

BL4820 BASIC BIOCHEMICAL TECHNIQUES

Lecture 5 - Glutamate Oxaloacetic Acid Transaminase (GOT) Purification -- Expt 3 Part B

Read in Text: p. 69-75

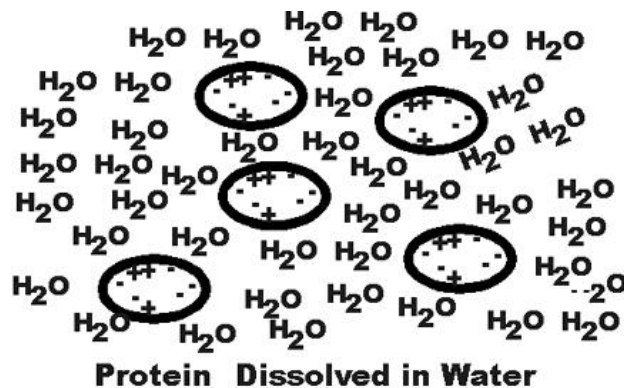
Lab Expt #3 - 2nd Part in 5th Week - Expt #10 p. 113-115

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:

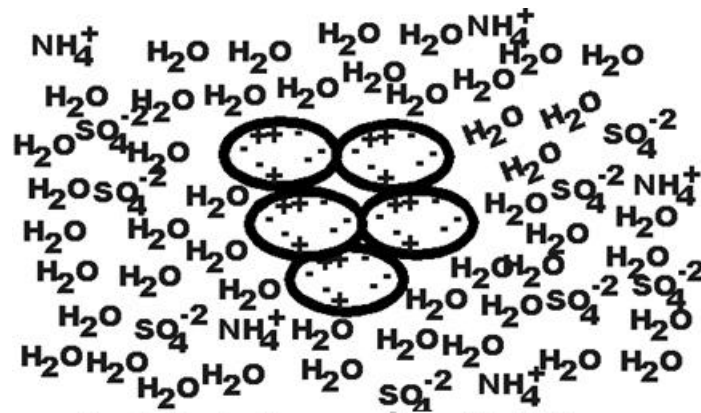
- **Week #5 Lab** - Ammonium sulfate precipitation and preparation from FII of week #4 of the ammonium sulfate fractions - ASI is first precipitate, ASII is second precipitate containing the bulk of GOT which will be dialyzed, and ASIII the supernatant of the second ammonium sulfate precipitation step. Carry out Folin/Lowry protein assays on FI and FII.

1. Ammonium Sulfate Fraction of Protein Mixtures

Increasing the salt concentration to a very high level will cause proteins to precipitate from solution without denaturation if done in a gentle manner. First, we want to understand why the protein precipitates. A protein in a buffer solution is very highly hydrated, in other words, the ionic groups on the surface of the protein attract and bind many water molecules very tightly:



This graphic illustrates how proteins in solution are hydrated by water molecules. When a lot of salt, such as ammonium sulfate, is added to the protein solution, the salt ions attract the water molecules away from the protein. This is partly since the salt ions have a much greater charge density than the proteins. So as the salt is added and these small ions bind water molecules, the protein molecules are forced to interact with themselves and begin to aggregate:



Protein in Ammonium Sulfate

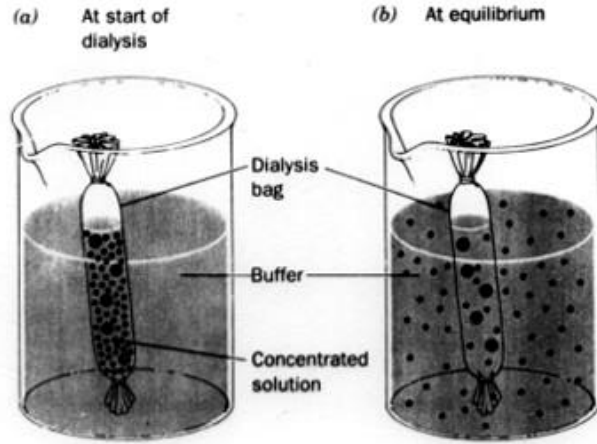
So when enough salt has been added, the proteins will begin to precipitate. If this is carried out at a cold temperature like in ice, the proteins will precipitate without denaturation. Thus, the proteins can be collected by centrifugation and then redissolved in solution using a buffer with low salt content.

This process is called "Salting Out" and works best with divalent anions like sulfate, especially ammonium sulfate which is highly soluble at ice temperatures (see Table 10-1 on p. 114 of your text where 100% saturation of ammonium sulfate is equivalent to a solution of 3.3 M ammonium sulfate at 0°C).

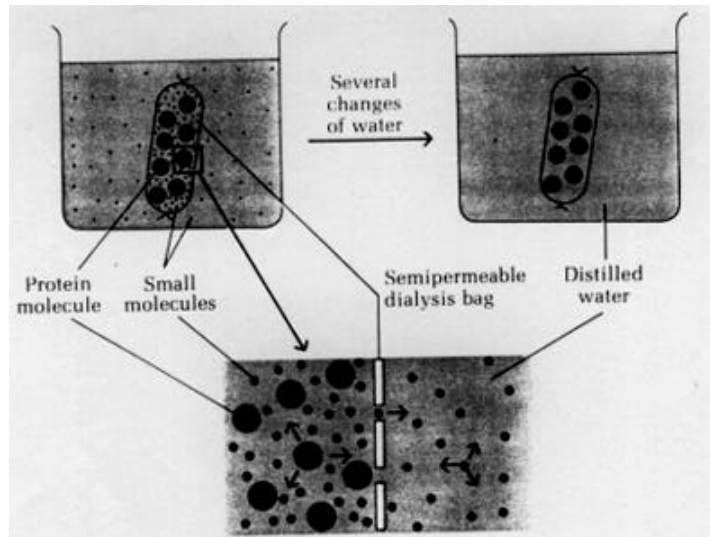
Salting out or ammonium sulfate precipitation is useful for concentrating dilute solutions of proteins. It is also useful for fractionating a mixture of proteins. Since large proteins tend to precipitate first, smaller ones will stay in solution. Thus, by analyzing various salt fractions, one can find conditions where the protein you are studying precipitates and many of the other proteins in the mixture stay in solution. The end result is that you are also able to increase the purity of your protein of interest while you concentrate it using an ammonium sulfate fractionation procedure.

2. Dialysis of Proteins

After a protein has been ammonium sulfate precipitate and taken back up in buffer at a much greater protein concentration than before precipitation, the solution will contain a lot of residual ammonium sulfate which was bound to the protein. One way to remove this excess salt is to dialyze the protein against a buffer low in salt concentration.



This graphic illustrates the dialysis process. First, the concentrated protein solution is placed in dialysis bag with small holes which allow water and salt to pass out of the bag while protein is retained. Next the dialysis bag is placed in a large volume of buffer and stirred for many hours (16 to 24 hours), which allows the solution inside the bag to equilibrate with the solution outside the bag with respect to salt concentration. When this process of equilibration is repeated several times (replacing the external solution with low salt solution each time), the protein solution in the bag will reach a low salt concentration:



The graphic illustrates the complete dialysis process, except for it suggests you do this with distilled water. Really you want to do this process with buffer to prevent the protein from denaturing due to the fact that distilled or deionized water is too low in salt and may have an undesirable pH for your protein, which may cause it to denature.

In fact, dialysis is a good way to exchange the buffer the protein is in at the same time you get rid of excess salt. For example, in the expt we are doing this week in lab, the GOT after ammonium sulfate precipitation contains a mixture of buffers as well as excess salt. So we use the buffer we want for the next step in the purification, which is ion-exchange chromatography, as the external solution during dialysis. After the 3 step dialysis process where the protein solution is dialyzed against the starting buffer for the ion-exchange chromatography step, not only will the salt be

removed but the protein will now be in the buffer needed for the next step and ready to go.

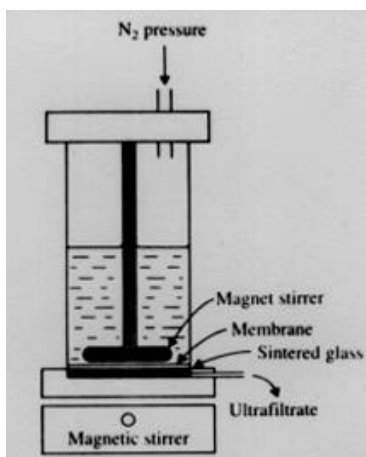
Sometimes, proteins will precipitate during the dialysis process and you will need to centrifuge the solution after dialysis to remove any particles which would interfere with the next step - such as ion-exchange chromatography where particles would clog the column and prevent the chromatography step from working.

In addition, you may lose enzyme activity during dialysis. So it is a good idea to keep some of your protein solution as a sample before it is put in the dialysis bag so that it can be assayed for enzyme activity before and after dialysis.

3. Alternative Methods for Desalting and Concentration of Proteins

There are several ways to get rid of excess salt in a protein solution. One rapid method is to use a small gel filtration column which contains a gel with very small pores which will only allow water and salt inside the gel particles and will exclude the protein. This method works very well and can be done at 4°C so that little or no enzyme activity is lost during processing. A small amount of dilution of the protein solution will take place during processing, but it is possible by this method to exchange the buffer and prepare the protein solution for the next step in the procedure. We will discuss the process of gel filtration in more detail in Week #8 in relation to estimating the native molecular weight of GOT.

Another way to both concentrate a protein and exchange the buffer, which completely avoids precipitation, is called ultrafiltration:



Ultrafiltration is done a device which can withstand high pressure. First, the ultrafiltration device is fitted with an ultrafilter membrane of the desired molecular weight cut off such that you protein of interest will be retain in the cell. Next, the pressure cell is filled with the protein solution and nitrogen gas at about 50 psi is applied while the cell is stirred gently at 4°C. After about 1 hour, the solution will be decreased in volume usually without loss of activity. To exchange the buffer the cell is filled with the desired buffer and the concentration process is repeated.

4. GOT Purification Steps done in Week #5

We will **not** do the pilot ammonium sulfate precipitation procedure described in the text since it takes too long to get done within the 3 hour lab period. We previously determined that the optimum ammonium sulfate precipitation procedure for concentrating GOT is to prepare the fraction between 50 and 70% saturation of ammonium sulfate.

So here is what you will do in the lab:

1. Put a known volume of your GOT F-II in a beaker on ice and slowly add solid ammonium sulfate to bring it to 50% saturation while stirring gently. You figure out how much ammonium sulfate to add using the chart in Table 10-1 in your text on p. 114.
2. Stir for 15 min to allow the precipitation process to come to completion and then centrifuge the solution to collect the protein precipitate. This protein precipitate is dissolved in buffer and assayed for GOT activity (and protein next week). This solution is called ammonium sulfate fraction #1 or AS-I.
3. Measure volume of the supernatant of the 50% ammonium sulfate precipitation and put it in the beaker in the ice on the stirrer. Now slowly add enough solid ammonium sulfate to bring the solution to 70% saturation (again use the chart in Table 10-1 on p. 114 in the text and be sure you use it correctly) while stirring gently.
4. After 15 min of stirring to allow the precipitation process to come to completion, centrifuge the solution. After centrifugation, the precipitate is dissolved in a minimum volume of buffer (try to keep it to about 2 ml) and this will be called ammonium sulfate fraction #2 or AS-II. The supernatant is called AS-III. Both of these should be assayed for GOT activity and samples saved for protein assays next week.
5. Finally, put the AS-II solution in a dialysis bag which has been prepared for you and clamp it carefully so that it will not leak during dialysis. **BE SURE TO PUT A LABEL ON YOUR AS-II SOLUTION IN THE DIALYSIS BAG SO THAT IT CAN BE IDENTIFIED AFTER DIALYSIS.** The TA will carry out the dialysis over the next two days so that the GOT ASII fraction will be ready to go for next week's ion-exchange chromatography purification step.
6. During the ammonium sulfate precipitation procedure, some members of your group should carry out Folin protein assays on your F-I and F-II samples from the first week. Be sure that the samples are sufficiently diluted so that the protein determinations fit on your standard curve which must be done along with the unknown samples. If they do not fit on the standard curve, then you can re-do them next week.