

BL483 BASIC BIOCHEMICAL TECHNIQUES

Lecture 8 - Denaturing/SDS PAGE and Subunit Composition of GOT -- Expt 4 Part B

Read in Text: p. 48-50

Polyacrylamide Gel Electrophoresis (PAGE) for Proteins

There are two types of PAGE which you will do:

- 1) Native PAGE - in Week 7
- 2) SDS-PAGE (Denaturing PAGE) - in Week 8

Lab Report - What to Turn In for Lab Report 4

Prepare report with usual sections: Introduction, Experimental Design, Methods, Results, Discussion

Native Page from Week 7

- Schematics of Native Gels for Protein Stain and GOT Activity Stain
- Table of Relative Mobilities of Bands for Protein Stain and Activity Stain

SDS-Page from Week 8

- Schematic of Protein Stain for Denaturing SDS Gel
- Mobilities of all bands in Gel for F-I, F-II, AS-II and CMC Fraction
- Plot of Std Protein MW (log) vs Relative Mobility on SDS-PAGE
- Table of Subunit MW estimates for all bands in CMC Fraction and Sigma GOT
- Native MW Estimate from Week 8
 - Plot of log MW of Std Proteins vs Relative Elution (V_e/V_o)
 - Estimate of Native MW of GOT
- Composition of GOT
 - Use Subunit MW for CMC Fraction and explain how you decide which band on the SDS gel represents the GOT Subunit
 - Divide your Native GOT MW by Subunit MW for finding GOT composition
 - Decide if GOT is a monomer, dimer or what for both your purified GOT and

Sigma GOT

• Discussion

- Compare Native Page and SDS-PAGE - which is more highly resolving electrophoretic method? Why do you think one is better than the other in resolution...What are the advantages of each gel method?
- Compare PAGE results with Purification results from Lab Report 3 using the number of protein bands found and specific activity of GOT, as well as total proteins in each purification step
- Describe how you were able to decide which protein band is GOT in Native Gel and how this helped to sort out the bands in SDS-PAGE gel.
- Compare SDS-PAGE results for your CMC fraction and Sigma GOT and describe any differences found in the subunit MW of the two samples. Why do you think there would be any difference between your CMC GOT and Sigma GOT?
- Compare the Composition of the Sigma GOT to your purified GOT fraction - do both GOT samples have the same subunit composition?

SDS-PAGE (Denaturing PAGE)

SDS-PAGE is an electrophoretic method for separating protein subunits after they have been denatured by heating under reducing conditions and bound with the detergent SDS. During denaturation by boiling, all disulfide bonds in the protein are reduced with 2-mercaptoethanol (some times called beta-mercaptoethanol) and the protein subunits (ie polypeptide chains) are uniformly bound with the detergent sodium dodecyl sulfate (SDS), which has the structure $\text{CH}_3(\text{CH}_2)_{11}\text{-SO}_3^-$ and the sodium is just a counter ion. The detergent gives the polypeptide a uniform negative charge and binds in proportion to size of the subunit. As a consequence, all the protein subunits in a mixture of proteins have the same charge density and will migrate in an electric field with the same mobility. However, in an SDS-PAGE system, the pores in the PAGE gel are small enough to cause molecular sieving during electrophoresis so that the polypeptides coated with SDS separate by size. Thus, an SDS-PAGE gel can be calibrated with proteins of known subunit size (or molecular mass - which is called Mr) and so the molecular mass of an unknown polypeptide can be determined. This is best done by preparing a plot of the log Mr of the protein bands representing the standard proteins versus electrophoretic mobility of the protein bands as compared to the dye front (called relative mobility). Then relative mobility of the unknown protein's subunit(s) can be compared to the standard curve and its Mr (ie. molecular size or Mr) estimated.

Since the proteins are denatured for SDS-PAGE, it is usually impossible to detect the enzyme activity after SDS-PAGE. However, since SDS-PAGE is a more highly resolving method than Native-PAGE and gives sharper protein bands after the gel is run and stained with Coomassie Brilliant Blue G, the same dye as used for the Native PAGE gel to stain for all proteins, the SDS-PAGE gel is better for detecting the presence of contaminants of the protein of interest. This, of

course, assumes that you have some way of knowing in advance what the expected Mr of your protein of interest is. In practice this is often the case, since you may be working with a cloned gene for the protein and have predicted the protein's subunit size by computer calculation of its molecular weight from the amino acid sequence. Or in some cases, you may have been purifying an enzyme from a new source and know the Mr of its subunit from another source (for example, the enzyme you are purifying is from spinach leaves and the enzyme has already been purified from corn leaves and its Mr determined). In the real world, one often only runs SDS-PAGE gels on proteins you are studying, but it is useful to know about the technique of Native PAGE and its utility as compared to SDS-PAGE.

For more details concerning the SDS-PAGE treatment of proteins is provided here from 401 Lecture Notes.

Determining the Number of Subunits by SDS-PAGE

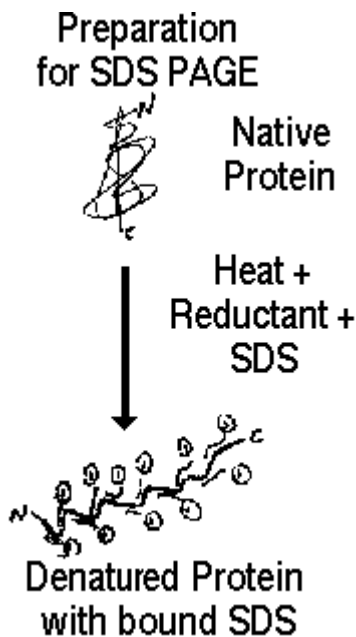
An easy way to analyze the subunit composition of enzyme is...

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



Native protein is unfolded by heating in the presence of a disulfide bond reducing agent 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.

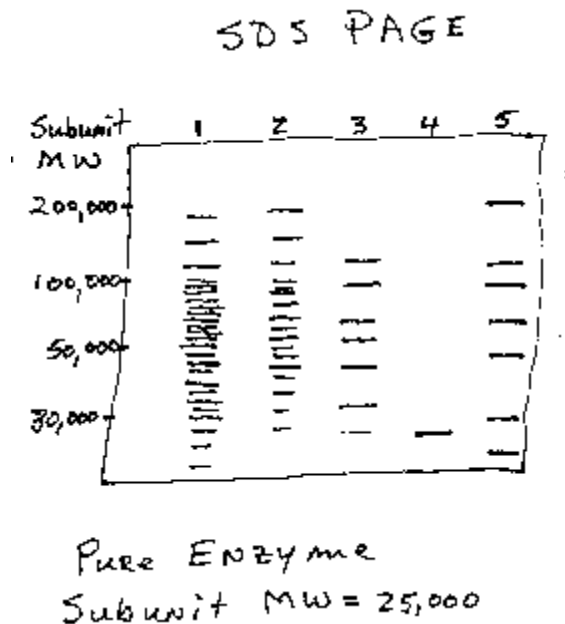
Large polypeptides bind more SDS than small polypeptides. So proteins end up with negative charge in relation to their size. During electrophoresis, large proteins move less distance in the gel than small proteins. This is because the protein/SDS ratio is constant for all proteins and the gel structure 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:



The SDS-PAGE gel illustrated here is for a complete purification of an enzyme. The protein mixtures obtained at each step in a typical purification are shown with the pure enzyme in lane 4 of the gel shown.

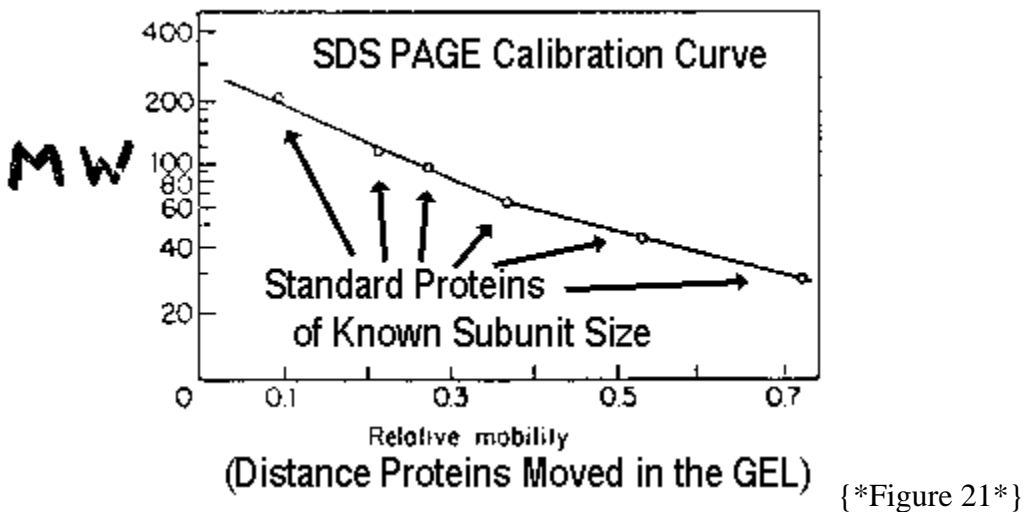
The Purification steps and the lane labels on the gel are:

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.

SDS-PAGE on GOT (Week 8)

An SDS-PAGE mini-slab gel will be prepared in advance of the labs this week and have already been run and stained prior to your class. This is necessary since it would take too long to prepare, load, run, stain and destain an SDS-PAGE during the 3 hour lab. So, your lab this week is to analyze the gels of your samples from the GOT purification. As in the Native PAGE gel, we will have run your GOT samples of FI, FII, ASII and CMC-13 (or whatever your hottest fraction

was) as well as the GOT from the Sigma Chemical Co. as a comparative standard. The gel will also have a lane with a mixture of standard proteins for calibrating the gel (you will be given a handout in lab which will identify the Mr of the standard proteins found on your gel). You should make a drawing of the gel, which shows the intensity of the stained protein bands and measure the electrophoretic mobility of all the bands in the lane with the standards, the lane with your CMC fraction and the lane with the Sigma standard. With this information, make a standard curve for the calibration of the gel using the given Mr values for the standard proteins and estimate the Mr of all bands in your CMC fraction as well as the Sigma GOT.

Since your CMC fraction will contain more than one band and as it turns out so does the Sigma GOT, try to use the staining intensity of the bands to decide which is the major protein and assume that this is the subunit of GOT.

So in the end, you want to report the size of all the protein staining bands found in your CMC GOT fraction and explain which one you think is the real GOT band. You can compare the results of this SDS-PAGE gel and your native PAGE, as well as the results of your GOT purification where the specific activity of GOT in each fraction obtained in purification can be compared to the PAGE gel patterns. As the specific activity of GOT goes up in your purification, you should find fewer bands on the SDS-PAGE gel.

Native Molecular Weight of GOT and Its Quaternary Structure

Estimate the native MW of GOT using the data handed out as shown here:

NATIVE MOLECULAR WEIGHT OF SIGMA GOT

ESTIMATION OF NATIVE MW BY FPLC GEL FILTRATION

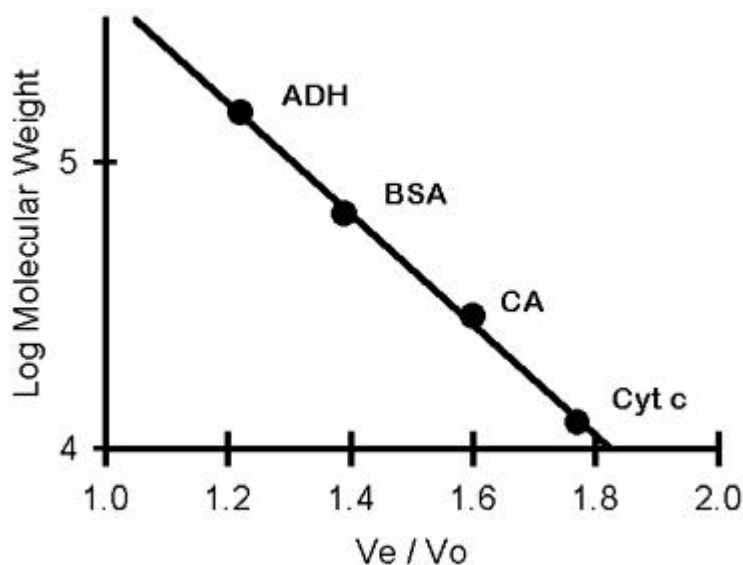
The native molecular weight (MW) of Sigma GOT will be estimated by gel filtration chromatography. The data to do this is provided here. You should treat this data as if you collected it in the lab running the FPLC system during your lab this week. I carried out the experiment for you and the data I collected are below.

CALIBRATION OF THE FPLC GEL FILTRATION COLUMN

To estimate the MW of GOT, the gel filtration column must be calibrated using proteins of known MW. . The standard proteins used for calibration and their native MW are:

Protein	Native Molecular Weight (MW)
Alcohol Dehydrogenase (ADH)	150,000
Bovine Serum Albumin (BSA)	66,000
Carbonic Anhydrase (CA)	29,000

Cytochrome c (Cyt c)	12,400
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Estimation of GOT Native MW

Make a plot of the log MW versus ratio of V_e/V_o (like that shown above). V_o (void volume of the column) is equal to the time it takes Blue Dextran to elute from the column since it has a MW of more than 1 million. V_e is the elution volume of the various standard proteins and GOT. Measure these values from the data on the chromatograms shown below. Volumes are given in minutes, which is equivalent to volume since the FPLC gel filtration column is pumped at a constant flow rate of 0.5 ml per min. The total volume of the column is 24 ml so things eluting at 45 to 50 min are very small. A typical calibration curve obtained with the Sigma MW-GF-200 kit used for this experiment is shown below.

Experimental Data for Your Calculations:

To estimate the MW of GOT, calculate its V_e from the data shown below and use your plot of the standard proteins to obtain the log of GOT's MW, then convert this log to MW. If you do not have Semi-Log paper, then take log of MW of standards and plot on linear graph paper (as shown above).

Chromatogram for Blue Dextran (use to find V_o):



Chromatogram for 2 of the standard proteins (use to find V_e for BSA and Cyt c):



Chromatogram for 2 other standards (use to find V_e for ADH and CA):



Chromatogram for GOT (use to find V_e for GOT):



This GOT was purchased from Sigma Chemical Co. A student sample of GOT eluted from the FPLC at the same position as the Sigma GOT except it gave a much lower absorbance.

Calculations of Quaternary Structure of GOT:

From these data determine what the quaternary structure of GOT is. By quaternary structure, I mean is GOT a monomer, dimer, or tetramer or what, according to your data. This is done by dividing the Native MW (obtained from the gel filtration estimate) by the subunit M_r or MW (obtained from the SDS-PAGE analysis) and rounding to the nearest whole number (since a protein can not contain a fraction of a subunit).

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