own samples.) Prepare a few dilutions of this sample. What will be the diluent?

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4. **ADD THE SAMPLES AND CONTROL TO Z BUFFER**. Add 10-50 µl of the diluted sample(s) to 1 ml of Z buffer. As always, remember to write down how much sample you used.

5. Prepare a control tube by adding a volume of buffer that is equal to the volume of sample used in step 4 (10-50 µl) to a tube containing 1 ml of Z buffer. This functions as a control for the spontaneous hydrolysis of your substrate, ONPG, and will serve as your calibration blank for the spectrophotometer.

6. **EQUILIBRATE.** Equilibrate the samples to be assayed to 37°C. Make sure the ONPG (4 mg/ml in 0.1 M sodium phosphate buffer pH 7.5) is also at 37°C. Prepare fresh ONPG solution each day.

7. **BEGIN THE REACTION**. Add 0.2 ml of ONPG to each tube and record the time.

8. **INCUBATE.** Incubate the samples at 37°C until yellow color appears. The color change should take at least 10 minutes but may take longer to result in an absorbance of 0.3 to 0.9. A very rapid color change (1-5 minutes) indicates that there is too much activity to measure accurately. Such samples should be repeated at a higher dilution. Samples containing low concentrations of ß-galactosidase may take significantly longer than 10 minutes to become yellow. (Why is a rapid color change (0.5 O.D. in 2 minutes) not accurate?)

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9. **STOP THE REACTION**. Add 0.5 ml of 1 M $NaC0_3$ (Stop Solution) to terminate the reaction and record the time.

10. **READ THE SAMPLES**. Transfer the samples to plastic cuvettes and read the A_{420} against the blank.

11. **DETERMINE THE ENZYME ACTIVITY**: Calculate the enzyme activity present in your sample using the formula shown below:

$$\text{Units of ß-galactosidase} = \frac{A_{420} \times 0.380}{\text{minutes at } 37^\circ \text{ C}}$$

One unit is the amount of enzyme that will hydrolyze 10^{-6} moles/minute of ONPG at 37°C and 0.380 in the above equation is the constant used to convert the A_{420} reading into these units. This constant is a function of the molar extinction coefficient for ONP+ that, under these conditions, is 4500 $M^{-1}cm^{-1}$. What is a molar extinction coefficient? (Refer to Seidman and Moore, chapters 19-21.)

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Sample Calculation: ß-galactosidase Assay

- Let's say that you started with an undiluted sample of ß-gal from which you made a 1/100 dilution. You took 10 of the diluted sample and added it to Z buffer to do your assay. After 11 minutes your sample had an A_{420} of 0.412 versus the blank.

- The A_{420} in this example equals 0.412. Time in this example equals 11 min. Plugging these values into the formula shown above results in 1.42×10^{-2} units of ß-galactosidase activity:

$$\text{Units of ß-gal} = \frac{0.412 \times 0.380}{\text{11 minutes at 37°C}}$$

  The answer is 0.0142 units.

- You then use a proportion to determine the number of units of ß-gal in the diluted sample from which you took your 10 µl.

$$\frac{1.42 \times 10^{-2} \text{ units}}{10 \text{ µl}} = \frac{? \text{ units}}{1000 \text{ µl}}$$

$$? = 1.42 \text{ units/ml}$$

- You have found that you have 1.42 units/ml in the sample you assayed. Now you need to account for your dilution. For the 1/100 dilution you made, you

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multiply 1.42 units/ml by 100. Therefore you have 142 units/ml of ß-galactosidase in the original undiluted sample.

Sample Calculation: Specific Activity

Specific activity is a convenient method of communicating the amount of enzyme activity you have in each milligram of protein. Specific activity is calculated after each purification step and can be used to monitor the success of that step. To do this calculation you need to know much enzyme activity there is in your sample as well as how much total protein is present. You can calculate the specific activity using the formula below:

$$\text{S.A.} = \frac{\text{units/ml}}{\text{mg total protein/ml}}$$

For practice, use the values from the two previous sample calculations, 142 units/ml for enzyme activity and 0.6mg/ml for total protein. The specific activity would then be 142 units/ml divided by 0.600 mg/ml or 237 units/mg.

##Note: Specific activity may be lower than expected if the enzyme has been denatured and therefore is no longer active. The protein assay will still detect denatured protein; therefore, the protein part of the calculation will not decrease but the enzyme activity will.

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Sample Calculation: Purification Factor

The purification factor (also called enrichment) is a comparison of the specific activity at some point in the purification process to the specific activity at the beginning of the purification. The formula for purification factor is as follows:

$$\frac{\text{Specific activity of separation step}}{\text{Specific activity of crude extract}}$$

Since the specific activity should increase with each separation step, the purification factor should always be greater than 1. The purification factor has no units since the units of specific activity are in both the numerator and denominator and will cancel out.

For example, the protein concentration for the crude extract may be 1000 mg/mL and the enzyme activity might be 30,000 units/mL, leading to a specific activity of 30 units/mg.

After the second step, the protein concentration may be 100 mg/mL and the enzyme activity could be 25,000 units /mL. So the specific activity would have improved from 30 units/mg 20 250 units/mg. Although you may have lost some, you removed a lot of contaminating proteins so the specific activity improves from 30 units/mg to 250 units/mg.

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To express this as a purification factor:

$$\frac{250 \text{ units/mg}}{30 \text{ units/mg}} = 8.33$$

So, the purity has improved by a factor of 8.33. Or, to say it a different way, the protein is 8.33 times more pure than it was when you started.

Sample Calculation. Yield

Yield is the total amount of product that you have. For enzymes, yield is best expressed in total units of enzymatic activity so you would use the results of your enzyme assay to determine the yield. Yield will decrease as the purification proceeds because losses are inevitable as you manipulate the extract.

Yield is **not** expressed as a concentration because this does not tell you the total amount you obtained. For example, If you are a strawberry farmer and you have 18 bushels of strawberries per row, what would be your strawberry yield if you have 50 rows of strawberries planted? If you report the yield as 18 bushels per row, it has no meaning since no one knows how many rows you have. It is the same idea with enzyme activity. Do not report the yield of enzyme activity in units/ml since no one knows whether you have 1000 ml or 0.25 ml.

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Yield of enzyme activity in units = $\frac{\text{units}}{\text{ml}}$ x total number mL

If your enzyme activity is 142 units/ml and you have 43 mls, then your yield of enzyme activity is 6106 units.

Sample Calculation Percent Yield

To report your yield as a percent yield, you need to know how many units of enzyme activity you have after this step and how many units of enzyme activity you started with. Suppose that you want to compare the amount of activity after the dialysis step to the total amount of enzyme that was originally present in the crude extract. In this case you divide the number of units in the dialysate by the total number of units in the crude extract and multiply by 100%. This is the percent yield of enzyme activity.
Suppose that you have 100,000 units of enzyme activity in the crude extract and you have 25,000 units left by the time you get to the dialysis step.

$\frac{\text{25,000 units}}{\text{100,000 units}}$ x 100% = 25% Therefore the percent yield is 25%

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How to summarize results of purification:

It is customary to communicate the results of a purification project by making a table such as the one below (Table 3.3).

The Yield of ß-galacosidase should go down the specific activity should go up as the purification proceeds. Why? Make sure you Understand this

Table 3.3: This is actual data from a student purification. Does your data look similar?

STEP	Enzyme Units/ml	Protein Mg/ml	Total Volume	Yield Units in total volume	Percent Yield	Specific Activity	Purification Factor
Crude Extract	343.9	5.53	37 mls	17,195	100%	62.24	1
AS Pellet	1240.7	6.99	4 ml	4962.8	29 %	177.66	2.85
Dialysate	478.8	2.24	4.8 ml	2298.2	13 %	213.83	3.45

References:

Bradford, M (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 722:248-254.

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Chapter 4
Purification Methods

This section will give you step-by-step procedures for purifying ß-galactosidase. In addition, it is designed to give you some background information for each of the different techniques in this purification strategy. For more detailed background information, and for information about other separation techniques, the textbook and resource materials in Appendix C.

Separation Methods: As described in Chapter 2, there are various techniques used to separate proteins from one another. Each of these techniques is based on a particular property by which proteins differ from one another. Some properties of proteins that are used as a basis for separation techniques include:

- **C**harge
- **H**ydrophobicity
- **A**ffinity- Biological affinity for another molecule (the ability to bind to another molecule)
- **S**olubility or Stability- Sensitivity to the effects of environmental conditions, such as heat
- **M**olecular Weight (size)

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The acronym **CHASM** is often used to summarize this list of properties. In order to purify a particular protein, it is usually necessary to use several separation techniques in a series. We will take advantage of differences in solubility when we perform the ammonium sulfate precipitation, procedure 4.2. We will use size as a basis for desalting and buffer exchange in dialysis, procedure 4.3. Size is also the basis for SDS PAGE analysis in Chapter Five. We will take advantage of differences in charge when we perform the ion exchange chromatography procedure, procedure 4.4. We will not be using hydrophobic properties or biological affinity as the basis for the methods in this purification strategy, although these techniques are widely used and will be discussed in lecture.

Resolution

Separation methods are often categorized according to the degree of resolution. Resolution refers to the resolving power of a method - its ability to separate (resolve) two proteins that are similar in properties. For example, a high resolution method that separates by size, is capable of separating two proteins that have very similar molecular weights, such as 50,000 daltons and 52,000 daltons. A low resolution method could only separate two proteins if they were very different in size such as 100,000 and 50,000 daltons. Ammonium sulfate precipitation is an example of a low resolution method while ion exchange chromatography is a high resolution method.

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Capacity

Separation methods are often described as being either low or high capacity techniques. As the name implies, this characterization refers to how much volume the technique can handle. Although, in principle, any method can be scaled up to increase the capacity, a low capacity technique would be more difficult (or prohibitively expensive) to scale up. Some forms of column chromatography are more amenable to scale-up than others. For example, ion exchange can be scaled up to a high capacity method quite easily whereas gel filtration chromatography is more suited for smaller sample volumes.

Preparative or Analytical

The same separation method can be used for either a preparative or an analytical purpose. An "analytical" procedure is performed primarily to obtain information: for example, to analyze the results of a purification step. For analytical procedures, aliquots of the protein mixture are used and are generally thrown away after the analysis is complete. In a preparative step, the entire protein mixture, not an aliquot, is used for the purification step since the purpose is to separate the target protein from contaminants in a non-destructive manner.

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Common Themes for Purification Strategies

Although each purification strategy is unique, there are some common themes. Generally, high capacity techniques with low resolution are used earlier in the purification strategy and higher resolution steps are used later in the process. Steps that result in dilution of the target protein sample are usually followed by steps that concentrate the volume of the sample. At some point, desalting and/or buffer exchange is usually necessary and either dialysis, gel filtration or ultrafiltration may be used for that purpose.

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Overview of our Purification Strategy:

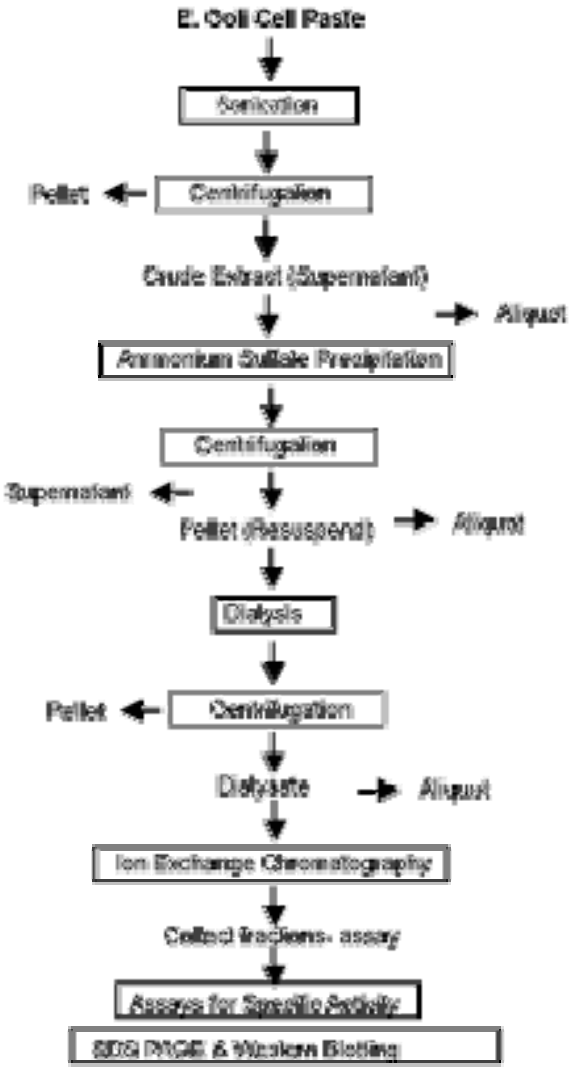

Figure 4.1. Flow chart.

http://matcmadison.edu/biotech/

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The ß-galactosidase purification strategy used in this manual is outlined in Figure 4.1 in a flowchart format. As a first step, a crude extract will be made using sonication, followed by ammonium sulfate precipitation as a high capacity, low resolution step to remove some contaminating proteins and concentrate the volume of extract. Excess salt is then removed by dialysis, and the sample is loaded on an ion exchange column for a high resolution separation step. We will analyze the success of the purification by specific activity calculations, by SDS PAGE analysis and finally by Western Blotting.

Although we will stop the purification after the ion exchange step, this would probably not be the final step in most research or industrial situations. Even if the product was judged to be "pure", the salt in the NTM buffer used for elution would probably be removed using ultrafiltration, tangential flow filtration or another "polishing" method appropriate to the scale of the purification process. If the product was to be used as a drug, more rigorous tests to determine purity would be used including HPLC methods, endotoxin testing, etc.

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Aliquots and Assays:

Aliquots will be saved after each separation step before proceeding with the next step. Some aliquots will be used right away for enzyme assay to verify that the majority of the enzyme activity has been captured by the technique.

The specific activity is usually determined after each purification step; however, it is possible to postpone protein assays until a more convenient time, as long as you save aliquots from each step. For example, you could begin dialysis before you complete the protein assay needed for specific activity determination of the AS pellet. However, do not start the dialysis until you know that the sample contains the majority of the enzyme activity.

Some aliquots will be stored and saved for later use after all preparative purification procedures are completed. These aliquots will be used for SDS PAGE and HPLC (optional) as described in section 4.

As you proceed through the purification procedures, the total mass of all proteins (as measured by the BioRad assay) should decrease since you are removing contaminating proteins at each step. The total enzyme activity will also decrease somewhat but should not decrease as dramatically since you are trying to keep as much of the target

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protein as possible. Therefore, the specific activity should increase as your protein becomes more pure.

Section 4.1 Making the Crude Extract

Background

The first step in protein purification is to make a cell-free extract from the source material so that the target protein is in solution. The source material consists of any biological material that contains an abundant source of the target protein. Source material may be a culture of microorganisms, cells grown in culture, plants, or tissues isolated from an animal, such as liver, lung, ovary, muscle, brain. When using cultured cells or microorganisms, if the target protein is secreted outside the cell, no cell disruption step is needed-- it is only necessary to spin down the cells and harvest the supernatant. However, most proteins are located inside the cell and must be released into solution by some type of disruption process. Disruption procedures vary from gentle (osmotic lysis) to harsh (sonication) depending on the cell type or tissue type that is the source of your target protein (Table 4.1). For example, making an extract from red blood cells requires only a mild disruption step while extracting plant tissue requires a much harsher process in order to break down the tough cell walls of the plant.

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ß-galactosidase is an intracellular protein made by *E. coli*. Like many bacteria, *E. coli* is encased in a tough cell wall to protect it from a potentially hostile environment. In this procedure, you will be using a method called sonication to break open the bacterial cells. Sonication damages the cell wall by high frequency sound waves. The cells are disrupted in a buffer that has been chosen to keep the target protein in an active form. After the cell disruption step, cells and other insoluble debris are separated from the extract by centrifugation. If the cells have not been completely disrupted, much of the protein will not be released into solution, and will be lost with the cell debris.

Table 4.1 Methods of Cell Disruption

Technique	Example	Principle
Gentle		
Enzyme Digestion	Lysozyme treatment of bacteria	Cell wall digested, leading to osmotic disruption
Hand Homogenizer	Liver tissue	Cells forced through narrow gap
Mincing, (grinding)	Muscle	Shear force
Moderate		
Blade Homogenizer	Muscle tissue, most animal tissues, plant tissues	Chopping action breaks up large cells, shears apart smaller ones
Grinding with abrasive (Alumina, sand)	Plant tissues Bacteria	Microroughness rips off cell walls

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Vigorous		
French Press	Bacteria, plant cells	Cells forced through small orifice at very high pressure; shear force disrupts cells
Ultrasonication	Cell suspensions	Microscale high-pressure sound waves cause disruption by shear forces and cavitation
Bead mill	Cell suspensions	Rapid vibration with glass beads rips cell walls off
Manton-Gaulin homogenizer	Cell suspensions	Same as French press but larger scale

Extraction Buffer: The composition of the extraction buffer is important for maintaining structure and function of the target protein and is based on the the unique characteristics of the target protein. The buffering pH is based on the pH stability range of the protein. Other components such as ionic strength, divalent cations (Ca^{++} and Mg^{++}), or reducing agents (dithiothreitol or ß-mercaptoethanol) may be needed to maintain activity. In making the extract, cells are lysed and proteases (enzymes that degrade proteins) are released from their intracellular compartments. These enzymes may damage the target protein. To prevent proteases from digesting the target protein, two strategies are commonly followed:

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The extract is kept cold. The activity of proteolytic enzymes is greatly reduced by cold temperatures. For this reason, the protein purification process is often conducted in cold rooms. At the very least, an effort is made to keep the extract at 4°C.

Protease inhibitors are sometimes added to the mixture to prevent degradation by proteases. The drawback to this strategy is that the inhibitors must eventually be removed, along with other contaminant proteins.

The extraction buffer for this ß-galactosidase purification is called Breaking Buffer and the recipe is found in Appendix A. The dithiothreitol (DTT) should be added just before use since it breaks down over time. Sonication generates a lot of heat and it will be necessary to use an ice bath with ethanol to preserve activity of the target protein.

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Procedure 4.1

1. Examine the sonicator. Find the probe of the sonicator within the soundproof box. Find the ring stand, clamps, etc that will be used to position the sample in the chamber of the sonicator.

2. You will receive a sample of frozen *E. coli* weighing approximately 8 grams. The exact weight should be marked on the tube. Thaw the cells on ice. Note: usually,100mLs of culture will yield about a gram of wet cell paste.

3. While the cells are thawing, choose a container for your sample. The container should be small enough so that the sonicator probe is immersed deeply in the sample but large enough so that the probe does not touch the sides or bottom of the container. (50 mL glass tubes with round bottoms work well.)

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4. Resuspend your thawed cells in breaking buffer. The necessary volume (in ml) of breaking buffer is determined by multiplying the weight of the cells (in grams) by a factor of five. (Remember that you are expected to add dithiothreitol (DTT) to the breaking buffer. Check the recipe in Appendix A to find out how much to add.) So if you have 8 grams of cells you will need 40 (8 x 5) mL of breaking buffer to resuspend the cells. The cell paste is viscous and a rubber policeman will probably be necessary – **gentle** vortexing is permitted at this stage only.

5. Prepare an ethanol ice bath in a beaker and place the beaker securely on a ring stand in the sound proof box of the sonicator.

6. Arrange everything so that the probe will be prevented from touching the sides of the glass while operating. It is useful to move the tube up so that the probe touches the bottom and then back off so that the probe does not touch the bottom. Make sure everything is clamped securely to prevent any slippage.

7. Lyse your cells with the sonicator using a 50% power setting for 12 minutes. We are using a Branson Sonifier 250. For other models follow the manufacturer's recommendations.

8. Pre-cool the centrifuge and rotor that you will be using to 4°C. Determine the appropriate rotor speed to obtain 10,000xg in the rotor.

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9. Clean thoroughly a centrifuge bottle or tube such as the 250 mL polycarbonate centrifuge bottle (hard clear plastic). Pour your sample into this bottle and prepare another bottle for balance or balance with another group's sample. Do not use polystyrene or polypropylene tubes (blue cap conicals) at these speeds.

10. Spin out the cell debris at 4°C for 30 minutes at 10,000 xg. (Don't forget to fill out the centrifuge log book.)

11. Remove the supernatant. (You may use a pipette or pour off the supernatant gently into a clean container.) This supernatant is your crude extract. Save the pellet until enzyme assay verifies that enzyme activity is present in the extract. Record the volume of the extract and save 3 ml in approximately 0.5 ml aliquots for assays. Store the aliquots in the refrigerator. Remember to write in your lab notebook where the aliquots are stored. Store the crude extract at 4°C – the entire volume of crude extract (except for the aliquots) will be used for the next purification step, ammonium sulfate precipitation.

12. **Optional: Compare the effect of storage temperature on enzyme activity over time**. Try storing one aliquot in the refrigerator and one in the freezer to see whether you enzyme is more stable over time at 4°C or -20°C.

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Assays

At this point, you will need to determine the enzyme activity of the crude extract according to the enzyme assay procedure outlined in Section 3.2. In the enzyme assay procedure, recall that you are adding 10-50µl of sample to 1 ml of Z buffer. After the sonication and centrifugation step, your sample is in breaking buffer so dilutions should be made in breaking buffer before removing an aliquot of 10-50µl and adding it to the Z buffer. What will be the composition of your blank tube?

- Once you have determined that you have enzyme activity in your extract, proceed with the next step in the purification, ammonium sulfate precipitation, Procedure 4.2.
- As time permits, determine the protein concentration and specific activity according to the assay methods in Chapter 3.

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Alternate Extraction Method

Procedure 4.1b; BugBuster™ reagent from Novagen,

1. You will receive a sample of frozen *E. coli* weighing approximately 8 grams. The exact weight should be marked on the tube. Thaw the cells on ice.

2. Verify that Dithiothreitol (DTT) has been added to the BugBuster™ buffer to a final concentration of 5 mM.

3. Resuspend the cell paste in room temperature BugBuster reagent using 5 ml reagent for each gram of wet cell paste**. DO NOT ADD** this volume all at once. The cell paste is viscous and a rubber policeman will probably be necessary – **gentle** vortexing is permitted if necessary **at this stage only.**

   Recommended method: Add a couple mLs of buffer to the cell paste and gently rock the tube back and forth (tapping is OK), work with a rubber policeman as needed. Repeat the process with another ml or two of buffer each time until you have suspended all the cell paste (or MOST of it). It is a good idea to avoid frothing and bubbles. (Why?)

4. Incubate the cell suspension on a shaking platform or rotating mixer at a slow setting for 10-20 minutes at room temperature.

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5. Pre-cool the centrifuge and rotor that you will be using to 4°C. Determine the appropriate rotor speed to obtain 16,000xg in the rotor. (check Appendix D for a centrifugation chart)

6. Clean thoroughly a centrifuge bottle or tube. Pour your sample into this bottle and prepare another bottle for balance or balance with another group's sample. Do not use polystyrene or polypropylene tubes (blue cap conicals) at these speeds.

7. Spin out the cell debris at 4°C for 20 minutes at 16,000 xg. (Don't forget to fill out the centrifuge log book.) (10,000xg for 30 minutes will also work)

8. Remove the supernatant. (You may use a pipette or pour off the supernatant gently into a clean container.) This supernatant is your crude extract. Record the volume of the extract and save 0.5 ml in approximately 0.1 ml aliquots for assays. Store the aliquots in the refrigerator. Remember to write in your lab notebook where the aliquots are stored. Store the crude extract at 4°C – the entire volume of crude extract (except for the aliquots) will be used for the next purification step, ammonium sulfate precipitation.

   Although you expect most of the activity to be in the supernatant, resuspend the pellet and save it for later enzyme assay to compare to the extract.

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Alternate Extraction Method: Procedure 4.1c

Acceptable disruption can also be achieved using a mortar and pestle and an abrasive, inert material such as glass beads or alumina. Alumina (aluminum oxide) is a fine white material similar in appearance to salt.

1. Freeze the *E. coli* cell pellets on parafilm (about 2 gms). After an hour at -20°C, place the cells in a mortar and pestle (prechilled) and grind them with 2-3 times the cell weight of alumina (Sigma #) for 20 minutes.

2. Add 5 cell volumes of breaking buffer and continue to work with the mortar and pestle for about 5 minutes. The volume (in ml) of breaking buffer is determined by multiplying the weight of the cells (in grams) by a factor of five. (Remember that you are expected to add dithiothreitol (DTT) to the breaking buffer. Check the recipe in Appendix XY to find out how much to add.)

3. Pour the slurry into a centrifuge tube and spin out the glass beads and cell debris for 20 minutes at 10,000 xg in a refrigerated centrifuge. (If a superspeed centrifuge is not available, a clinical centrifuge may also be used for 30 minutes at top speed although it will not be as effective.)

4. Remove the supernatant and take aliquots as described in procedure 4.1 a step 11.

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Section 4.2 Salting Out: Ammonium Sulfate Precipitation

Background

Proteins vary greatly in their solubility depending on their amino acid composition and the characteristics of the solvent such as ionic strength and temperature. A common approach for separating proteins is to add increasing concentrations of a salt in order to "salt out" the proteins. Salting out is often used as an early step in a purification strategy. Ammonium sulfate is commonly chosen as the salt since high concentrations can be achieved and since it does not usually denature proteins. Even if a significant increase in purity is not achieved, an ammonium sulfate "cut" can be used to concentrate a large volume of extract for the subsequent purification steps.

"Salting out" is thought to work by "dehydrating" the environment around the protein. When ammonium sulfate is added to the protein solution, a large number of water molecules bind to the sulfate ion, which reduces the amount of water available to bind to the protein. If a protein is not hydrated by binding to water molecules, it will precipitate. Different proteins precipitate at different concentrations of ammonium sulfate. We can take advantage of this difference to remove some contaminating proteins from our crude extract.

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In this ß-galactosidase purification, we will use precipitation with ammonium sulfate as our first step in the purification process. We know from published papers that ß-galactosidase precipitates when the concentration of ammonium sulfate reaches 33% of saturation. (We could also determine this on our own by trial and error.) It has been determined that 100 ml of water is saturated with ammonium sulfate when it contains 70 g. 33% saturation would be 23.1 g per 100 mls or 231 mg per ml. That is the amount we will use in step 3 below.

Procedure 4.2: Ammonium Sulfate Precipitation

1. Pour the cell free extract (the supernatant from procedure 4.1) into a beaker with a stir bar, allowing for a 50% volume increase. Prepare an ice bath with water (an ethanol ice bath can actually freeze the sample) to contain the beaker with the sample in it.

2. Add crystalline ammonium sulfate **slowly** over a period of 30-45 minutes with gentle stirring, adding a total of 231 mg $(NH_4)_2SO_4$ per ml of starting solution. **Keep your sample on ice at all times.**

3. Add 1 ml of 1 M NaOH for every 10 grams of ammonium sulfate. Let stirring continue for 1-3 hours.

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4. Split the volume of your sample equally into two centrifuge tubes so that you can balance them against each other. (Why?) Centrifuge the entire sample volume at 10,000 xg for 10 minutes. Use the swinging bucket rotor with 40 ml centrifuge tubes. (If you use larger bottles, the pellet can spread out too much to recover easily.)

5. Although the ß-galactosidase activity should be in the precipitate, decant and save the supernatant. Refrigerate the supernatant after labeling it. (It is always good practice to save both the supernatant and precipitate until you verify the location of your target protein)

6. Resuspend the AS pellet in 1/10 the original volume of breaking buffer (step 4.2).

7. Remove 0.2-0.4 ml in 0.1 ml aliquots for later assay and analysis steps.

8. The resuspended pellet may be stored at 4°C. (Did you remember to record the volume of resuspended pellet?) The entire volume of the AS pellet will be used in the dialysis step in procedure 4.3 below. Therefore, you need to save aliquots before moving on to dialysis.

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Assays

- Perform enzyme activity assays. Calculate the % of total units in the pellet and in the supernatant. Determine the % yield.

- Once you have determined that you have enzyme activity in your AS pellet, proceed with the next step in the purification, dialysis, Procedure 4.3. As time permits (after dialysis is started), determine the protein concentration using the Bio-Rad assay and calculate specific activity according to the assay methods in Chapter Two.

Section 4.3: Dialysis: Desalting and Buffer Exchange

Background

At this point your target protein, ß-galactosidase, is in a solution with a high concentration of salt, ammonium sulfate. The excess salt must be removed because it will interfere with the process of ion exchange chromatography, the next purification step. In addition, a different buffer is needed for the ion exchange chromatography step. There are several methods that can be used for desalting and/or buffer exchange.

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We will use dialysis, because it is relatively simple to set up and our sample size is small.

Dialysis tubing is a semi-permeable membrane available in a wide range of size dimensions and pore sizes (molecular weight cut-offs). Our procedure uses dialysis tubing with a 10,000 Dalton molecular weight cut-off. Our extract is placed inside the tubing and the ends are sealed off. The tubing is then suspended in a large volume of the desired buffer solution. The pores in the membrane allow molecules that are smaller than the pores to move freely across the membrane. Therefore, the ammonium sulfate ions will cross out of the tubing into the buffer. Eventually, an equilibrium is achieved where the concentration of ammonium sulfate is equal inside and outside of the tubing. However since the volume outside the tubing is much greater than inside, and this outside volume is replaced with fresh buffer, over time, most of the salt will leave the tubing. Larger molecules (such as most proteins) are retained within the membrane. Since most buffer components are small molecules and can therefore pass through the pores, dialysis is also used as a method for changing buffers.

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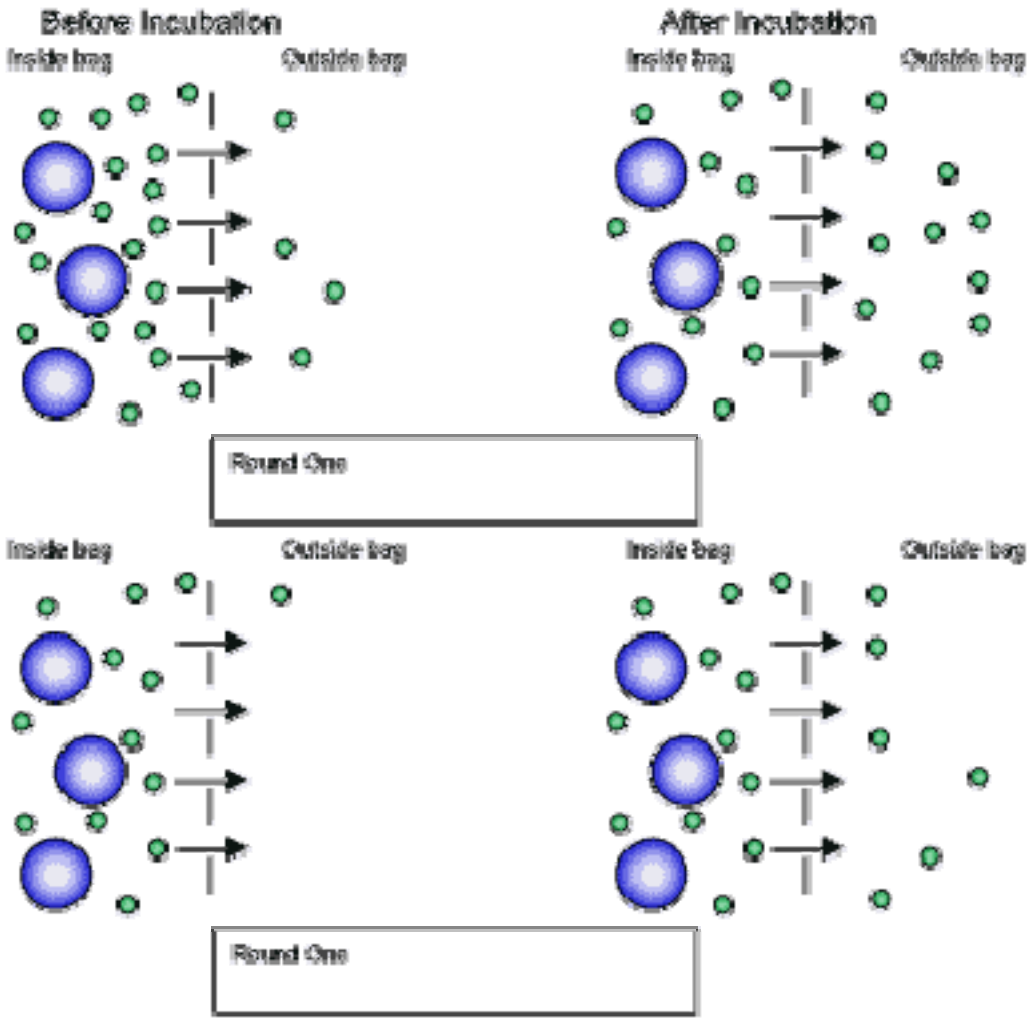

Figure 4.2.
Mechanism of Dialysis

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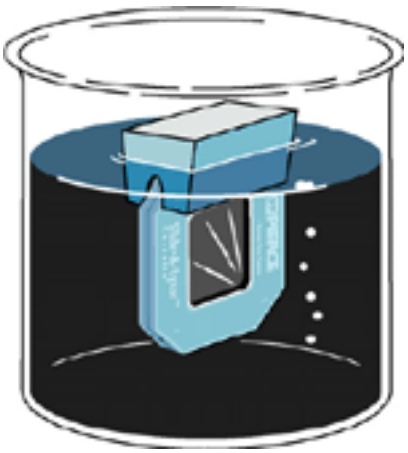
Other methods for desalting and buffer exchange: Gel filtration chromatography is another method of desalting because it separates molecules on the basis of size. Smaller molecules can move inside the gel beads and therefore progress more slowly through the column than larger molecules, such as proteins, which remain outside of the gel beads. If the column has been equilibrated with the new buffer, the protein will elute in the new buffer, leaving the salts from the old buffer in the column. Tangential flow filtration is a high capacity method that is similar, in principle, to dialysis but large volumes can be applied to the same membrane system since the volume containing the target protein is recirculated through the same membrane system using a pump.

Procedure 4.3: Dialysis

Procedure 4.3a: Slide-A-Lyzer™ Dialysis (Pierce)

1. Read over the protocol in the Pierce product literature before you start. Locate materials, including large beaker with buffer, syringe (10-20 mls) with 18-Gauge, 1 inch beveled needle. Hold the cassette by the plastic frame only – do not touch membrane. You will need to locate the ports that are located at each corner of the cassette. Mark the ports as you use them and do not use them more



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than once. Do not allow needle to touch membrane. CAUTION: The beveled portion of the needle should penetrate the gaskets to only a slight degree – avoid overextending the needle into the cavity as it may puncture the membrane.

2. Hydrate membrane by placing cassette in 0.2M NTM buffer according to directions.

3. Fill syringe with sample, leaving a small amount of air in the syringe. (As noted in the product literature, maximum sample size for (NH4)2SO4 samples is 80% of maximum or 12 mls.)

4. With the bevel sideways, insert the tip of the needle through one of the syringe ports located at a top corner of the cassette.

5. Inject sample slowly. With the syringe needle still inserted in the cavity, draw up on the syringe piston to remove air from the cassette cavity. This will compress the membrane so that the sample contacts the greatest window surface area.

6. Remove the syringe needle from the cassette while retaining air in the syringe. Label the port that you have used and do not use this port again.

7. Attach the cassette to the buoy and place unit in beaker with buffer and stir bar.

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8. Place the beaker or flask on a stir plate and stir at least two hours (overnight is OK) **in the cold**.

9. Change the buffer after allowing at least another two hours of stirring. Repeat this process two more times for a total of three changes over at least an 24 hour period. Ideally, you should not take longer than 2 days to accomplish three changes.

To remove sample after dialysis is complete:

10. Fill the syringe with a volume of air equal to the sample size and, with the bevel sideways, insert the tip of the needle through a different syringe port located at a top corner of the cassette.

11. Inject air slowly into the cassette to separate the membranes.

12. Turn the unit so that the needle is on the bottom and allow the sample to collect near the port. Withdraw sample into the syringe.

13. Centrifuge the dialysate at 10,000 xg for 10 minutes to remove any insoluble material. Carefully separate the supernatant from the pellet.

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14. Record the volume of the supernatant and remove 0.2-0.4 ml in 0.1 ml aliquots for assay. The supernatant that you will use for further purification may be stored in the refrigerator.

Procedure 4.3b: Dialysis Tubing:

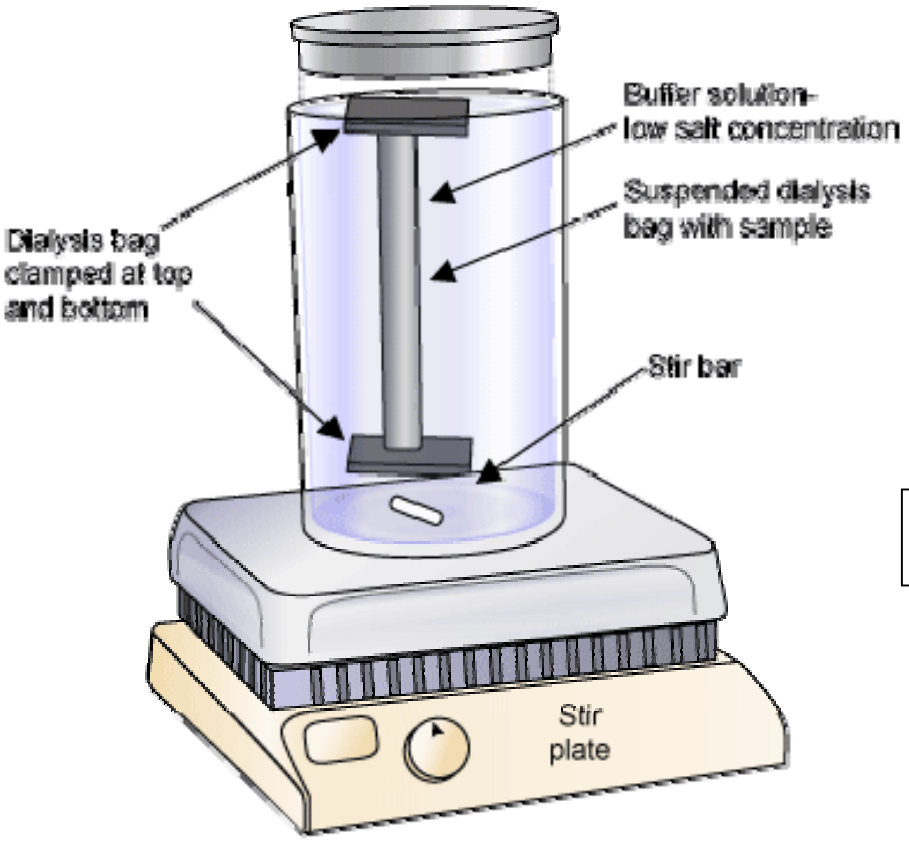

Figure 4.4 Dialysis Tubing

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1. Read over the protocol completely before you start.

2. Figure out how much dialysis tubing you will need to hold your sample and cut a piece that has an extra couple of inches. Follow manufacturers directions. **Always handle dialysis tubing with gloves.**

3. Wash your dialysis tubing. Place the tubing in a beaker with water. Place the beaker in the cold and stir for at least 2 hours. This will remove impurities, such as antifungal and antibacterial agents with which the tubing has been treated. **Never let your dialysis tubing dry out once it has been wetted.**

4. When washing is completed, remove the water carefully from the tubing. Clip the bottom of the tubing with a dialysis clip. Carefully pipette your sample into the dialysis tubing, being careful not to puncture the tubing. Also, don't spill your sample! Clip the top and attach a string to the top of the tubing.

5. Place the tubing in a beaker or flask containing approximately 1000 ml of 0.2 M NTM buffer and tape the string to the side of the container. Did you remember to add DTT to the NTM? (You may share beakers with other groups.)

6. Place the beaker or flask on a stir plate and stir at least two hours (overnight is OK) **in the cold**.

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7. Change the buffer after allowing at least another two hours of stirring. Repeat this process two more times for a total of three changes over at least an 24 hour period. Ideally, you should not take longer than 2 days to accomplish three changes. Note:

8. Carefully remove the dialysate from the tubing with a pipette and squeeze out as much as possible of whatever is left into a centrifuge tube.

9. Centrifuge the dialysate at 10,000 xg for 10 minutes to remove any insoluble material. Carefully separate the supernatant from the pellet.

10. Record the volume of the supernatant and remove 0.2-0.4 ml for assay. The supernatant that you will use for further purification may be stored in the refrigerator.

Assays

- Determine the enzyme activity of the dialysate using the method outlined in Chapter 3. (Procedure 3.2)

- As time permits, determine the protein concentration using the Bio-Rad assay and calculate specific activity according to the assay methods in Chapter 3. (Procedure 3.1)

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- Summarize the results of the purification so far by making a table as shown in Table 4.2. Include the data from each of the purification stages listed below.

    - The crude extract
    - The ammonium sulfate supernatant
    - The redissolved ammonium sulfate pellet
    - The dialysate

What do you think should be happening to specific activity as you go from crude extract to dialysate?

Table 4.2 This is actual data from a student purification. Does your data look similar?

STEP	Enzyme Units/ml	Protein Mg/ml	Total Volume	Yield Units in total volume	Percent Yield	Specific Activity	Purification Factor
Crude Extract	343.9	5.53	37 mls	17,195	100%	62.24	NA
AS Pellet	1240.7	6.99	4 ml	4962.8	29 %	177.66	2.85
Dialysate	478.8	2.24	4.8 ml	2298.2	13 %	213.83	3.45

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ASSIGNMENT: Turn in a progress report of your purification procedure according to your instructor's directions.

Section 4.4. Ion Exchange Chromatography

Background

The term "chromatography" applies to a wide range of separation techniques that are based on the differential interaction of molecules between a moving phase and a stationary phase. The interaction of molecules can be based on any of the protein properties, charge, hydrophobicity, affinity, solubility or molecular weight (size). As the name indicates, column chromatography is performed with the stationary phase packed in a cylindrical container, the column. The mobile phase passes through the column driven by gravity or by a pump. Fractions of the mobile phase are collected as they leave the column. Each fraction can then be assayed to determine the location of the target protein.

Column chromatography is a commonly used technique for protein purification. Column chromatographic methods are generally more complicated to set up than batch methods such as ammonium sulfate precipitation; however, they are capable of high resolution

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separation and are usually necessary for a successful purification. Like other separation techniques, different column chromatography methods exploit different properties of the protein. Ion exchange chromatography separates molecules based on charge. Gel filtration chromatography separates according to size. Hydrophobic Interaction chromatography separates based on hydrophobic properties of proteins. Affinity chromatography separates based on biological affinity for specific molecules, such as antigen-antibody interaction, metal binding capability, hormone receptor interactions, etc. Although the focus of this chapter is on ion exchange chromatography since that is used in the current purification, other types of chromatography will be discussed in lecture.

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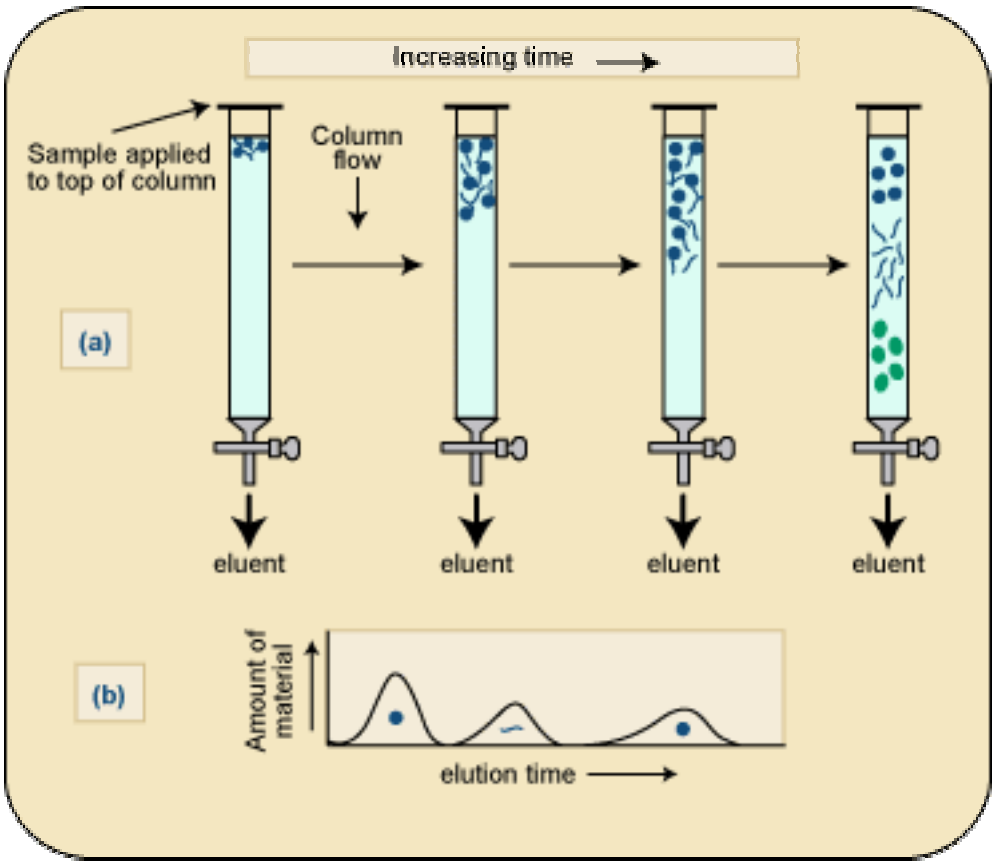

Figure 4.5. Elution of a sample mixture from a chromatographic column a) A sample containing 3 different molecules is loaded onto a column. As the mobile phase carries the sample components through the column, their rate of movement is affected by the relative tendency to adsorb to the stationary phase, slowing their elution. B. The three molecules are graphed according to their elution times

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Ion Exchange Chromatography: Ion exchange chromatography (IEC) is a high resolution technique for separating proteins according to their charge. It is the most commonly used chromatographic method of protein separation due to its ease of use and scale up capabilities. Large volumes of protein solution can be applied to ion exchange columns, often much greater than the volume of the column itself. Therefore, IEC is both a high resolution and a high capacity method.

In IEC, the packing material (resin) of the column has many charged molecules that are securely bound to it by covalent bonds. Manufacturers make and sell many different types of packing materials. Some have negatively charged groups and some have positively charged groups. When the packing material is suspended in buffer, the charged groups become loosely associated with ions of the opposite charge, since the buffer contains NaCl that dissociates into Na+ and Cl-. The loosely bound ions are called mobile counter-ions. As you can see in Figure XY, positively charged beads associate with, and therefore "exchange" with negatively charged counter ions. Since negatively charged molecules are also called "anions", this type of column resin is called anion exchange. Negatively charged beads associate with and exchange positive counter-ions (cations) and these types are called cation exchangers.

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Figure 4.3. Two Types of Ion Exchange Resins.

	Functional Group	Counter-ion
Anion Exchanger		
Diethylaminoethyl (DEAE)	$-O-CH_2-CH_2-N^+H(CH_2CH_3)_2$	Cl^-
Cation Exchanger		
Carboxymethyl (CM)	$-O-CH_2-COO^-$	Na^+

Since proteins are charged molecules, proteins in the mixture will interact with the column resin depending on the distribution of charged molecules on the surface of the protein, displacing mobile counter ions that are bound to the resin. The way that a protein interacts with the packing material depends on its overall charge and on the distribution of that charge over the protein surface. The net charge on a given protein will depend on the composition of amino acids in the protein and on the pH of the buffering solution. The charge distribution will depend on how the charges are distributed on the folded protein.

Proteins carry both positive and negative charges and the overall, net charge is dependent on the pH. The isoelectric point (pI) is the pH at which a particular protein is overall electrically neutral -- in other words, the number of positive charges is equal to the number of negative charges. Below its pI, a protein has more positively charged amino acids and therefore an overall positive charge. Conversely, above its pI, a protein has more negatively charged amino acids and an overall negative charge. Above its pI,

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the protein will bind to anion exchangers; below its pI it will bind to cation exchangers. At its pI, a protein will not bind to either a cationic or an anionic exchanger. In principal, one could use either a cation or an anion exchanger to bind to the target protein by selecting the appropriate pH. For example, the pI of ß-galactosidase is 4.6. At a pH below 4.6, ß-galactosidase carries a net positive charge and would bind to a cation exchanger (Figure 2.5) However, we know that the pH stability range for ß-galactosidase is pH 6-8. Therefore, it would not be prudent to use a buffer at pH 4.6. For this purification strategy, an anion exchange resin, DEAE Sepharose, will be used with a buffer of pH 7.2

The above explanation makes it sound like protein behavior can be completely predicted based on pI; in practice, separation conditions are often determined by trial and error. It is also wise to allow a 1 pH point "margin of error" on either side of the pI when determining whether to use anion or cation exchange.

Equilibration: The first step in the process is to "equilibrate" the column. Since the packing material of the column contains charged molecules, these will interact with the ions in the buffer. Just as the pH of a solution changes when you add charged molecules to it, the pH of the column-buffer environment will change as buffer is added to the column. This process should be stabilized before your protein mixture is applied to the column. When the pH of the buffer coming out of the column is the same as the pH going into the column, the column is said to be equilibrated.

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In our procedure, we will first apply our sample protein mixture to the IEC column using buffer conditions that allow ß-galactosidase to bind to the packing material. After washing the column thoroughly to remove any unbound molecules, we will change the incoming buffer by connecting a gradient maker that will slowly and progressively increase the ionic strength of the buffer, adding back the mobile counter-ion in the buffer at increasingly higher concentrations (gradient from 0.2 to 0.5 M NaCl). As we do this, ions in the buffer begin to exchange with and displace proteins that had adsorbed to the beads. The displaced proteins then flow out of the bottom of the column. At a certain point, the concentration of ions in the buffer is such that ß-galactosidase is no longer adsorbed and it elutes from the column.

As the liquid exits the column, the A_{280} is read by a UV monitor and these readings give an indication of where protein is found in the eluent. All the material that elutes from the column is collected in a numbered series of test tubes (1 ml each). These 1 ml aliquots are called “fractions”. Certain fractions will contain our ß-galactosidase but most of the fractions will not. After assays, we can combine the fractions containing ß-galactosidase by removing those fractions that do not contain our tarfet protein, we will significantly improve the purity of our ß-galactosidase preparation.

Chromatography is often performed using instrumentation including a pump to force liquid through the column, a UV detector with chart recorder to automatically measure and record the absorbance at 280 , and a fraction collector to automatically move the tubes after a volume of liquid has been collected. A typical configuration is shown in

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Figure XY. Although convenient and expedient, such instrumentation is not required and the procedures can be performed adequately (but slower) by manually collecting fractions, reading their absorbance on a UV spectrophotometer and plotting the results on graph paper.

Procedure 4.4 Ion Exchange Chromatography

Summary of IEC Procedure: It is helpful to break the column chromatography phase into several steps:

- **Set up**: Assemble the column, ring stand and any accessory equipment (pumps, chart recorders, fraction collectors, etc.) and perform necessary calibration steps.
- **Equilibration:** Prepare column resin, pour and equilibrate the column.
- Loading sample
- Washing unbound
- Elution (Gradient)
- Regeneration of column (Optional)

Description of the Overall Process: The column must first be equilibrated with the buffer that will be used to run the column. Equilibration of the column ensures that the column environment is stable at the correct pH. After the column is completely equilibrated, the protein sample is loaded on to the column. Next, the column is

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washed thoroughly to remove all unbound proteins. **SAVE** all column flow through until you verify that there is no enzyme activity present. Finally, after the column has been washed extensively with 0.2 M NTM to remove unbound protein (and the A280 of the flow-through is less than 0.1), the gradient maker is connected to start the elution process. At this point, slow the flow rate and collect 1 ml fractions. Store the fractions on ice as soon as they are collected.

Procedure 4.4A: Equipment Set up:

- Mount the column vertically on a suitable ring stand and determine the volume of your column. If you are planning to use 10 ml of ion exchange resin, you can fill your column with 10 ml of water and mark it at the 10 ml level. Empty the column.

- If you are using an automated column chromatography set up you will need to connect all the equipment in sequence according to the diagram.

- Calibrate the pump according to the procedure in the manual.

- Calibrate the fraction collector – How many drops are in one ml?

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- Test that the UV monitor and the chart recorder are communicating.
- You will need to zero the UV monitor on 0.2M NTM buffer.

- The chart recorder is a relative scale; how will you know what it means?

- Attach a short piece of Tygon tubing a few inches long to the outlet of the column. Replace the air in the tubing with buffer by filling the bottom of the column with buffer and running a few ml of 0.2 M NTM buffer through the tubing. Close the column outlet after all the air is removed from the tubing.

Check list:

___ Verify that the pump is connected to a buffer reservoir and the top of the column.
____ Verify that the UV monitor is hooked up to the column output (the tube coming from the bottom of the column)
____ Verify that the Fraction collector is hooked up to the tubing coming from the UV monitor.
____ Make sure that the tubing volumes are not excessive.
____ Verify that the chart recorder is hooked up to the UV monitor and that it is responding. (Use the event marker button on the UV monitor to check this.)
___ Familiarize yourself with all buttons on the equipment.
___ Does the pump reading (ml/minute) correspond to the volume collected in a given time?

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____ Has the UV monitor been zeroed on 0.2M NTM buffer?

____ Verify the number of drops in a 1ml fraction so that you can set the drops/fraction on the fraction collector.

Some settings: Pump speed should be no more than 1 ml a minute with the column hooked up. If you are flushing the tubing with the column disconnected, higher speeds may be used.

Chart recorder speed should be set at __**5**__ mm/minute. (Arbitrary, but it helps if students are consistant.)

UV RANGE should be set to 1.0 AUFS

Chart recorder input should be set at 1 V

Note: While running the columns, it is important to mark fraction numbers on the chart recorder so you can later associate the chart peaks with specific fractions. Use the event marker to mark when one fraction starts and then stops.

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Procedure 4.4B: Equilibration

- In preparation for ion exchange chromatography (IEC), calculate the amount of ion exchange resin you need, based on the amount of protein you have. The resin we will be using is DEAE Sepharose CL-6B. Check the product literature for the binding capacity of this resin. Binding capacity is reported in milli-equilavents of charge and in mg of protein for a specific protein like bovine serum albumin (BSA) or hemoglobin. Since the number of charged groups varies from protein to protein, the amount of protein that the column resin can "hold" varies depending on the charges present on the proteins in your sample.

  Although it is possible to determine the amount of ß-galactosidase that would bind experimentally by loading more and more on the column until ß-galactosidase activity is present in the flow through, we will adopt a best guess approach. Since you don't know exactly what percentage of the proteins in your sample will bind to the resin, assume that you need resin capacity for l/3 to l/2 of all the protein in your sample in milligrams.

- DEAE Sepharose CL-6B comes pre-swollen in an ethanol/water mixture that must be removed and replaced with 0.2 M NTM buffer. An aliquot of resin has been placed in a tube for you, so the resin will have settled, leaving the ethanol at the top. Pour off excess ethanol and add some 0.2 NTM buffer with 10X Tris.

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(We have found that the addition of 10X TRIS speeds up the equilibration process.) The resin should be about 75% of the total volume of the final slurry. Check the pH with pH paper.

- The final slurry of ion exchanger and buffer should be fairly thick but it should not so thick as to retain air bubbles. Usually about 75% settled gel is suitable. **Optional:** Put the slurry into a flask and degas it.

- With the column outlet turned off, pour the slurry into the column in an even, continuous motion. A glass rod can be used to prevent splashes. Let the slurry settle.

- **STOP POINT:** When all the suspension has settled, you may store the column at 4°C.

- Open the column outlet and allow the slurry to pack (settle) as the buffer drains out of the column. **Do NOT let the column run dry.**

- Begin equilibrating the column with 0.2 M NTM **with 10X TRIS**. If the suspension does not fill the column, buffer should be carefully added until the level is 1 cm from the top of the column before connecting the headpiece. Connect the tubing attached to the headpiece to a beaker containing 0.2 M NTM with 10X TRIS. (If

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you are using a pump, connect the tubing to the buffer reservoir and turn the pump on at 1 ml/min.)

- Let equilibrating buffer flow through the column at a rate of approximately 1 ml/min. Washing the column will go faster or slower depending on the hydrostatic pressure, which can be regulated by raising or lowering the height of the beaker containing the buffer. (If a pump is available, this step is unnecessary.) Some matrices are more resistant to pressure than others. Check the product literature for recommended flow rates.

- After washing the column for about 30-60 minutes, check to see if the pH of the buffer going into the column is the same as the pH coming out of the column, by pipetting a drop of solution onto pH paper. If the two pH readings are identical, change the column buffer to 0.2M NTM with **1X Tris** (NOT 10x Tris). **This is very important. If you have any doubt about whether or not your column has equilibrated completely, it is better to keep running buffer through it.** The column is ready when all the 10X Tris has been washed out using 2-3 column volumes of 1x Tris. You can do other tasks while it is equilibrating such as making a detailed flow chart for column loading and sample elution, measuring the flow rate under different conditions and deciding on the methods you want to use to collect fractions, etc. When we are equilibrating the column, the counter ion for the DEAE resin will be one of the ions found in the buffer

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(What ion is that?) (What would happen if you apply your protein before the column is fully equilibrated?)

STOP POINT: After the column is equilibrated, it should be stored at 4°C unless you are planning to apply your protein sample right away.

Procedure 4.4C: Loading the column:

Note: If at all possible, Procedure 4.4c, 4.4d and 4.4e should be done in the same day, without stop points. You should allow at least 3 hours (four is better).

OVERVIEW: In this procedure, you are applying your sample to the column and therefore allowing any proteins that can bind to the column to do so. Then, you are washing the column thoroughly with buffer to remove any unbound proteins BEFORE YOU START THE GRADIENT. After you have verified that all the unbound protein has been removed from the column, then you may begin the elution process by connecting the gradient**.**

- Remember to add dithiothreitol (DTT) to your buffers before starting. Apply the sample to the column with a flow rate no higher than 1ml per minute. In the interests of time (or if you are using a manual set-up without the pump), it is

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helpful to close off the column outlet, remove excess buffer from the top of the column and pipette the sample into the column. Then connect the buffer reservoir to the top of the column and open up the column outlet. If you are using a pump, you simply need to stop the pump and then start it again.

Procedure 4.4D: Washing the column:

- After the sample has been loaded on the column, connect the buffer reservoir containing 0.2 M NTM buffer and start running the column, again at a flow rate no greater than 1 ml/minute. Collect the effluent (flow-through) in a beaker or test tube and save it. Check the A_{280} regularly. Continue to run buffer through the column until the A_{280} is less than 0.1 with 0.2M NTM as the blank. (Check this on the DU 64 spec, not just the chart recorder.) Save this effluent. It contains any protein that did not stick to the column and will need to be checked for enzyme activity.

- Stop the column when the A_{280} is less than 0.1 and prepare to connect the gradient.

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Procedure 4.4E: Elution Phase

Note: Elution speed should be 0.5-0.8 ml/minute. Your resolution will be much better, i.e., you will get tighter peaks, if you maintain a slow flow rate during the elution phase. Monitor the flow rate and adjust it by carefully raising or lowering your gradient apparatus, or changing the pump speed.

- Prepare your gradient maker. If a commercial gradient maker is available, check it to make sure it is working and close off the outlet between the two cells. After verification, add 30 mls of 0.2 M NTM to one side and 30 mls of 0.5M NTM to the other side. Add a small stir bar to the side with 0.2M NTM. The reservoir that is directly hooked up to the column is the reservoir that has the 0.2M NTM buffer.

Home made gradient makers: If a commercial gradient maker is not available, a home-made gradient maker can be made with two small beakers and a glass U tube. The U tube is a piece of glass tubing a few inches long that has been bent into the shape of a U. Place 30 ml of 0.2 M NTM in one beaker and 30 ml of 0.5 M NTM in the other. Fill the U tube with 0.2 M NTM buffer. Then carefully invert the filled U-tube so that it straddles the two beakers, being very careful not to introduce **any** air bubbles into the tube. The tube should reach close to the bottom of each of the two beakers. Place

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a small stir bar in the 0.2 M NTM beaker and place the beaker setup on top of a stir plate. The gradient maker set-up is depicted below in Figure 4.2.

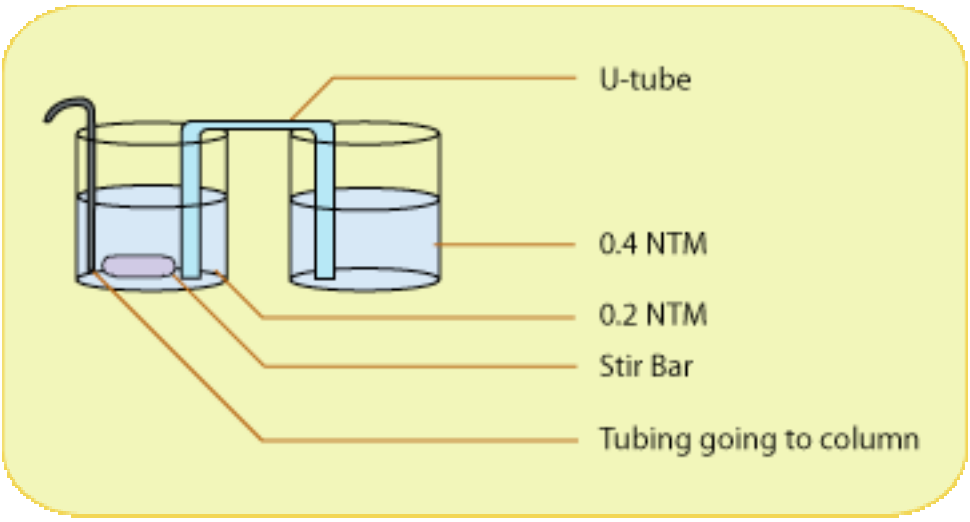

Figure 4.6. A gradient maker made with a U-tube.

- Assemble and label enough small test tubes to collect 1 ml fractions from the column(80+ tubes). (If you are collecting them manually, it is helpful to mark the 1 ml mark with a Sharpie.) Your fractions will need to be kept on ice once they are collected.

- To start running your column, place the tubing connected to the column headpiece into the gradient maker by securing the open end of the tubing in the bottom of the beaker containing the stir bar. Use a syringe to get the buffer

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flowing if needed and connect the tubing to the column. Make sure there is enough buffer on top of the resin bed so that the drops coming from the gradient maker do not disturb the resin. To ensure a smooth gradient make sure the stirrer is turning fast enough to mix the solution but not so fast as to make bubbles or be unstable. When you open the column outlet, the column will start running. The gradient will form automatically in the beaker containing 0.2 M NTM. As the buffer flows out of the low salt beaker, buffer from the high salt beaker will be siphoned into the low salt beaker through the U-tube, increasing ionic strength in a linear fashion..

- Store all fractions at 4° C until time to assay. Make sure all tubes are properly labeled

Procedure 4.4F:

Regeneration: When the gradient has been run completely, if the column is to be reused, you need to clean the column with 2-3 column volumes of 1 M NTM buffer until the A_{280} of the effluent drops below 0.05. Then run 2-3 column volumes of 0.2M NTM buffer to re-equilibrate the column. Store the column at 4° C.

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4.5 Results and Data Analysis:

After the column has been run, you need to have an estimate of the amount of ß-galactosidase activity as well as the total protein in each fraction. Although the chart recorder gives a useful estimate of protein peaks (A_{280}), you will need to assay each fraction for ß-galactosidase activity. This task may be speeded up by the plate reader assay below.**

Plate Reader Assay for ß-galactosidase. This assay is identical to the ONPG assay in Chapter 2 except that the volumes have been cut to 1/10 of their original amount.

1. Determine how many fractions you have – you will need to designate one well of the 96 well plate for each fraction, leaving well A-1 for the blank.

2. Fill each of the wells of a 96 well plate with 100 µl of Z buffer.

3. Reserve well A-1 for your calibration blank. Do not add any sample to that well.

4. Add 1-10µl of sample from each of the column fractions to each of the wells in order.

5. Add 20 µl of ONPG (4 mgs/ml in phosphate buffer) to each of the wells, including the blank.

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6. After the formation of yellow color (it should go fairly quickly), add 50 µl of STOP solution to each of the wells.

7. Read the plate at a wavelength of 4 05 – 420 nm on the plate reader. Although many of the readings will probably be off scale, this is still a useful way to estimate the location of peaks of ß-gal activity. A more accurate, but more time-consuming, estimate can be obtained by diluting all fractions 1/100 to begin with and then assaying fractions in the plate reader.

8. Plot your data for A_{420} and the chart recorder data on one graph with the fraction number on the x-axis and Absorbance on the y-axis. One Y-axis is A_{280} and one Y-axis is A_{420} or enzyme activity units.

9. After plotting the data, decide which fractions to pool. Do not pool any of the fractions until ALL data has been graphed! Let your instructor check your graphs before pooling fractions. (Remember that you can choose to pool for maximum purity or maximum yield.) Save 0.1 ml of the activity pool for assay. We have sometime noted 2 peaks with enzyme activity; usually one is larger than the other. Pool these peaks separately since they usually have different specific activities.

TIP: For final assay, it is generally necessary to dilute column fractions to 1/50 or 1/100 in order to measure enzyme activity accurately.

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** **Note to instructors:** If a plate reader is not available, you may want to have students plot the A_{280} results first and then choose fractions to assay for enzyme activity based on the protein peaks. For example, if there were no significant protein elution until fraction 25, it would be unnecessary to assay enzyme activity until fraction 25. Another strategy is to have students assay every third fraction to get an idea of the location of the ß-galactosidase activity and then come back and assay again to clarify the location of the peaks.

Table 4.4 Sample Data from Student Purification:

Purification Step	Enzyme Units/ml	Protein Mg/ml	Volume of fraction	Units Total Volume	Yield %	Specific Activity Units/ mg	Purification Factor
Crude	107	5.04	38 mls	4,058	100 %	21	1.0
Ammonium Sulfate Pellet	531	7.45	4.2 mls	2,231	55%	71	3.4
Dialysate	366	7.25	2.5 mls	914	22.5	50	2.4
IEC pool I	128	0.5	5.0 mls	641	15.8	251	11.9
IEC pool 2	57	0.425	2.0 mls	114	2.8	134	6.3

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Chapter 5
Analytical Procedures

Although specific activity gives an indication of purity, additional procedures are necessary to verify that the target protein has been purified to the desired level. Analysis of any mixture to look for contaminants requires a technique with high resolving power. Two procedures offer the kind of sensitivity and resolving power needed for protein analysis and verification, HPLC (High Performance Liquid Chromatography or High Pressure Liquid Chromatography) and polyacrylamide gel electrophoresis (PAGE).

HPLC differs from traditional liquid chromatography in that the column bed is made of particles that are extremely small (8-40 microns) and therefore pack together very tightly. Because of the densely packed matrix, high pressures are required to force the sample through the column. The advantages to HPLC include rapid run times and excellent resolution. The disadvantages of HPLC are the expense of the equipment and the high maintenance required to keep it running optimally. Because of this, HPLC systems are not standard equipment in all labs.

More sophisticated methods for analysis and verification, such as mass spectrometry, will not be described in the lab manual, as the use of these methods in teaching laboratories is generally cost prohibitive. Consult the additional Resources section in Appendix C

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Polyacrylamide gel electrophoresis (PAGE) is one of the most powerful methods for protein analysis and is widely available. There are several types of PAGE that are discussed in the textbook (Rosenberg) and resource materials for the course; but in the laboratory, we will concentrate on SDS-PAGE.

Determining the degree of purity of a protein preparation is somewhat ambiguous because it depends on the criteria used to define purity. If purification to homogeneity is necessary, the criteria used to specify homogeneity must be defined. For example, if your protein sample is run on SDS PAGE and only one band is present, you might conclude that you had purified the protein to homogeneity. However, two- dimensional SDS PAGE of the same sample could show multiple bands, indicating the presence of multiple proteins with similar molecular weight but with different isoelectric points. For one protein, the purification goal might be a certain specific activity value together with the presence of one band at a particular molecular weight on an SDS PAGE gel. For another protein, the purification goal might be the presence of one band at a certain MW and pI on a 2-D gel. Further confirmation by other methods (such as immunoblotting or HPLC) might also be necessary to ensure that the desired level of purity had been reached.

In analyzing our purification of ß-galactosidase, we will compare the number of protein bands present on an SDS PAGE gel, stained with Coomassie Blue, for each of the different stages in the purification procedure and examine how this relates to the specific activity at each point in the purification procedure. Then, in procedure 5.4, immunoblotting will be used to determine which of the protein bands on the gel are actually ß-galactosidase.

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For each of the steps listed below there is a detailed procedure in this section of your manual:

- concentration of samples as needed: Procedure 5.1
- Polyacrylamide gel electrophoresis: Procedure 5.2
- Staining the gel: Procedure 5.3
- Western Blotting (Immunoblotting): Procedure 5.4

Procedure 5.1 (OPTIONAL) Concentration of the Sample

Decide which, if any, samples will require concentration. For example, the crude extract and ammonium sulfate pellet fraction may not need concentration but the ion exchange pooled fractions may. For SDS PAGE analysis, the sample volume in each well should contain approximately 20-100 µg of protein, if there are many proteins present, but should only contain 1-20 µg if only 1-2 bands is expected. Since you have already determined the protein concentration of your fractions, you can do some calculations to determine if any of them needs concentrating. Do not concentrate the entire sample, only what you will need for electrophoresis and HPLC, etc.

There are many ways to concentrate proteins. Most involve the removal of water and salts by using a membrane with a defined pore size, similar to a dialysis membrane, through which the low molecular components are removed, leaving the more concentrated

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protein solution behind. Water and salts can be forced out of the protein solution by physical means such as centrifugation or pressure and also by osmotic forces, for example, by placing the protein solution in a dialysis bag and covering the bag with a substance such as polyethylene glycol that has a higher affinity for water than does the protein.

We will be using small ultrafiltration devices from the Millipore Corporation, the Ultrafree-MC, with a molecular weight exclusion limit of 10,000 Daltons. Refer to product literature on these devices for specific instructions. Each ultrafiltration device is a microfuge tube containing an upper chamber with a filter membrane forming the bottom of the upper chamber. The sample is loaded into the top chamber of the tube. As the sample spins in the microfuge, the buffer is forced through the filter, while the sample protein, which is too large to pass through, remains above the filter. Note: that the pore size is the same as that dialysis in purification procedure 4.3.

1. Put 400 µl of the sample to be concentrated in the cup at the top of the microfuge concentrator apparatus.

2. Place the tube in the microfuge and spin at 1200 xg (or according to product literature.) The microfuge can be placed in a cold room or large refrigerator. Otherwise, periodically remove the tube and place it on ice. Check the progress of your concentration every ten minutes or so when you place the tube on ice.

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3. Stop spinning the sample when approximately 50 µl of sample is left in the cup. **Do not allow the top chamber to completely empty.** You may find it more efficient to transfer the supernatant to a second filter apparatus if, after about half an hour, the first one has slowed down. Some samples may require more concentration than others.

4. Collect your sample from the cup but save the filtrate until you are sure your concentration worked.

5. Repeat for each sample desired.

6. Store your concentrated samples properly until you are ready to run your polyacrylamide gels.

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Procedure 5.2 Polyacrylamide Gel Electrophoresis

Background

Running polyacrylamide gels is part of working in any modern laboratory where protein or nucleic acid samples are analyzed. Typically, when purity needs to be monitored closely, for example, when proteins are being purified for the first time, PAGE is used before and after each purification step to evaluate the success of that step. We will use it to evaluate the success of our purification by running samples on the gel from each stage of our purification. PAGE is an indispensable analytical tool for protein chemists and there is no other technique with the resolving power of PAGE that is as inexpensive and widely available.

SDS Polyacrylamide gels (SDS-PAGE) that we will be using in this procedure, are called "denaturing gels" because they contain sodium dodecyl sulfate (SDS), an ionic detergent that binds to the amino acid residues in the proteins. Due to its ionic properties, SDS confers a net negative charge on all the proteins, overcoming any intrinsic charge; in this way the proteins uniformly migrate toward the positive electrode. SDS also disrupts the secondary and tertiary structure of the proteins, essentially destroying their globular configuration and making them into linear molecules that then migrate in the electric field on the basis of their size. PAGE is a very powerful technique because even small differences in molecular weights produce distinguishable bands on a gel.

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REMEMBER THAT ACRYLAMIDE IS A POTENT NEUROTOXIN. ALWAYS WEAR GLOVES WHEN WORKING WITH IT.

There are many different types and brands of electrophoresis equipment. There are also many different methods for casting gels, including commercially prepared gels that are purchased "ready to use". As you encounter these in the workplace, remember that none of these methods is necessarily better or worse although some methods are easier than others. They all accomplish the same goals.

Before you begin, make sure you understand the basic principles of SDS-PAGE as discussed in lecture. In addition, read all the instructions and watch the instructor's demonstration before attempting this procedure. Bear in mind that there is no substitute for repetition. The more gels you run, the better your gels will turn out.

You will prepare and run at least three gels. The first gel will be a practice gel. For the practice gel choose samples that are not scarce or valuable such as the commercial ß-galactosidase and the molecular weight markers. Do not use samples from your purification until the second gel. Hopefully, you will make all the mistakes you are going to make on the first gel and won't have to waste your valuable samples.

For the second gel, you will need the following samples:

- pure ß-galactosidase (commercial preparation)
- crude extract

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- dialysate or column start (sample you loaded on your column)
- pooled samples from your column
- any other samples you wish to run

For the first and second gels, protein bands will be detected using Coomassie Blue staining. The third gel will not be stained and be used for the immunoblotting procedure, which is discussed later. Additional gels can be run for silver staining or other staining methods if time permits.

Preparing your samples:

Note that the sample buffer has ß-mercaptoethanol in it. Handle samples in the fume hood if possible.

1. Prepare your samples. Place 18µl of each sample in a microfuge tube and add 6µl of sample buffer (4x) to each sample. If you have less than 24µl of a sample, make up the difference with water. These instructions are based on a 10 well comb and a 1.5 mm thick gel. Note that the well volumes formed by different combs and different gel thickness accommodate different sample volumes. Refer to Table 5.1 for details.

2. Using a pushpin, poke a hole in the top of the sealed microfuge tubes containing your samples. Place the tube in a boiling water bath for 2 minutes. After removing the tubes from the boiling water, spin down any condensed water or disturbed

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sample in a microfuge for a few seconds. Boiling your samples in sample buffer changes your proteins into long strands, disrupting their three-dimensional structure and making all the amino acids available to bind the negatively charged detergent, SDS. Note that the sample buffer contains ß-mercaptoethanol (ß-ME).

3. Check to see that each well has roughly the same volume; in this case, approximately 25 µl. Use a gel loader tip to carefully layer each sample in its well. The sample will sink to the bottom of the well, displacing the buffer. It is usually a good idea not to use the first and last wells.

Table 5.1 Sample Well Volumes for Different Combs

	Comb Thickness		
	0.50 mm	**0.75 mm**	**1.5 mm**
5-well comb	23 µl	33 µl	65 µl
10-well comb	8 µl	13 µl	25 µl
15-well comb	5 µl	8 µl	5 µl

Place the safety lid on the unit and attach the leads to the power supply.

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Loading the gel:

1. Check for availability of all needed reagents and equipment. See Appendix A for recipes.

    a. Sample buffer
    b. Running buffer (Laemli buffer)
    c. Staining solution or fixative (depending on visualization method)
    d. Apparatus
    e. Precast gels (make sure they fit with your apparatus and do not leak)
    f. Power supply

2. Remove pre-cast gels from packaging and assemble in apparatus according to manufacturer directions (instructor hand-out optional here). Gently remove the comb. Rinse the sample wells with running buffer and drain by inverting and shaking the unit over the sink.

3. To facilitate sample loading, a 'well location decal' can be used. The decal is a clear plastic square that adheres to glass when wet. The outline of three different combs, corresponding to 5, 10 and 15 wells respectively, is marked along three separate edges of the decal. Simply wet the decal and place it against the front of the sandwich with the appropriate edge outlining the sample wells. Alternatively, you can take a marker and put a dot under each of the wells.

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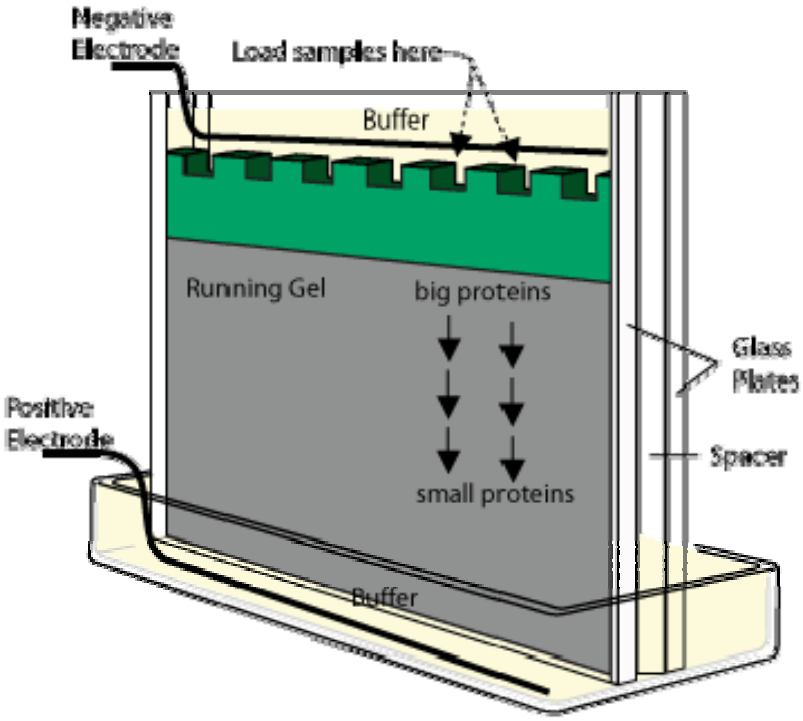
4. Fill the sample wells and the upper and lower buffer chambers with electrophoresis buffer (Laemli buffer).

5. Using a micropipettor (extra long tips can be used), carefully load each well with the appropriate sample. DO NOT forget to write down the contents of each well.

SAFETY NOTE: The electrophoresis unit is designed to prevent you from contacting the gel or buffer while voltage is applied. Never by-pass this safety feature. Also, never touch a puddle around a gel box while voltage is applied to the apparatus.



http://matcmadison.edu/biotech/

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Running the Gel

Gels may be run at either constant current or constant voltage. We are using what is known as the discontinuous (Laemmli) system because the upper and lower gel buffers are different. Constant current is usually used with a discontinuous buffer system so that the rate of electrophoretic migration will remain constant throughout the run. We have obtained good results at a constant current of running 15-20 mA. per gel. If you have two gels, you will need to double the mA. However, we have also found it convenient to run Laemmli gels at constant voltage of 90 V –125 volts using a small power supply such as the Hoeffer PS 150. (Although you do double the current if running two gels, do NOT double the volts for 2 gels. Why?) Refer to the product literature for the power supply for help. (What might happen if you run your gel at too low a voltage or amperage? What might happen if you turn it up too high?)

Electrophoresis is governed by Ohm's Law, V = I R, where the gel is the resistor and the power supply determines both the current (I) and the voltage (V). If the current is held constant, the voltage will increase during the run as the resistance goes up. (The resistance of the gel goes up because the ions in the gel run out reducing its conductivity.) Would you expect the resistance of a 0.5 mm thick gel to be the same as our 1.5 mm thick gels? What if the gel were twice as long?

1. Place the safety lid on the gel box and attach the electrode leads to the power supply. Turn on the power to the power supply and set it to the appropriate voltage or current level. Check to make sure that bubbles are forming around the electrode wires.

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2. Periodically check your gel to make sure the running buffer has not leaked out of the upper buffer chamber. This is especially important when using precast gels from some manufacturers. (What will happen if the upper chamber is empty?)

3. When the tracking dye reaches the bottom of the gel, i.e., below the surface of the lower buffer, turn off the power supply, disconnect the leads and remove the lid of the unit. Do not run the dye front off the gel.

4. Pour out the buffer by inverting the entire unit over a sink.

5. Pry open the gel sandwich. Remove the spacers and peel the gel off the plate into a tray of stain. Wetting the gel helps to loosen it from the plastic plate.

6. Rinse the lower buffer chamber and upper buffer chamber pods with distilled water after each use. Be careful not to damage the platinum wires in your electrophoresis apparatus. Be sure to clean everything carefully and put all the parts away properly.

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Procedure 5.3 Staining the Gel

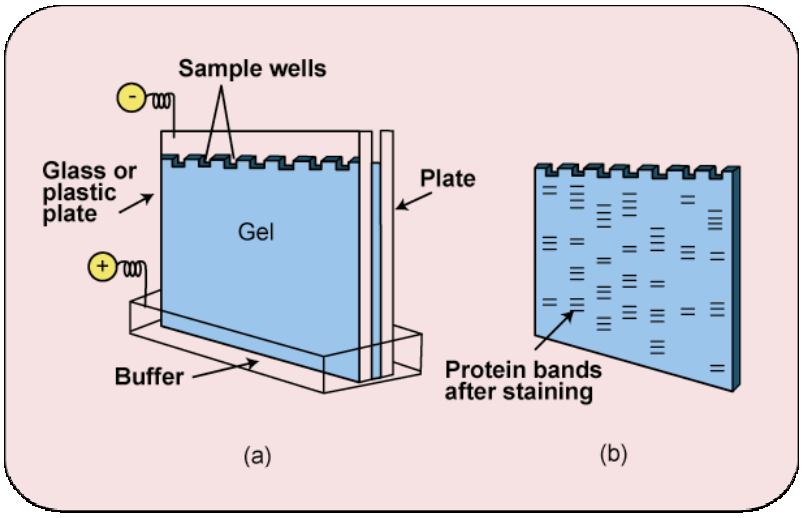

Figure 5.2 Polyacrylamide Gel Electrophoresis (PAGE) a) The gel is poured vertically between two glass plates. b.) Protein bands are separated on the basis of relative molecular weight and visualized with stains. Figure from Seidman and Moore. Basic Lab Methods for Biotechnology. Prentice Hall, New Jersey

There are several methods for visualizing the proteins in polyacrylamide gels. These staining methods are not specific for the target protein- they stain all proteins. Coomassie Blue Stain is the most common method and it works well when the detection of minor bands is not necessary. Generally, 10-20 µg protein/cm^2/band provides good detection by Coomassie staining. When sensitivity is important, silver staining is the method of choice because it can detect bands that contain nanogram quantities of protein. Proteins in acrylamide gels can also stained with heavy metal salts. While not as sensitive as

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silver staining, heavy metal staining has the advantage that it allows quantitative recovery of resolved proteins from the gel.

Method A. Coomassie Blue Staining:

- Transfer your gel to a Pyrex dish containing Coomassie Blue Stain. The recipe for the stain is found in Appendix A. Stain your gel for approximately 1 hour; overnight staining will also work but it will take longer to destain. Do not use plastic dishes because they permanently take up the stain.

- 2.Transfer your gel to destain solution 1 (50% methanol and 10% acetic acid) and incubate for approximately 30 minutes. Destaining can be left overnight or longer. To speed up destaining add a small piece of foam (or even a wadded up kimwipe) to the destaining tray. It will absorb the stain.
- Note: If the gel shrinks too much to view the bands, you may swell it by putting the gel in DI water with 1% glycerol for 30 minutes or so.

- 3.View the gel and interpret your results. Make a diagram in your notebook or take a picture. Alternatively, you may dry the gel according to the procedure below.

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Drying the gel:

Kits for air drying gels are commercially available at a nominal cost and these work quite well, although overnight drying is usually required. Follow the directions on the kit for setting up the apparatus.

For all gel drying methods, soak the gel for 30 minutes in water containing 1% glycerol to prevent cracking of the gel during the drying process.

Method B. Silver Staining:

- Read the Bio-Rad silver stain directions and watch the instructional video.

- You will need to prepare five different solutions for this procedure: fixatives, oxidizer, silver stain reagent, developer and stop solution. You may make the fixatives, developer and stop solution ahead of time but you must prepare the oxidizer and silver stain the day that staining is performed. Prepare the reagents as follows:

  Fixatives and Stop Solution. The recipes for the fixatives and stop solution are contained in Table 5.3 (not in the appendix).

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Oxidizer and Silver stain. The concentrates for the oxidizer and silver stain are provided in the kit and must be diluted 1/10 in water. Suggested volumes are provided in the kit.

Developer. The concentrate for the developer is also provided in the kit and must be diluted in water. To prepare the developer dissolve one bottle of concentrate in 3.6 liters of deionized water by stirring for 15 minutes at room temperature. This is enough for 12 mini gels like those we are using. To prepare smaller amounts, use 32 grams of developer per liter of deionized water. Be sure to shake the bottle thoroughly before weighing out the concentrate since the components settle out. Failure to mix the contents can result in very slow or no development. Store the diluted developer solution at room temperature for up to 1 month. Keep the solution tightly covered to avoid evaporation of paraformaldehyde. Use the volumes for mini gels.

- Perform silver staining as shown in Table 5.3 below. Do not start the procedure until you have the stop solution ready. We have observed that students have a tendency to overstain. Gels that are overstained will have high background or surface deposits of silver. We advise you to monitor development of your gel closely.

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Table 5.2 Reagents and Times for Silver Staining.

Reagents	Volumes	Times
1. Fixative (40% methanol/10% acetic acid)	200 ml	30 min
2. Fixative (10% ethanol/5% acetic acid)	200 ml	15 min
3. Fixative (10% ethanol/5% acetic acid)	200 ml	15 min
4. Oxidizer	100 ml	5 min
5. Deionized water (milli-Q)	200 ml	5 min
6. Deionized water (milli-Q)	200 ml	5 min
7. Deionized water (milli-Q)	200 ml	*
8. Silver Reagent	100 ml	20 min
9. Deionized water (milli-Q)	200 ml	1 min
10. Developer	100 ml	**
11. Developer	100 ml	5 min
12. Developer	100 ml	5 min
13. STOP (5% acetic acid)	200 ml	5 min

* Repeat washes 5, 6 and 7 until all the yellow color has been removed from the gel.

** Develop 30 seconds or until the solution turns yellow or until a brown "smoky" precipitate appears. Then pour off the developer, proceed to the next step and add fresh developer.

View the gel and interpret your results. Sketch, photograph or dry the gel. .

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Procedure 5.4 Western Blotting

Background

The gel staining methods described in the previous procedures are methods that stain all proteins. Identification of a specific protein must be verified by more specific techniques, such as immunoblotting, also called Western blotting.

Western Blotting is a method for identifying proteins that combines the resolving power of PAGE with the specificity of antibodies. Because of the power of this technique, Western blotting has become an increasingly important tool, not only for protein chemists but also in clinical labs where Western blotting is now considered the “gold standard” for identifying HIV-positive individuals.

Western blotting is similar to Southern (DNA) and Northern (RNA) blotting in that target molecules are first separated on a gel and then transferred from the gel to a membrane, or blot, which binds the molecules. The membrane is then incubated with reagents that specifically react with target molecules on the membrane. In Western blotting, proteins are detected instead of nucleic acid and the reagent used to detect proteins is an antibody, also a protein, instead of a nucleic acid probe. This antibody specifically binds the target protein. In our case, we will use an antibody to ß-galactosidase, our target molecule of interest. The antibody is visualized because it is labeled by a tag that makes it visible, either by radioactive, chemiluminescent , enzymatic or other means. Most often

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the “tag” is actually on a second antibody, an antibody that recognizes the first antibody to ß-galactosidase.

Try to make an educated guess as to which bands on your Coumassie-stained gel are ß-galactosidase. The Western blot that you will do now will either prove or disprove your hypothesis regarding the identity of the band(s).

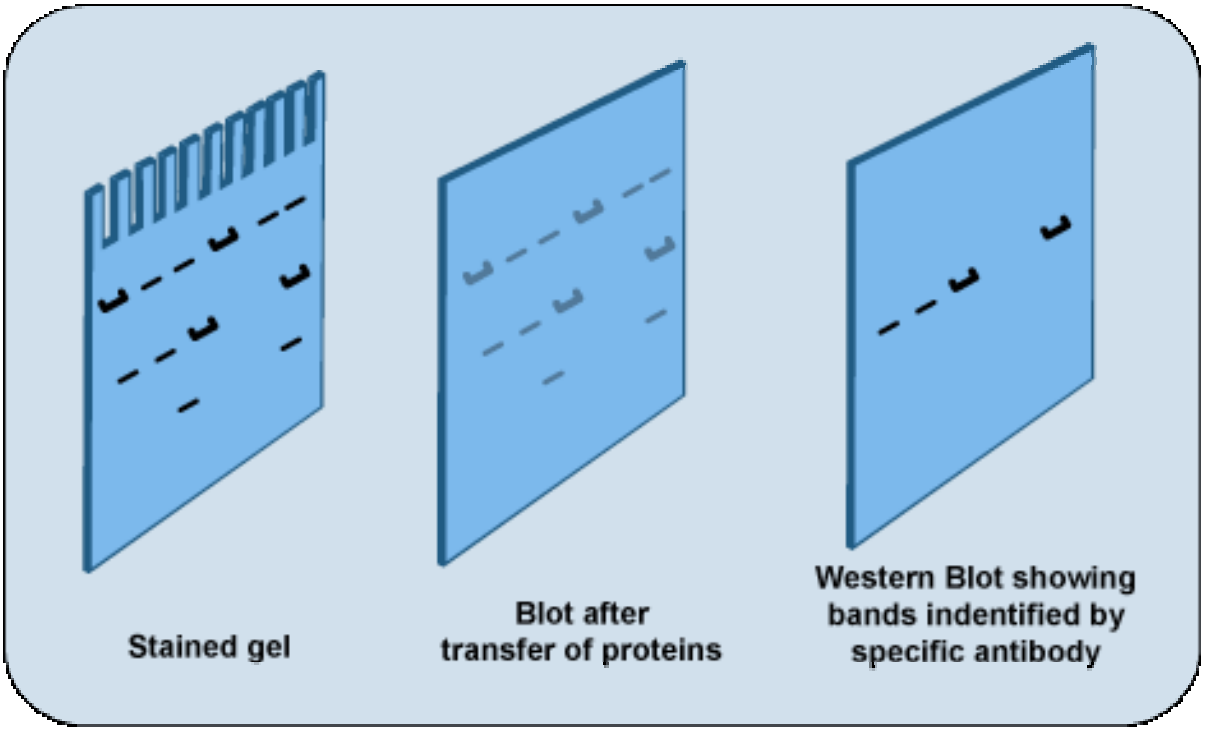

Figure 5.3. The concept of Western blotting. CAUTION: This figure is misleading because the gel is not stained before transferring to a blot

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To understand the specificity of antigen-antibody reactions, the analogy is sometimes made to the fit between a lock and key. While useful for visualization, this comparison falls short. The lock and key fit is an interaction between two essentially flat surfaces whereas an antigen antibody complex is more complicated; in part because it is a three dimensional structure but also because of the electrostatic and hydrophobic interactions between the amino acid residues of the two proteins. With this in mind, it is not surprising that antibodies can be used to distinguish even very similar proteins and even to identify other antibodies.

Separation and Transfer of Proteins

1. Run all your samples on a PAGE gel just as you did in the previous procedure, beginning with the crude extract and all the succeeding purified fractions. Load the same amounts of protein that are appropriate for Coomassie Blue detection. Also run at least one ß-galactosidase standard purchased from a commercial supplier and a lane of molecular weight standards. It is preferable to use pre-stained molecular weight standards as they make it easy to determine that the transfer has occurred.

2. The membrane used for the blot is nitrocellulose. Cut one sheet of nitrocellulose and four sheets of Whatman 3MM filter paper to the size of the gel. Always handle membranes with gloves.

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3. Soak the nitrocellulose membrane in distilled water. Nitrocellulose should be wetted by carefully laying it on the surface of the water. Allow the nitrocellulose to wet by capillary action for several minutes and then submerge the sheet for 2 minutes. Move the membrane to transfer buffer for 5 minutes. Wet the filter paper by soaking it in transfer buffer. The recipe for this buffer and the other buffers used in this procedure are given in appendix A.

4. Immerse the gel, membrane, filter papers and support pads in transfer buffer to be sure they are thoroughly soaked.

5. Assemble the transfer sandwich so that the membrane is in direct contact with the gel, making sure there are no air bubbles trapped between the gel and the nitrocellulose. Keep all the components wet and make sure the sandwich is tightly assembled. The assembly is shown in Figure 5.4

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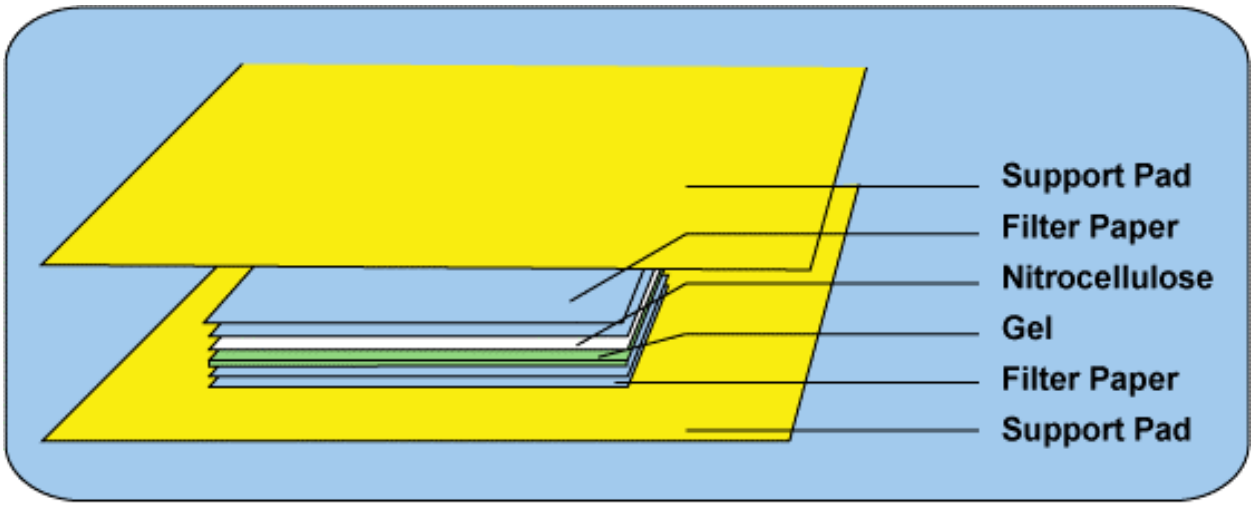

Figure 5.4. Arrangement of the gel, blot, filter paper and support pads for electrophoretic transfer of proteins for a Western Blot.

6. Place the complete sandwich in the transfer tank with the membrane closest to the anode or positive (red) electrode. Fill up the transfer tank with buffer, making sure that the gel and blot are completely submerged. Put a stir bar in the bottom of the tank.

7. The rate of transfer of proteins is a function of their molecular weight. Larger proteins generally require higher voltages and longer transfer times than smaller proteins. A convenient and reliable method is to transfer overnight. We will follow the recommendations that accompany the Trans-Blot unit, 30 volts at 40 mA, for overnight transfer. [If you want to do your transfer in a shorter time, consult the Trans-Blot (or your equipment) literature].

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8. Disconnect the power supply. Disassemble the sandwich and mark the membrane by clipping off approximately 1 cm of the upper left hand corner. Place the blot into PBS and rinse several times.

Blocking the Membrane

9. Place your membrane in blocking buffer (3% BSA or nonfat dried milk in PBS) and incubate at room temperature for 2 hours with agitation or leave the blot overnight at 4°C in blocking buffer. The purpose of blocking is to keep the membrane from adsorbing antibody molecules nonspecifically. The membrane has a high affinity for protein and we want the antibody to bind only at the sites where the ß-galactosidase is located. What would happen if we forgot to block the membrane or, if for some reason, the blocking step did not work?

Antibody Binding

10. Rinse the membrane twice for 5 minutes each in PBS.

11. Prepare the first antibody, the antibody against ß-galactosidase by diluting it according to the supplier directions in a solution of 3% BSA/PBS (usually l/2000 dilution or greater). The volume of the antibody solution should be enough to cover the blot. Large plastic weigh boats (about 4 inch square) work well and require about 20 mls. **Note: To minimize the volume of solution needed, the blots can be sealed in plastic wrap with heat sealable plastic bags (Fisher). Immerse the membrane in the diluted

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antibody solution. The anti-ß-galactosidase antibody is a mouse monoclonal antibody that was prepared by a commercial supplier.

12. Incubate at room temperature for 1 hour with agitation. (Can be overnight if necessary)

13. To remove unbound antibody, wash the blot with four changes of Tris buffered saline (TBS) for 5-10 minutes each. Note that we are also changing the buffer in this step. TBS is the preferred buffer for the steps that follow.

14. Prepare the secondary antibody solution. The secondary antibody is a conjugate that consists of an antibody that recognizes and binds to antigenic determinants on the mouse anti-ß-galactosidase and has the enzyme, alkaline phosphatase, coupled to the antibody. The enzyme and the antibody are each prepared and purified separately from different sources by the supplier. They were then conjugated chemically by covalently coupling the enzyme to the antibody. Dilute the conjugate as directed by the supplier in a solution of 3% BSA/TBS (usually at least 1/1000 dilution.)

15. Immerse the membrane in the diluted solution of conjugate. Incubate 1 hour at room temperature with agitation.

Detection

16. To remove unbound conjugate, wash the blot with four changes of PBS for 5-10 minutes each.

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17. Prepare the alkaline phosphatase substrate solution and use 10 ml per blot. The substrate solution contains the substrate for alkaline phosphatase, bromo-chloro-indolyl-phosphate (BCIP) and a dye, Nitro Blue Tetrazolium (NBT). This solution is light sensitive and should be made the day of use and stored at 4°C. Develop blots at room temperature with agitation until the bands are suitably dark. Cover the blots with foil while they are developing. Check often as the reaction can happen quickly but can take 30 minutes. Expect to see a brownish-purple-colored band in lanes where ß-galactosidase has been loaded.

18. To stop the reaction, rinse with a PBS solution containing 20 mM EDTA.

19. To save the blot dry it on a sandwich of filter paper, between paper towels, away from the light.

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Chapter 6
Reports

Now that the project is completed, it is time to write the project report. Due to the length of time spent on this project, writing the report may seem an overwhelming task. Remember that no matter how well the purification project worked, and even if it disintegrated into total chaos, you can still write an excellent report.

You will be working in pairs or groups to present your oral reports but you will prepare your written reports individually. The instructor will provide you with guidelines for your oral reports. A suggested format for the written report follows.

Note: Generally we prefer to have oral presentations first, then allow about a week before final written reports are due. The oral reports allow for discussion, analysis and comparison between groups of results and that information can be used to write the discussion section of the written report.

SUGGESTIONS FOR WRITTEN REPORTS: The written report should be organized into four distinct, non-overlapping sections as follows:

- **INTRODUCTION.** The introduction gives the background to the project, starting with the most general statement of the project goals, leading up to more specific

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information such as the types of separation procedures that will be used and which assay methods will be used for analysis. This section should be one-two pages long. No recipes or technical details are given in this section.

- **MATERIALS AND METHODS**. Due to the length of this project, this section should be organized into subsections; for example, extraction, salting out, ion exchange chromatography, Western Blotting and so on. You should report the methods that you performed in **past tense** and in **third person.** You should state exactly how you did them, not how they are written in the lab manual. Do not make a list of the methods - they should be written out in paragraph form. The purpose of this section is to permit someone who is not familiar with the project to repeat all of the procedures exactly as you carried them out. You may assume that your reader is familiar with the laboratory and does not have to be told such information as the fact that you turned the spectrophotometer on in order to use it.

- **RESULTS.** The most common error for a results section is to string together tables and graphs without text explanations. First, organize all the data and assemble all the tables and graphs (part A). Then explain them to your reader (a knowledgeable someone who wasn't in the lab with you).

TABLES AND GRAPHS:

You need at least three tables. Each table must have a title and a legend. The legend, or caption, should explain the table and include any notes

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necessary to understand your data. A sample calculation must be included for each type of calculation.

- **Enzyme assay table**
- **Protein assay table**
  Include a sample standard curve

You need to include the raw data from each assay that you did if you used the information from that assay in the project. But if you threw out the protein assay and did another one, you do not need to report that one. However, the first protein assay should be in your notebook, but it can have a large X through it, to indicate that you did not use the data. Explain in your notebook why you did not use that data.

- **Summary table showing Specific Activity and Yield Data.** You need a table that includes the following data for each step of the purification: (see Table 4.2 in chapter 4)

  - total protein in µg or mg
  - total enzyme activity in units
  - specific activity
  - percent yield
  - purification factor

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- **Ion Exchange Chromatography Data**. You need a properly labeled graph that shows the relationship between enzyme activity and A_{280} as well as fraction number. Use a double Y axis to include both functions. Indicate which fractions were pooled for further analysis. Don't forget the title and legend.

- **PAGE and Western Blot Data.** You need some sort of diagram, photo, drawing, photocopy or other visual representation of your dried gels and Western. Make sure that you label all of the lanes and remember to include a title and legend for each figure. Normally photographs of gels and Westerns are presented in papers.

EXT EXPLANATIONS of RESULTS. You must make statements about the Results. Describe the overall results, not each separate measurement. Look at the data in the tables and graphs and decide what they communicate about the project. Then state in words what that information is. For example, the statement might go something like this "As shown in Figure 5 (Ion exchange graph) the peaks of protein elution were at fractions 20-25 and 45-55. Both of these protein peaks were peaks of ß-galactosidase activity, as demonstrated by enzyme assay results, although peak 20-25 had considerably more enzyme activity than peak 45-55. "

Graphs and tables present data- they do not state results so do NOT simply say " The data is present in Table X and Graph Z."

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The results should state only what you found, not how it would have been better if only ..... That kind of speculation is for the Discussion section.

DISCUSSION

- A brief summary of your results.

- A complete discussion of each section of your results. For example, you might discuss the possible reasons for a decrease in yield at a particular step. Did your specific activity increase as the purification proceeded? Did the total enzyme activity go up or down? You might discuss why you chose to pool the particular fractions you pooled. You might speculate on how you would make different decisions if your goals were different or if your previous purification step(s) had turned out differently.

- A discussion of similarities and differences in procedures followed and results obtained between your group and other groups or individuals. This information will be available to you during the oral presentations. Be prepared to ask questions when your fellow students are presenting.

- A discussions of various aspects of the protocols. Describe ways you would change the protocols and why you think the change would be an

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improvement. Speculate on improvements you would make if you were to repeat this project.

- A description of what you learned about how proteins are isolated and purified. Be specific about the important concepts of protein purification and how different strategies can be used to isolate different proteins. For example, include different methods of extraction, different kinds column chromatography that might be appropriate for purifying various proteins.

- A discussion of any other results you might have. Did you compare the activity of a frozen aliquot with a refrigerated one?

- Conclusions

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Appendix A: Reagents

Molecular Weights of Some Common Atoms and Compounds

MW NaCl = 58.44

MW ß-mercaptoethanol = 78.13, density ß-mercaptoethanol = 1.1 g/ml

MW Tris Base = 121.1

MW Mg Acetate (MgAc) = 214.4

MW NaH_2PO_4 = 120

MW Na_2HPO_4 = 141.96

MW KCl = 74.56

$MgSO_4$ = 120.37

Hints for Making Your Solutions

Always use the C1V1 = C2V2 equation when you are diluting stock solutions.

Always use the purest water available for your solutions. In this class it will be Milli-Q water. In other laboratories it may be de-ionized or distilled water.

DTT is expensive and it gradually evaporates from solutions in which it is dissolved. Therefore, add DTT to your solutions just before you use them. Do this only in the hood. Stock solutions of 0.5 M DTT are stored in the freezer. Remember to pH all Tris solutions

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with the calomel electrode. Also, do not place the electrode in your solution if it contains SDS. Adjust the pH before you add the SDS stock.

Necessary Stock Solutions

1 M Tris-HCl pH 7.6 at 4°C Note that this means you should pH the solution when it is at this temperature.

1 M MgAc

1 M NaCl

Recipes for ß-galactosidase Assay (Procedure 2.2)

Z Buffer (500 ml)

4.26 g Na_2HPO_4

2.40 g NaH_2PO_4

0.373 g KCl

0.06 g $MgSO_4$

5 mM DTT **

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Bring to volume of 500 ml with water. Adjust pH to 7

0.1 M Phosphate Buffer for ONPG pH 7.5 (100 ml)

0.2 M $Na_2HPO_4 \cdot 2H_2O$ (MW = 178.05; use 35.61 g/l)	41 ml
0.2 M $NaH_2PO_4 \cdot 2H_2O$ (MW = 156.01; use 31.21 g/l)	9 ml
water	50 ml

Recipe for Extraction (Procedure 3.1)

Breaking Buffer (200 ml)

0.2 M Tris	40 ml of 1 M Tris pH 7.6 stock
0.2 M NaCl	40 ml 1 M NaCl stock
0.01 M MgAc	2 ml 1 M MgAc stock
5% glycerol	10 ml 100% glycerol
5 mM DTT	2 ml 0.5 M DTT (**add just before using**)

Bring to volume of 200 ml with water.

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Recipes for Ion Exchange Chromatography (Procedure 3.4)

0.2 M NTM Buffer (1 liter)

0.2 M NaCl	200 ml of 1 M NaCl stock
0.01 M Tris	10 ml of 1 M Tris pH 7.6 stock
0.01 M MgAc	10 ml 1 M MgAc stock
5 mM DTT	10 ml of 0.5M DTT (**add just before using**)

Bring to volume of 1 liter with water.

0.5 M NTM Buffer (1 liter)

0.5 M NaCl	500 ml of 1 M NaCl stock
0.01 M Tris	10 ml 1 M Tris pH 7.6 stock
0.01 M MgAc	10 ml 1 M MgAc stock
5 mM DTT	10 ml 0.5 M DTT (**add just before using**)

Bring to volume of 1 liter with water.

0.2 M NTM with 10X Tris (1 liter)

0.2 M NaCl	200 ml 1 M NaCl stock
0.1 M Tris	100 ml 1 M Tris pH 7.6 stock

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0.01 M MgAc 10 ml 1 M MgAc stock

5 mM DTT 10 ml 0.5 M DTT (**add just before using**)

Bring to volume of 1 liter with water.

Recipes for SDS-PAGE[1] (Procedure 4.2)

Running Buffer (1X Laemmli Buffer) 0.025 M Tris pH 8.3 (1 liter)

3.03 g Tris base

14.4 g glycine

10 ml 10% SDS stock

Add most of water, adjust pH to 8.3, bring to volume of 1 liter.

Sample Buffer (4X Laemmli Buffer)

2.4 ml 1 M Tris pH 6.8 (upper gel buffer)

0.8g SDS stock

4 ml 100% glycerol

0.01% bromophenol blues .02%

1 ml ß-mercaptoethanol (electrophoresis grade)

2.8 ml water about

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ßTV 10 mls

[1] SDS-PAGE reagents were taken from Antibodies, A Laboratory Manual.

Recipes for pouring your own gels (Not used in this manual)

Resolving Gel 8% acrylamide (15 ml)

3.8 ml 1.5 M Tris pH 8.8 (lower gel buffer)
4.0 ml 30 % acrylamide stock
7.0 ml water (degassed)
150 µl 10% SDS stock
150 µml APS stock
9 µl TEMED

Stacking Gel (8 ml)

1 ml 1 M Tris pH 6.8 (upper gel buffer)
1.3 ml 30 % acrylamide stock
5.5 ml water (degassed)
80 µl 10% SDS stock
80 µl APS stock
8 µl TEMED

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Recipes for Gel Staining (Procedure 4.3)

Coomassie Blue Stain

400 ml methanol
100 ml acetic acid
2.5 grams Coomassie Brilliant Blue
500 ml water (add water last)
Filter with Whatman paper #1 -
Stain can be reused several times but staining will lose intensity over time.

Coomassie Blue Destaining Solutions :

40% methanol + 10% acetic acid (v/v) (30 minutes)
To speed up the destain process, a small piece of foam rubber can be placed in the container with the gel.
For 1 liter:
400 ml methanol
100 ml acetic acid
500 ml water

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Silver Staining Reagents:

Follow Bio-Rad Kit directions

Recipes for Western Blotting (Procedure 4.4)

Transfer Buffer 25 mM Tris (1 liter)

3.03 g Tris base
14.4 g glycine
200 ml methanol
Bring to volume of 1 liter with water, store at 4°C. Do not add acid or base to adjust pH. The buffer will range from pH 8.1 to 8.4 depending on the quality of Tris, glycine and methanol.

Ponceau S Stock/Working Solution

2% Ponceau S
30% trichloroacetic acid
30% sulfosalicylic acid
For staining, dilute concentrated dye solution 1 in 10 with water to make working solution, store at room temp. This stock is stable for 1 year.

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Phosphate Buffered Saline (PBS) (1 liter)

8 g NaCl

0.2 g KCl

1.44 g Na_2HPO_4

0.24 g KH_2PO_4

Dissolve in 800 ml water. Adjust pH to 7.2 with H_3PO_4. Adjust volume to 1 liter.

10% Sodium Azide Stock

2 g sodium azide

Dissolve in 20 ml water. Sodium azide is poisonous. Avoid inhalation of the powder and use caution when handling the liquid.

3%Blocking solution:

3 grams of BSA **(**non-fat dry milk can be substituted and is cheaper)

ßTV 100 mls with PBS

Tris Buffered Saline (TBS) (1 liter)

8.0 g NaCl

0.2 g KCl

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3.0 g Tris base

Dissolve in 800 ml water. Adjust pH to 8.0 with 1 M HCl. Adjust volume to 1 liter, store at room temp.

Nitro Blue Tetrazolium (NBT) Stock (10 ml)

0.5 g NBT

10 ml of 70% dimethylformamide (DMF)

Store at 4°C for 1 year.

BCIP (Bromo-chloro-indolyl-phosphate) Stock Solution (10 ml)

0.5 g BCIP

10 ml of 100% DMF

Store at 4°C for 1 year.

Alkaline Phosphatase Buffer

100 mM NaCl

5 mM $MgCl_2$

100 mM Tris

Adjust pH to 9.5, store at 4°C for 1 year.

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Alkaline Phosphatase Substrate Solution (NBT/BCIP)

10 ml alkaline phosphatase buffer

66 µl NBT Stock

33 µl BCIP Stock

Use within 1 hour. Wear gloves when using NBT and BCIP.

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Appendix B: MATERIALS AND ORDERING INFORMATION

This appendix is intended primarily for instructors.

Below is a list of materials which are necessary or helpful for the course as well as for each procedure. Note that some of the solutions are marked with an asterix. This indicates that the recipe for that solution is included in Appendix A.

General materials

Means of growing and harvesting bacterial cells of *E.coli*
Incubator, Centrifuge (Clinical centrifuge), Autoclave (?)
Materials for reagent preparation
Balances (standard and micro), graduated cylinders, pipettes
Storage facilities -- Refrigerators, freezers
Ice machine
Source of purified water, distilled or RO water
pH meter

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Procedure 3.1 Bradford Microassay

Visible Spectrophotometer set to 595 nm

Cuvettes with a 1 cm path length

Test tubes for a volume of 1ml

Test tube rack

Vortex mixer

Pipette to accurately measure 10 µl (micropipettors)

Graph paper or graphing program

Bio-Rad protein determination kit # 500-0002, or Sigma Bradford Reagent #B6916

BSA standard at 1 mg/ml (Sigma sells BSA premixed at 1mg/ml or 2 mg/ml # P0914, P0959)

Stock of ß-galactosidase made at ____ U/ml and _____mg/ml protein purified enzyme available from Sigma # _____

Procedure 3.2 ß-galactosidase Assay

Pipettes and micropipettors

Visible spectrophotometer that reads at 420 nm

(Plate reader may also be used for smaller volume assay)

Cuvettes

37 °C water bath or incubator

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Test tubes

Test tube rack

Vortex mixer

Timer

ONPG (o-Nitrophenol -D-Galactopyranoside) Sigma #N1127

ß-galactosidase made up to _____ U/ml commercially available Sigma #

0.1 M Phosphate buffer for ONPG

1 M Na2CO3

*Z buffer

Procedure 4.1 Extraction

E.coli strain ATCC 15224_____ ml overnight culture, centrifuged and harvested in cell paste pellets of approximately 8 g and stored in a freezer at -20°C

Ice Bath

Balance to determine cell weight

Timer

Beakers/flasks

Refrigerated Centrifuge (high speed)

Centrifuge bottles and tubes to harvest bacteri

Branson Sonifier (Sonicator with probe)

Ring stand and sound proof chamber for sonicator

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Storage refrigerator/freezer

Graduated cylinders and pipets for measuring volumes

Source of distilled/purified water

*Breaking buffer

Procedure 4.2 Ammonium Sulfate Precipitation

Beakers

Stir plate and magnetic stirrers

Ice bath

pH meter

Balance

Centrifuge (High Speed)

Centrifuge tubes/bottles

Ammonium Sulfate (NH4)2SO4

1 M NaOH

*Breaking buffer

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Procedure 4.3 Desalting and Changing Buffers -- Dialysis

Dialysis tubing with 10 kD MW cut off

SlideALyzer casettes and buoys from Pierce

8-10 ml syringes and 18 gauge needles for loading and unloading casettes

Latex or Nitryl gloves

Dialysis clips or string (not needed for SlydeAlyzer)

Beakers and flasks

Stir plate/magnetic stir bars

Refrigerator or cold room

Refrigerated high speed centrifuge or refrigerated clinical centrifuge

Pipettes/graduated cylinder

pH paper

Tape

*0.2 M NTM Buffer

Procedure 4.4 Ion Exchange Chromatography

DEAE Sepharose CL-6B (Pharmacia Corp. #_____

Glass columns size _____ with reservoir, stopcock or tubing/clamp setup

Ring stand to hold column

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Beakers/flasks

Aspirator/vacuum source to degas gel matrix

tubing ____ type

UV Spectrophotometer that can read at 280 nm

Glass U tubing or gradient maker

Stir plate/magnetic stir bars

Test tubes and rack

pH paper

Ice bath/ice

*0.2 M NTM Buffer

*0.2 M NTM with 10X Tris

*0.5 M NTM

Procedure 5.1 Concentration of Sample

Millipore ultrafiltration devices #_____

Microfuge

Ice bath

micropipettors

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Procedure 5.2 Polyacrylamide Gel Electrophoresis

Gel casting system such as Hoeffer SE 200 series minigel apparatus_____

- Gel plates notched and entire plates
- Casting Stand
- Spacers/combs
- Clamps for holding casting chamber closed
- Celloseal grease (source_____
- Wax paper

25 ml pipette

Pasteur pipettes

Power supply to run electrophoresis. _____

Flasks/beakers with stoppers

Aspirator/vacuum source to degas gel matrix

Micropipettors

Saran wrap

Razor blades

Pure ß-galactosidase for marker

TEMED N,N,N',N'-Tetramethylethylenediamine Sigma #T8133

Molecular weight markers Source____

- Pre-cast gels
- 10% APS
- 10% SDS

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- Upper Gel Buffer
- Lower Gel Buffer
- Running Buffer
- Loading Buffer
- Resolving Gel
- Stacking Gel

Procedure 4.3 staining the gel

Pyrex dishes

Saran wrap

Bio-Rad silver stain #____

40% Methanol 10% Acetic Acid

10% Ethanol 5% Acetic Acid

10 % ethanol 5% Acetic Acid

Oxidizer

Silver Reagent

Developer

Stop solution (5% Acetic Acid)

*Coomassie Stain

10% glycerol

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Procedure 5.4 Western Blotting

Nitrocellulose or PVDF
3MM paper source
Electro-Transfer apparatus
Molecular weight markers:
Power supply
Saran wrap
Anti ß-galactosidase antibody Sigma # G8021
Alkaline phospatase conjugate Sigma # A3562
Alkaline phospatase substrate,bromo-chloro-indolyl-phosphate (BCIP), Sigma # B0274
Nitro Blue Tetrazolium (NBT) Sigma # N5514

- Ponceau S stain (source _____ *Optional*
- PBS
- 10% Sodium Azide
- Tris-Glycine Transfer Buffer
- Tween Blocking Buffer
- TBS
- NBT Solution
- BCIP Solution
- Alkaline Phosphate Buffer
- Alkaline Phosphate Substrate Solution
- PBS with EDTA

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APPENDIX C: Resources:

Proteins Textbook:

The text we use is Protein Analysis and Purification by Ian Rosenberg. I do not require it of students since It is a fairly advanced text but very well-written.

Reference Textbook:

Seidman, LA. And Moore, CJ. Basic Laboratory Methods for Biotechnology. Prentice Hall, New Jersey. The entire book is useful for biotechnology laboratory work but these chapters are especially important for protein purification and assays:
Chapter 27- Introduction to Bioseparations
Pages 573-591- Solutions to support the activity of proteins
Chapters 19-20: Spectrophotometry and Standard curves

Computer Simulation:

This very important resource can be downloaded free of charge from the following web-site.
http://www.tlsu.leeds.ac.uk/courses/bioc2060/proteinlab102/proteinlab.html

http://matcmadison.edu/biotech/

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http://instruct1.cit.cornell.edu/courses/biobm330/protlab/

Special thanks to the author of this program, Andrew Booth, for updating this excellent program and making it available to all free of charge.) This program is extremely helpful to allow newcomers a chance to get beyond the details of individual techniques and get a sense of the overall process of a protein purification strategy.

We also provide students with copies of any instructions which come with purchased products like Slide-A-Lyzers (Pierce) and Bio-Rad protein assay (Bio-Rad).

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