Experiment #5
Ion Exchange Chromatography

Introduction

Ion-exchange chromatography separates molecules on the basis of their charged groups, which cause the molecules to interact electrostatically with opposite charges on the stationary-phase matrix. Therefore, the procedure is limited to purification of ionizable molecules. The stationary phase carries ionizable functional groups coupled to an inert matrix material. Because of the principles of electroneutrality, these immobilized charges are electrostatically associated with exchangeable counterions from solution. Charged molecules to be purified compete with these counterions for binding to the charged groups on the stationary phase and are thereby retarded on the basis of their charge. Different types of molecules will bind to the matrix with affinities that depend on both the conditions used and the types and number of individual charged groups. These differences lead to resolution of various molecule types by ion-exchange chromatography. In typical protein purification using ion exchange chromatography a mixture of many proteins derived from a bacterial cell is applied to the column. After molecules that do not bind are washed away, conditions can be gradually adjusted, such as by increasing the concentration of a simple counterion or by altering the pH, to release the molecule of interest from the stationary phase. Molecules with different charges will elute at specific points in the chromatography as adjustments are made. Thus, the protein of interest can be separated from many others.

Ion-exchange chromatography is named on the basis of the exchangeable counterion. When the stationary phase bears a positive charge and the exchangeable ion is an anion, the process is referred to as **anion-exchange** chromatography. When the stationary phase bears a negative charge and the exchangeable ion is a cation, it is referred to as **cation-exchange** chromatography.

Nearly any molecule that has an exposed ionizable group can be purified by ion-exchange chromatography. Whether an ionizable group is charged depends on the pH of the solvent and the pK_a of the ionizable group. Proteins consist of many different amino acids, and the overall charge is caused by the composite effect of many different ionizable groups. The pH at which the protein has no net charge is called the isoelectric point, and is termed pI. The pI of most proteins is in the range of 5-9. **Ion-exchange of proteins is usually performed at least 1 pH unit away from the pI of the protein of interest to assure that it is charged.** When the pH is below the pI, the molecule will be positively charged and you would use a cation-exchange resin. When the pH is above the pI, the molecule will be negatively charged and you would use an anion exchange resin. Since interactions of ion-exchange groups with proteins depend on the surface (accessible) charges of the protein, even a protein at its pI may bind to the column matrix. Interactions in ion-exchange chromatography can be complex.

By altering the mobile phase, this will allow for selective elution of bound molecules in fairly small volumes of elution buffer. Increasing the concentration of counterion will elute proteins as the counterions compete with the proteins for ions of opposite charge on the stationary phase. For example, Na^+ and H^+ are counterions frequently used for cation-exchange chromatography, and Cl^- and OH^- are commonly used for anion-exchange chromatography. In practice, proteins are usually fractionated by allowing adsorption to the stationary phase at a low concentration of KCl or NaCl and then are eluted from the stationary phase by increasing the concentration of KCl or NaCl in elution buffer. When the concentration of counterions is continuously varied during the elution process, it is referred to as **gradient elution**, whereas when the concentration is altered in a

stepwise fashion during elution, it is referred to as **step elution**.

In this experiment you will use a Q-Sepharose anion exchange column to separate a mixture of proteins. Based upon the charge of cytochorme c (pI 12.4), myoglobin (pI 7.25), and ovalbumin (pI 4.7) you will efficiently separate the proteins using the aforementioned column and the state of the art GradiFrac system.

Experiment

You will be using the GradiFrac system for purification. Please do not operate the instrument until your TA has checked you.

Before you begin be certain that enough test tubes are present in the fraction collector. Also be sure that the entire system is ready, including a sufficient amount of both buffers A and B, all components are turned on, the pens have been inserted into the recorder, and the column is attached properly.

First, set the recorder to the following settings:

- Set the Int.-Ext. in the Int. position (=up).
- Select a chart speed, 10mm/min on dial and button down.
- When running the system the rec. off-on key should always be in the on position (=down). Recorder will then start when flow is started and stopped when the flow is stopped.
- Press the pen up-down keys to down position (=down). The blue pen will show the UV signal and the red pen the gradient.
- Press the zero keys down to zero the pens. Now, adjust the position of the pens to the right hand zero of the paper with the zero thumb wheel. Then release the zero keys, as they must be in the up position when the system is running.
- Additionally, you can adjust the position of the blue marker on the recorder with the baseline adjust potentiometer on the control unit of UV-1. This is done after buffer has equilibrated the system before a run is started. Essentially, this is done to zero the UV signal for running buffer.
- Leave the pen up-down keys in the down position. The pens will move up automatically 30 seconds after the run is finished. When starting the pump again, the pens will go down automatically.

Second, before protein is added system needs to be equilibrated:

- Be sure the valve is in the load position.
- Press the 'down arrow' on the control panel of the GradiFrac to move down through the display until the "Main Menu Manual Run" is shown. Press 'Enter'.
- You are now in the manual block. Press the 'down arrow' once and the display shows "Manual Flow ml/min. Press '2' ml/min and 'Enter'.
- Check that the buffer is flowing properly out of buffer A and the recorder is moving uninhibited.
- You should equilibrate for 10 minutes (20ml).
- After this time press 'End'.
- With the syringe, wash out the sample loop by injecting 10ml of buffer A into port #3. Be sure not to inject any air bubbles.

Finally, add protein sample:

- The 1ml protein sample has already been filtered, so inject this 1 ml into the sample loop **without injecting any air bubbles**.
- Press the 'down arrow' on the control panel of the GradiFrac to move down through the display until the "Main Menu Manual Run" is shown. Press 'Enter'.
- At this time, press the 'down arrow' key on the GradiFrac and press 'Enter' for Manual Run. Also enter flow rate of 2 ml/min, 0% [B], 2ml fractions
- **IMMEDIATELY** after these functions are entered switch the valve to the inject position.
- Note that the recorder marks each fraction, however, it is advisable to closely monitor where each fraction is on the recorder so elution time and volume can be calculated.
- Allow the sample to be loaded to the column and unbound protein washed out for 15 minutes (30ml).

- After this time, you can start a gradient to elute bound proteins. To do so, cycle the 'down key' to gradient and press 'Enter'. For target concentration of B enter 100% and reach this gradient after 50ml.
- After the gradient is over, press 'End', and switch the valve back to the load position. Also wash the column with 50 ml of buffer A at 2ml/min. Do not collect these fractions.
- Lastly, wash the sample loop (port #3) with 10ml of buffer A.

Data Analysis

From the printout of the GradiFrac you should see 3 distinct peaks corresponding to the three proteins you were given. By now you should be able to explain the principles of ion-exchange chromatography and why the elution pattern you have occurs. Be sure to include your elution profile, elution times and volumes. Additionally, a detailed discussion of the elution of the three proteins should be included describing why each is eluted at a particular salt concentration. Be sure to calculate the salt concentration each protein was eluted at.