

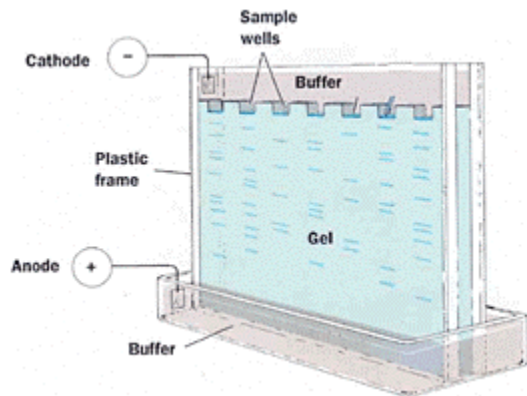
BL/CH 401 Lecture 7

Protein/Enzyme Purification -- Part II

Determination of Purity of Enzyme after Purification Procedure

Although several methods are available for determining purity, the easiest to apply and best method is PolyAcrylamide Gel Electrophoresis (PAGE).

Thus, determination of purity is done by PAGE:



{*Figure 13*} from Voet & Voet Biochemistry Text

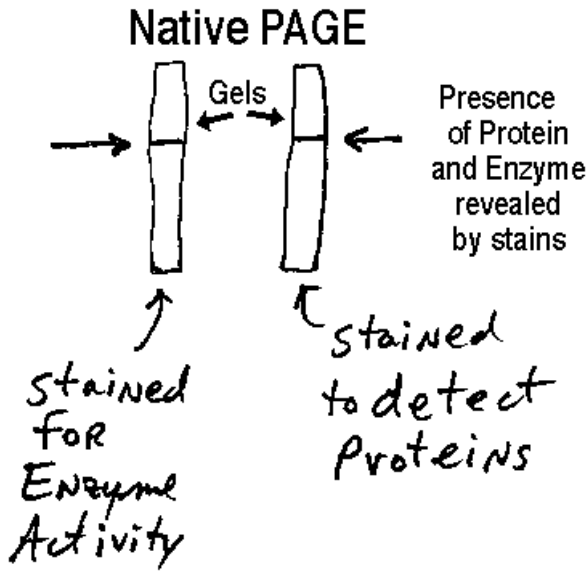
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Illustration of the setup of a PAGE apparatus and concept of using electric charge to drive protein separation. In electrophoresis, the proteins are applied to the gel and separated by applying a high voltage across the gel for ~1 hour. Proteins migrate in an electric field because they are charged at the pH of buffer used in PAGE. After the separation is completed, the gel is stained with a dye to reveal the positions of the proteins in the gel.

NATIVE PAGE

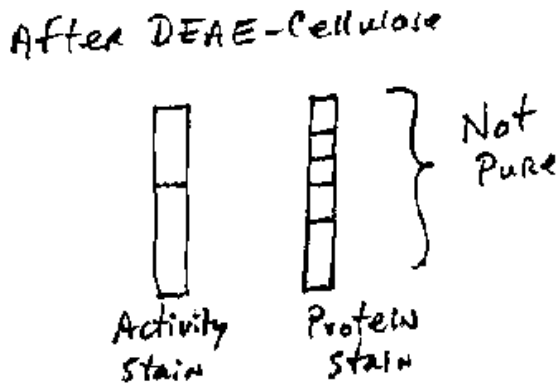
The proteins are maintained in their **Native, Active form during Native PAGE**

Then after electrophoresis the gel can be stained for enzyme activity and for protein content.



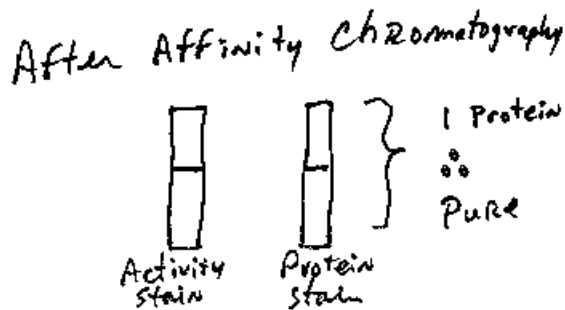
Native Gels to test Purity:

After ion-exchange column chromatography (DEAE cellulose, for example), the enzyme/protein is usually not completely pure:



{*Figure 14*}

The enzyme activity stain reveals the position of the enzyme after PAGE, while the total protein stain reveals the position of all the proteins on the gel after PAGE



{*Figure 15*}

If you find only one band of protein, it is pure and called a homogeneous protein or

enzyme.

Protein Characterization

Determine:

1. Native molecular weight (MW)
2. Subunit composition (ie. is the protein a dimer, trimer, tetramer, etc.) by finding subunit MW
3. AA composition (see lecture 5)
4. Enzyme kinetic properties (To be discussed in Part II of this course)

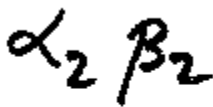
Native Molecular Weight and Subunit Composition.

Some proteins/enzymes are composed of a single polypeptide chain, which are called **monomers**.

Many have multiple copies of the same polypeptide chain -- homodimers, homotetramers. Others have more than one polypeptide chain -- for example hemoglobin has an α and β chains with 2 of each to form a heterotetramer.



Tetrahedral Shape



Subunit Type

{*Figure 17*}

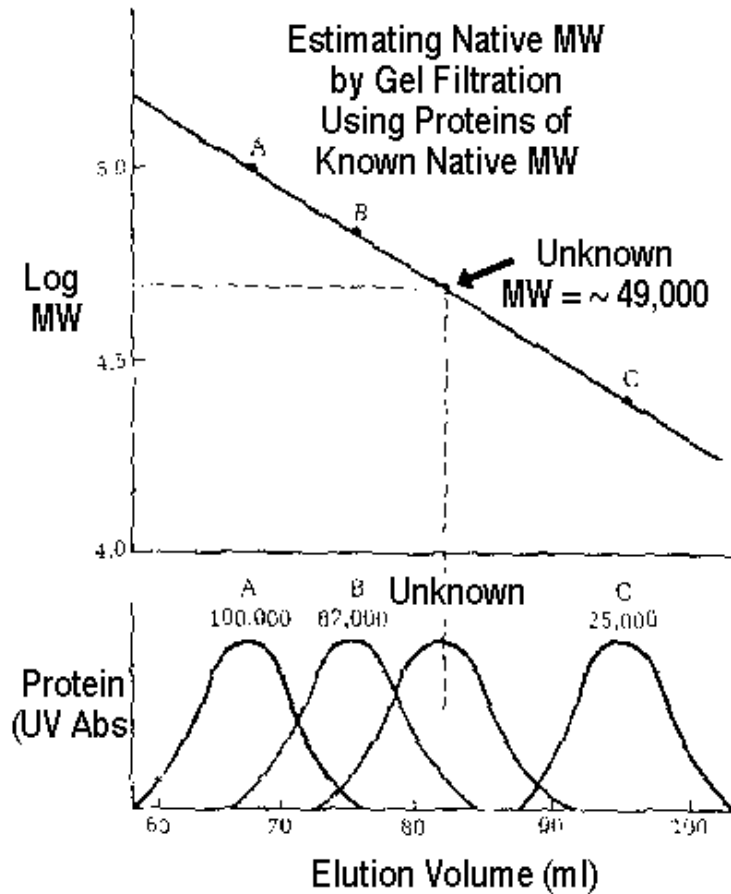
List of Native Molecular Weight and Subunit Composition.

Protein/Enzyme	***** MW (daltons)	** # Subunits	*** Type
Glucagon	3,000	1	Monomer
Insulin	11,500	1	Monomer
Cytochrome c	13,000	1	Monomer
Ribonuclease A	13,700	1	Monomer
Lysozyme	13,900	1	Monomer
Myoglobin	16,900	1	Monomer
Chymotrypsin	21,600	1	Monomer
Carbonic Anhydrase	30,000	1	Monomer
Hexokinase	102,000	2	Dimer
Glycogen Phosphorylase	194,000	2	Dimer
Hemoglobin	64,500	4	Tetramer
Lactate Dehydrogenase	140,000	4	Tetramer
Pyruvate Dehydrogenase	140,000	4	Tetramer
Asp Transcarbamoylase	310,000	12	Dodecamer
Glutamine Synthetase	600,000	12	Dodecamer

Native MW is estimated by gel filtration and...used together with subunit MW to figure out

the subunit composition of the enzyme/protein.

Estimating Native MW:



{*Figure 18*} from Lenigner

Biochemistry ©19?? Worth

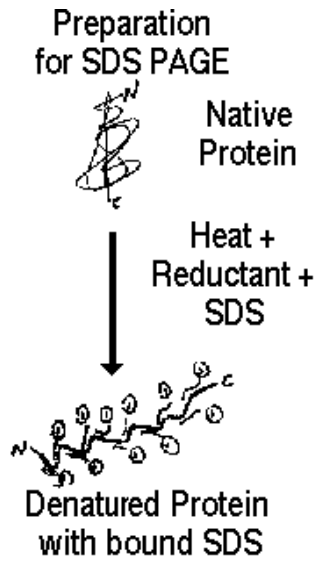
Plot of the calibration for a Gel Filtration Column. The MW of an unknown protein can be estimated using this plot.

Since proteins/enzymes are usually not perfectly symmetric, Gel Filtration provides only an approximate MW. For more accurate MW determinations, more sophisticated methods must be used. But these other methods will not be discussed in this course.

Determining the Number of Subunits by SDS-PAGE

An easy way to analyze the subunit composition of enzyme is denature the protein in the presence of detergent **Sodium DodecylSulfate (SDS)** and run a polyacrylamide gel on the denatured polypeptide. This is called SDS-PAGE or Denaturing PAGE.

Illustration of Protein Treatment with SDS and Heat for Denaturation



{*Figure 19*}

Native protein is unfolded by heating in the presence of a disulfide bond reductant and SDS. Many proteins contain disulfide bonds (Cys-S-S-Cys) joining the polypeptide backbone and to remove these bonds, a disulfide reducing agent (like beta-mercaptoethanol) is used. Disulfide reducing agents convert disulfide bonds (Cys-S-S-Cys) to thiols (Cys-SH). During heating the protein would normally precipitate, but the SDS binds to the backbone and provides a negative charge to make the denatured protein soluble. SDS, like all detergents, has a hydrophobic tail and a charged polar ion. The hydrophobic tail (ie the dodecyl part of SDS) binds to the hydrophobic backbone of the protein, and the ionic sulfate group projects out into solution making the denatured protein soluble.

Binding of SDS to the protein is in proportion to protein size.

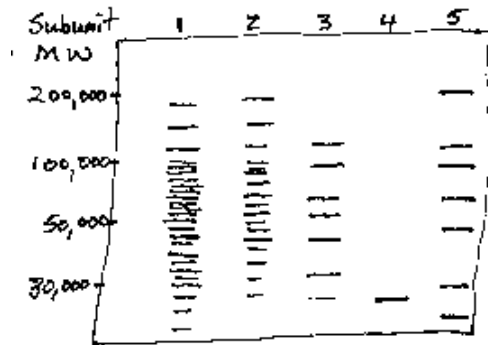
Large polypeptides bind more SDS than small polypeptides. So proteins end up with negative charge in relation to their size. During electrophoresis, large proteins move less distance in the gel than small proteins. This is because the protein/SDS ratio is constant for all proteins and the gel structure controls protein movement by friction. So bigger polypeptides move slower during the electrophoresis. Overall, this results in electrophoretic mobility for polypeptides in the SDS PAGE gel in relation to subunit molecular weight (MW).

The MW of Protein Subunits Estimated by SDS-PAGE

Separate denatured polypeptides by PAGE, which is usually called SDS-PAGE. Estimate MW of subunit by comparison to polypeptides of known MW, which have also been denatured in presence of SDS.

Illustration of an SDS-PAGE gel:

SDS PAGE



PURE ENZYME
Subunit MW = 25,000

{*Figure 20*}

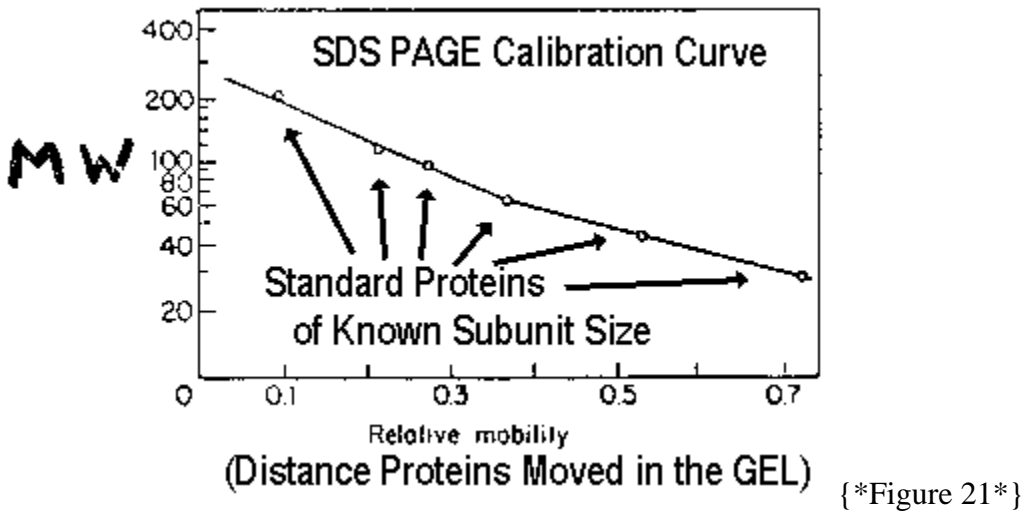
The SDS-PAGE gel illustrated here is for a complete purification of an enzyme. The protein mixtures obtained at each step in the purification are run in a lane of a slab gel (see Fig. 13 above).

The Purification steps and the lane labels on the gel are:

1. Crude Extract -- total mixture of all proteins at the start of the purification.
2. After Ion Exchange Chromatography -- containing enzyme activity of interest and a mixture of proteins.
3. After Gel Filtration Chromatography -- containing enzyme activity of interest and a mixture of proteins.
4. After Affinity Chromatography -- containing enzyme activity of interest and a single protein.
5. Standard Proteins of known molecular weight for their subunits.

The SDS-PAGE gel can only be stained for protein since the proteins are denatured and... no longer have any biological activity.

Calibration Plot for an SDS-PAGE Gel



The log of the molecular weight (MW) is plotted versus the electrophoretic mobility of the standard proteins. (Electrophoretic mobility means how far the protein moved in the gel during electrophoresis). The standard proteins have known subunit molecular weights. Using the plot, the MW of the unknown pure protein is determined. Only pure proteins can be used for estimating subunit MW, since the presence of contaminating proteins would lead to confusion.

Determining the Subunit Composition with Native MW and Subunit MW

By combining the information on the native MW, the subunit MW and the number of different polypeptide chains - you can figure out what the subunit composition of the enzyme is. For example, by gel filtration Native MW = 49,000. One subunit polypeptide was found with MW = 25,000. Therefore, the protein/enzyme exists in solution as a dimer (ie $49,000/25,000 = 2$). In other words, divide the Native MW by the Subunit MW to find an integer.

Summary

Two types of polyacrylamide gel electrophoresis are used in protein purification/characterization.

1. Native PAGE to test for purity.
2. Denaturing or SDS-PAGE to determine subunit MW.

One can also determine by SDS-PAGE if the protein contains subunits of different size. For example, a protein may contain two kinds of subunits and these may have a different size. Some enzymes have a catalytic subunit and a regulatory subunit.