

BL483 BASIC BIOCHEMICAL TECHNIQUES

Lecture 6 - Glutamate Oxaloacetic Acid Transaminase (GOT) Purification -- Expt 3 Part A

Read in Text: p. 69-75

Lab Expt #3 - 1st Part in 6th Week - Expt #10 p. 115-117 (Procedure will be done differently than in text)

What you will do in Lab: The purification of Glutamate Oxaloacetate Transaminase (GOT) from pig heart will be done over a 3 week period. **BE SURE TO SAVE SAMPLES AT EVERY STEP OF THE GOT PURIFICATION SO COMPLETE ANALYSIS CAN BE DONE ON ALL PARTS OF THE EXPERIMENT.** The following scheme will be used for the purification:

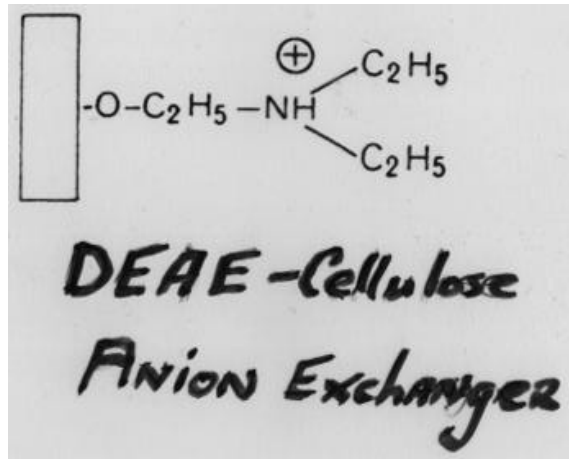
- **Week #6 Lab** - Ion exchange (carboxyl methyl cellulose or CMC) chromatography on dialyzed ASII is done and fractions collected are assayed for GOT activity and absorbance at 280 nm for protein. Folin/Lowry protein assays on ASI, ASII, ASII after dialysis and ASIII are done. The peak fraction CMC fraction with the most GOT activity is saved for analysis by gel electrophoresis in the Week # 7 and 8 labs.

1. Ion Exchange Chromatography - Part A - Types of Ion Exchangers

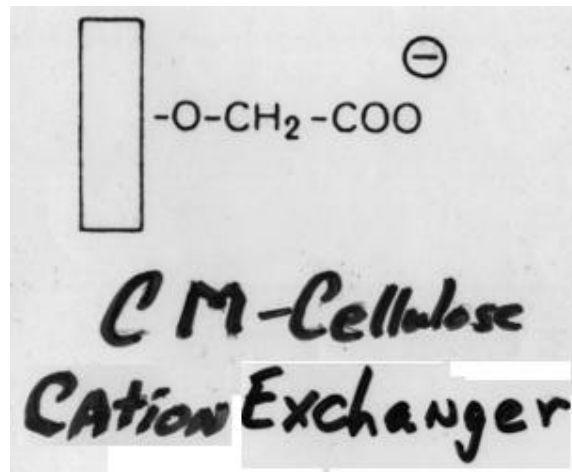
Ion exchange chromatography depends on the ionic character of a protein/enzyme and since this property of an enzyme will change with the pH, it also depends on the pH of buffer used to dissolve the enzyme. In addition, the ionic strength of the solution or its total salt concentration is important in making ion exchange chromatography work.

There are two basic types of ion exchangers: those for binding positively charged ions or cations, which display on their surface negatively charged groups; and those for binding negatively charged ions or anions, which display on their surface positively charged groups.

The ion exchanger is composed of the solid support material, which for enzymes and proteins must be a "hydrogel" or polymer composed for easily hydrated groups like cellulose consisting of polymers of sugar molecules. On the surface of the polymeric support material ionic groups are displayed which have been covalently linked to the polymer support. Here are models of ion exchangers:

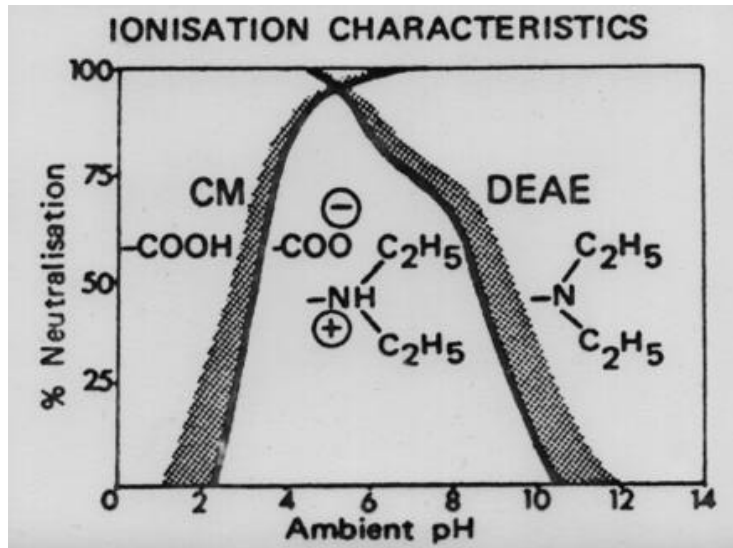


DEAE-cellulose or DEAE-C has a cellulose bead as the support material which has been derivatized with a positively charged amino group displaying a positive charge into the solution.



CM-cellulose or CMC has a cellulose bead as the support material which has been derivatized with a negatively charged carboxylic acid group displaying a negative charge into the solution.

Both these ion exchangers have changing degrees of ionization depending on the pH:



Thus, the pH range over which you can use the ion exchangers is limited by these properties. For example, the effective pH range for CMC is above pH 4, while it is below pH 9 for DEAE-C.

2. Ion Exchange Chromatography - Part B - Using Ion Exchange for Protein Purification

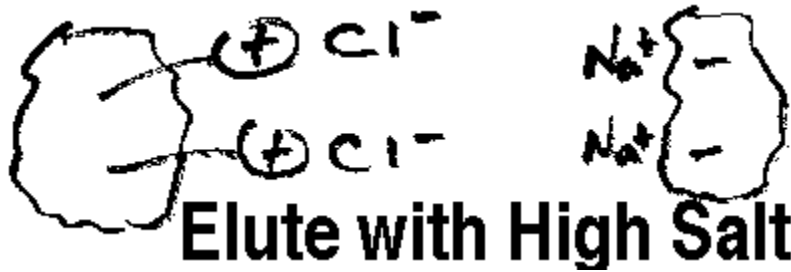
The process of using the ion exchanger, for example a cation exchanger, in a protein or enzyme purification goes like this:

1. Equilibrate the cation exchanger with low salt concentration buffer of desired pH
2. Apply the enzyme in same buffer at same pH and salt concentration
3. Wash the unbound ions away using the equilibration buffer
4. Elute the bound enzyme by increasing the salt concentration in the buffer

Here are illustrations showing these steps:

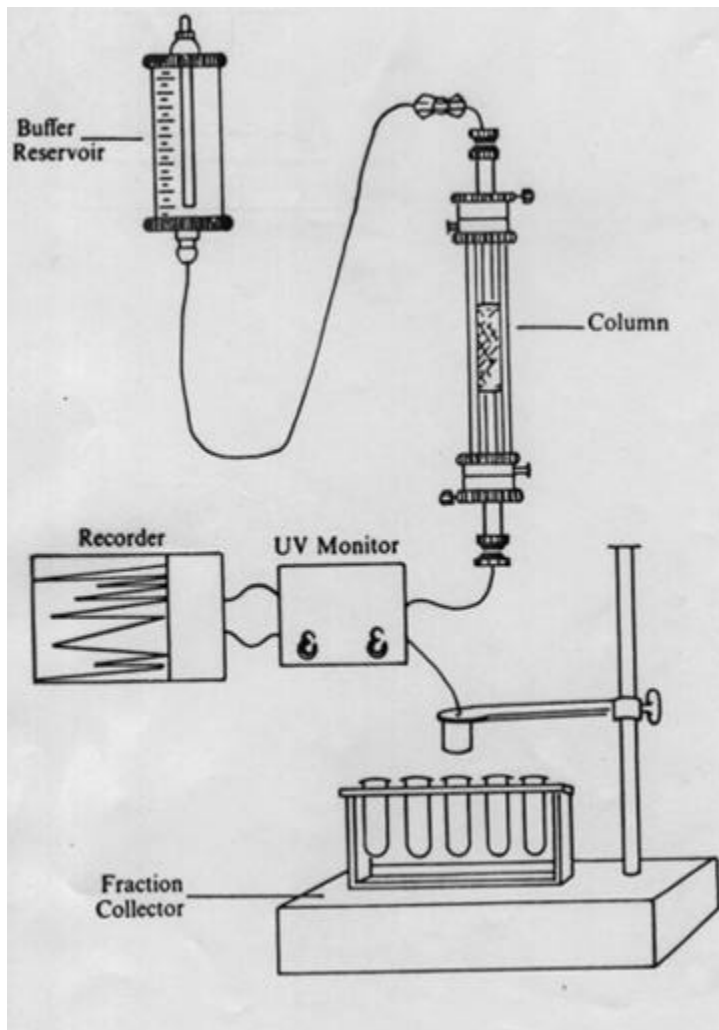


- For enzyme binding, the protein solution is applied to Ion Exchanger at fixed pH and low [salt]



- For elution of enzyme from Ion Exchanger, a high [salt] is applied to column.

Generally, the ion exchanger is poured into a column before the protein or enzyme is applied and then after the binding and washing steps, the enzyme or protein is eluted into a fraction collector with the effluent or solution eluting from the column being monitored by an absorbance device for measuring the protein concentration in the effluent.



We will use a similar set up in the lab this week as described in other sections of this lecture.

3. Applying Beer's Law to Continuous Enzyme Assays

To determine the rates of GOT (and GDH) assays, we will apply Beer's law to the continuous enzyme assay:

Rates obtained directly from spectrophotometer outputs in change in A-340 nm/min

- To convert delta A-340nm/min to $\mu\text{mol}/\text{min}$, use Beer's law; where $c = A/E$
- For this Expt we say, amount NADH oxidized/min = (delta Abs/min)/extinction coefficient
- Extinction coefficient for NADH = 6.2/ml of assay

Conversion absorbance readings to amount of NADH is complicated by several factors of your assay conditions:

- Total volume of assay = 3 ml
- Volume of purification fraction used in the assay = 0.1 ml
- Dilution factor for each fraction analyzed = variable (different for each assay)

These factors are then combined with the delta A-340 nm/min for each assay, to yield rates in terms of μmol OAA formed/min/ml purification fraction analyzed (remembering that for each NADH oxidized an OAA is reduced).

$\mu\text{mol}/\text{min}/\text{ml} = (\text{delta A-340 nm}/\text{min}) \times (3 \text{ ml assay volume}/6.2) \times (1/0.1 \text{ ml extract volume}) \times (\text{dilution factor})$

Defining 1 unit of GOT enzyme activity = 1 μmol OAA formed/min simplifies the reporting of your results, which can then be given as units GOT activity/ml purification fraction.

Example of how to calculate GOT Activity:

- Assume for purification fraction F-1, you obtained delta A-340 nm/min = 0.1 and the F-1 had been diluted 1:100 before the assay was done
- GOT units/ml F-1 = $(0.1/\text{min}) \times (3/6.2) \times (10/\text{ml}) \times (100)$
- GOT units/ml F-1 = 48 μmol OAA formed/min/ml F-1
- This simplified to 48 units GOT activity/ml F-1, assuming 1 unit of GOT activity = 1 $\mu\text{mol}/\text{min}$

Calculation of Specific Activity (ie. units of enzyme activity / mg total protein):

Let's carry this example one step further. You need to calculate specific activity for each step in the GOT purification to make a judgment about the improvement in purity of GOT achieved by each step in the purification.

Example of how to calculate GOT specific Activity:

- Assume for purification fraction F-1, you got Folin assay A-500 nm = 0.200 for the 0.1 ml of F-1 centrifuged extract diluted 1:10
- Total protein for F-1 is found using the Folin standard curve and we'll assume it is (150 $\mu\text{g}/0.1 \text{ ml}$) x 10 or 15 mg/ml
- Specific Activity GOT units/mg F-1 = (48 $\mu\text{mol OAA formed}/\text{min}/\text{ml F-1}$) / (15 mg protein) or 3.2 $\mu\text{mol}/\text{min}/\text{mg protein}$
- This simplified to 3.2 units GOT activity/mg for F-1, assuming 1 unit of GOT activity = 1 $\mu\text{mol}/\text{min}$

Summary: To determine Specific Activity for each step in the purification -

1. **Determine the GOT activity in $\mu\text{mol}/\text{min}/\text{ml}$ of the fraction**
2. **Determine the amount of total protein in that fraction in terms of mg/ml of the fraction**
3. **Then divide the GOT activity/ml by the total protein/ml to yield the specific activity in GOT activity units/mg total protein.**

4. Summary Table of an Enzyme Purification

A key part of any enzyme purification is to make a summary table which brings together all the results from the various steps of the procedure into a single place for easy comparison. A summary table can be found in almost any paper on purifying an enzyme in a journal in the library. An example is given in the text in Expt #10, p. 116. You can use the text example as a guide for the one in your Lab Report, but I want one additional column in the Summary Table in your Lab Report, which is one that shows how much your specific activity has improved at each step - I will explain this below.

Your Purification Summary Table for Expt #3 - Purification of GOT should have the following columns:

1. Purification Step (called "Fraction" in text example)
2. Volume of the total sample obtained at each step of purification in ml
3. Protein for each sample in mg/ml - obtained in your Folin assays for most steps in purification, but use the A-280 nm for the last step (CMC fractions) where you arrive at mg/ml by assuming that an A-280nm = 1.0 is 1 mg/ml

4. GOT Activity in units/ml where units are defined as $\mu\text{mol OAA formed/min/ml}$
5. GOT Specific Activity in units/mg protein -- obtained by dividing column 4 by column 3
6. GOT Total Activity in units -- obtained by multiplying column 2 times column 4
7. Yield in Percent -- treat GOT Total Activity units for Fraction I (crude extract) as 100 % and find the Yield for other steps in purification by dividing their GOT Total Activity units by Fraction I GOT Total Activity Units to obtain a fraction which is then multiplied by 100 to get the percent Yield -- Overall Yield will decrease at each step in the purification
8. Fold Purified -- treat GOT Specific Activity for Fraction I as "1" and basis for calculating other steps increase in Purification -- find the Fold Purified for each step by dividing their GOT Specific Activity by Fraction I GOT Specific Activity, which should give a number greater than 1 if the GOT is more purified after that step -- Overall, Fold Purified should increase at the key steps of the purification.

Tricky Part of the Purification Summary Table: For Fraction I, you only took 5 ml of the total of 75 ml of pig heart extract you were given to obtain the data for Fraction I. However, you must treat this step in the purification as if you centrifuged all 75 ml of pig heart extract or your table will look messed up. In addition, you do not actually get 75 ml of Centrifuged Crude Extract, which is what we call Fraction I, since there is a large pellet after centrifugation. As shown below in the example Purification Summary, 45 ml is the volume of Fraction I, which is arrived at by taking the volume of Fraction I after you have centrifuged the 5 ml of pig heart extract to decide how much of the 75 ml would be Fraction I. In the data for the example Table below, 3 ml of Fraction I was obtained so the real volume = $(3/5) \times 75 \text{ ml} = 45 \text{ ml}$.

Getting a Better Yield from CMC Step: You may find that in the last step (ie. Carboxy-Methyl Cellulose Column Chromatography), the GOT did not come out in one fraction. In this case, you still want to report your best fraction (ie. one with most GOT Activity) to get the Specific Activity. But you will get a better Yield if you sum up all the CMC fractions containing GOT Activity. To do this you multiply the volume (5 ml) times the GOT Activity (units/ml), then add up the total units of these fractions and calculate the Yield...**Do Not calculate a Specific Activity for the Summed Total Units or a Fold Purification.**

Example of Purification Summary for Pig Heart GOT (typical data - yours should be different):

Step	Volume	Protein	GOT Activity	GOT Total Activity	GOT Specific Activity	Yield	Fold Purified
	ml	mg/ml	Units/ml	Units*	Units/mg	%	
Fraction I (Centrifuged Crude Extract)	45	17	48	2160	2.8	100	1.0
Fraction II (Centrifuged Heated Extract)	43	4	21	903	5.3	42	1.9
Ammonium Sulfate II	4	13	194	776	14.9	36	5.3
CMC #13	5	0.1	60	300	600	14	210

***Units are defined as μmol OAA produced/min**

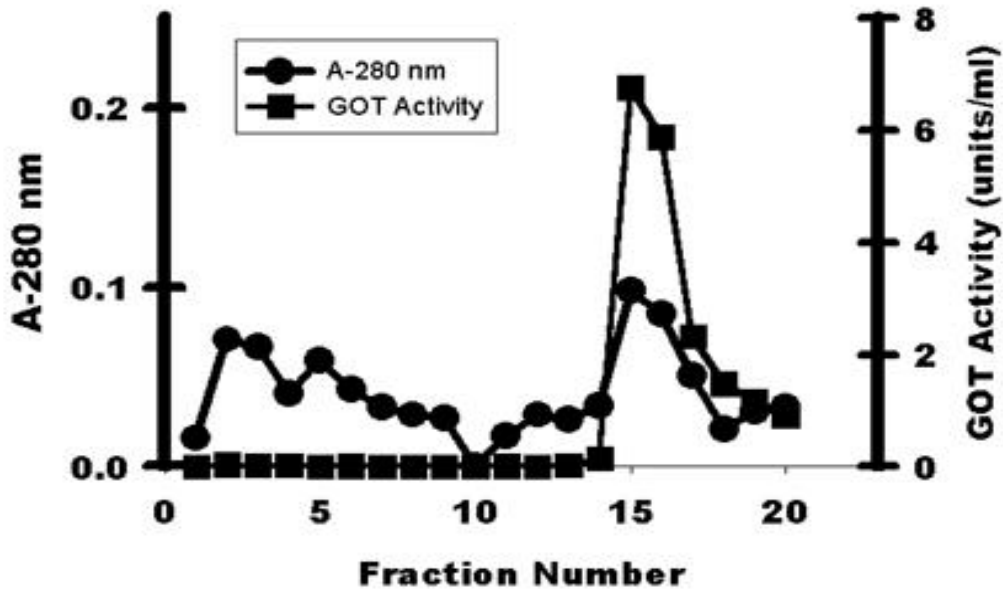
5. Graphic Illustrating the CMC Purification Step

You must make a plot of the elution of the CMC column where you plot two parameters vs. fraction number:

1. Absorbance at 280 nm for each fraction
2. GOT Activity in Units/ml (ie. μmol OAA formed/min/ml)

This is a bit of a complicated graphic and so you should make it by hand using at least 1/2 a sheet of graph paper to make it sufficiently large to easy read. Be sure to use different Y-axis for each of the parameters as well as different symbols to plot them.

Here is an example graphic like the one you should put in your Lab Report:



6. Changes to Week #6 Lab Procedures

These procedure changes will be handed out in class and also in the Lab (in case you miss the class) - be sure to put this information in your Lab Report in the Methods section.

CarboxyMethyl Cellulose (CMC) Column Chromatography

1. The dimensions of the CMC column will be different from those given in the text. We will use a column 2.5 x 1.7 cm. The column will be ready when you arrive in the Lab. It will have a small layer of glass wool on top the CMC to make it easier for you to layer your GOT sample and the various elution fractions without disturbing the CMC bed.
2. Set up 20 test tubes numbered 1 to 20. All test tubes must be of the same diameter. Also set up two test tubes: one containing 2.5 ml of water and one containing 5 ml water. These tubes will be used for comparison to your fractions during the elution where you collect the column effluent so that you can collect fractions of 2.5 and 5 ml volumes.
3. The first step will be to drain off excess buffer from the top of the column by opening the outlet and letting the buffer flow into a beaker. This buffer is discarded.
4. Next apply 1.5 ml of your centrifuged AS-II fraction which has been dialyzed. Be sure to save some of this sample for analysis of GOT activity and protein content. As you layer the sample on, be sure to avoid upsetting the CMC bed and get the sample evenly distributed over the column. While the sample runs into the CMC bed, begin to collect fraction #1. Let it run in slowly!!! Allow all to run in before you do the next step.
5. Apply the 25 ml of 0.03 M Na-Acetate, pH 5.0, wash buffer which will wash the column

of non-binding proteins, to the top of the column very carefully so you do not disturb the CMC bed. Be sure your sample has completely entered the column before applying this buffer. Continue to collect fraction #1 until you have 2.5 ml and then collect 2.5 ml fractions until all the wash buffer has entered the column.

6. Apply 50 ml of 0.08 M Na-Acetate, pH 5.0, elution buffer, which will elute the GOT from the CMC column, to the top of the column and begin to collect fraction #11. This will be a 5 ml fraction and you continue to collect 5 ml fractions until all the elution buffer has passed through the column.
7. Read the A-280 nm of each fraction and record it in relation to fraction #. This will provide a measure of the protein content of your fractions. The concentration of the protein in the fractions can be calculated using the following equation: $1.0 \text{ A-280 nm} = 1.0 \text{ mg protein/ml}$. Thus, if the A-280 nm of a fraction is 0.2 then it has 0.2 mg protein/ml.
8. Assay GOT activity of every other fraction beginning with fraction #2. The most active fractions will need to be diluted 1:9 or more in some cases, to get the assay to be on scale. If you get a very active fraction when you have the 1/10 dilution, even if you can read the rate, try a 1/20 dilution since you may get a better result with this dilution. In the regions of the elution fractions, do an assay on every fraction so that you find all those with high GOT activity. So if fraction 12 has GOT activity, then do fraction 11, 12, 13, 14 and 15 for GOT activity.

Save the fraction with the highest GOT activity, which will be analyzed in Weeks #7 and 8 using gel electrophoresis. Give this fraction to your TA with a label with your lab group name on it.

Lab Report for Expt #3 - What to turn in for this Lab Report

Here is a list of what you need to turn in for your Lab Report - use the usual 4 part style: Introduction, Methods, Results, and Discussion.

In the Results be sure to include the following:

1. **Flow Chart** showing how the Purification was done and here define the Abbreviations to be used for various steps - for example, define F-I as Fraction I - Pig Heart Centrifuged Crude Extract. If you do not define the various abbreviations like F-I, F-II, AS-I, AS-II, AS-III, CMC etc., then the report is confusing and incomplete. Think about your Lab Report as being read by someone who is not as familiar with the procedure as you expect me to be.
2. GOT rates for NADH based activity assays for all fractions analyzed and calculations illustrating how these were converted to GOT Activities as described in Part 3 of this

lecture.

3. GDH rates for assays to detect contamination by this enzyme
4. Protein Assay results, including standard curve for Folin assay (identify standard protein used), volumes of fractions, dilution of these fractions, absorbances for the assay results and calculations of concentration of protein in the fraction in mg/ml.
5. Graphic for CMC column elution as illustrated in Part 5 of this lecture.
6. Purification Summary Table, as illustrated in Part 4 of this lecture.

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