

Experiment 4: Column Chromatography.

Reading: Chapter 5 and pages 121 – 148 in NB&B.

Goal: In this experiment we will learn, in a “*tour de force*”, how to use three types of column chromatography: Gel Filtration or Size Exclusion (SEC), Ion Exchange (IEC), and affinity (AC) in order to purify proteins and enzymes based on the physical properties of these biomolecules. These experiments require that you be thoroughly prepared **prior** to coming to class in order to finish in the allotted time. *Please make sure you have completed the cytc_Hb_expsy assignment (course home page) prior to Tuesday’s experiment, it will make the rationale behind everything we do much more understandable.*

Introduction

When purifying proteins from native sources, different chromatographic resins are often employed and there are several important decisions that have to be made when designing, or implementing, a sequence of chromatographic steps.

1. What level of purification do you hope to obtain with a given column and will the specific type of column be suitable to achieve that goal.
2. Will the size of the column be appropriate for the amount of material you are loading? This is more critical for size exclusion columns.
3. How will the nature of the buffer from the first column (specifically, pH and/or ionic strength) affect the behavior of the sample on the second column? This point is most critical when going from a size exclusion column to an ion exchange column, where electrostatic interactions are responsible for attraction (or repulsion) of a protein to the resin. If the buffer is inappropriate, it is necessary to exchange buffers by either dialysis (pressure or bag) or Sephadex G25 “desalting” columns? Optimally, it is best to avoid this complication because it lengthens the time required to purify the protein, and often the amount of protein you purify is inversely proportional to the time required to purify it. It is better to choose a buffer for the first column that will allow the protein to be loaded directly onto the second column (as we shall do in the experiment that follows).

The authors of your text, who “have been there and done that”, have one of the best descriptions of both the theoretical and practical aspects of gel filtration or SEC (pp. 125-133), IEC (pp. 143-148), and AC (133 -139) that you can find anywhere. In addition, they adequately describe two other recently developed column chromatography methods under **Gene Fusion** (pp. 139- 141) and **Metal Chelate Chromatography** (141-142). Both of these techniques are specialized forms of affinity chromatography and involve the expression of your protein of interest from a commercially available bacterial plasmid, which “**Tags**” your protein with either another protein or a short peptide sequence that has a high degree of affinity for something that has been covalently cross-linked to a column matrix. Simply put, “**Tagging**” means covalently attaching a polypeptide to either the N- or C- terminus of the protein you want to purify using a bacterial expression system. Generally this technique falls into two categories. First, plasmids that tag your protein with another protein, as shown for

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the gene fusion product coding for malE or maltose binding protein (MBP; see Figure 5-11) or another commonly used gene fusion plasmid coding for glutathione S transferase (GST), which has a high affinity for glutathione. For either of these tags, the substrate analog for the tag is covalently cross-linked to the column, and as the gene fusion product flows through the column, highly specific interactions (H-bonding, electrostatic, van der Waals) between the tag and the substrate allow for rapid purification on the gene fusion product. Elution is accomplished by passing free substrate or product through the column, which disrupts the interactions of the gene fusion construct with the column matrix. In most cases, the short peptide sequence between the tag and your protein contains an amino acid that serves as a recognition site for a specific protease, thereby allowing you to remove the tag by proteolytic cleavage.

The second type of tagging system uses plasmids that tag your protein with a short peptide sequence that codes for either 7-8 His residues (poly-His tags) or a short peptide sequence that is bound very tightly by a β -barrel biotin binding protein called streptavidin (Streptavidin tags). His-tagged proteins are purified by passing the cell lysate through a column to which has been attached Ni (metal chelation chromatography). The poly-His tag on your protein is chelated by the fixed Ni resulting in strong interactions between the protein and the column. Elution is accomplished by adding free imidazole (His) to the column. It is worth noting that Professor Jerker Porath, of the University of Arizona, developed this chromatographic technique. A streptavidin tag is a short peptide (8 – 10 amino acids) sequence that is added to the N-terminus of your protein, which binds very tightly to streptavidin that has been covalently cross-linked to a column matrix. Elution of bound protein from this column requires addition of a biotin analog, HABA (we will determine the K_d for avidin-HABA binding latter in the semester).

It should be obvious that in order to use these techniques, it is necessary to clone (or remove it from the native organism's genome) the gene for the protein you wish to purify or have access to a cloned gene. It also requires that you have some molecular biology knowledge AND ability to sub-clone your gene into the appropriate tagging vector. Fortunately, most of the companies have kits which make this process much less intimidating than it may seem. Once you have made your plasmid construct, the primary advantage of "tagging" is that proteins can be purified to a fairly high degree of purity with just one chromatographic step. As you will see in this experiment, affinity columns are usually very short and small in volume so there is minimal effect of dilution of the protein. However, there are some distinct drawbacks to tagging proteins that we will discuss in class.

Proper preparation, pouring, and storage of resins are also important topics since these materials are usually very expensive and time consuming to prepare. Pharmacia has published booklets that cover virtually all types of chromatography and can be obtained easily. These books are invaluable tools in the laboratory:

- **Gel Filtration. Principles and Methods.**
- **Ion Exchange Chromatography. Principles and Methods.**
- **Affinity Chromatography. Principles and Methods.**

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Refer to a current Pharmacia catalog in order to obtain the current catalog number and ordering information. We will have these books on hand for your reference in the lab.

In Experiment 4.1a and b, you will separate a mixture of blue dextran, bovine hemoglobin (Hb), bovine cytochrome c (cyt c), and riboflavin using a combination of size exclusion (SEC) and ion exchange (IEC) chromatography. The blue dextran and riboflavin are considered dye indicators for determination of the void and total volumes, respectively, of the SEC. Hb and cyt c are both heme-containing proteins with very different physical properties that we be taken advantage of in order to:

1. **Separate based on size.** The size of a protein, or more correctly its molecular diameter, is related to its molecular mass for globular proteins.
2. **Separate based on charge.** The ability to do this will be dependent on the pI of both proteins, which is in turn a function of the number of charged amino acids and their extent of de-protonation (hence their charge) at a given pH. In this experiment, we will use an initial pH that is $< pI$ for both proteins (**What is the net charge on either protein when $pH < pI$?**). We will then use an increasing salt AND pH gradient that will weaken the interaction between the resin and the protein with less charge, while not significantly affecting the binding of the other protein due to the fact that it has a much greater pI ($\gg 7$).
3. Use UV-visible spectroscopy to determine both the purity and the identity of the proteins eluting from the column. Based on the spectra obtained in the previous experiment, you can identify the two proteins based on their visible spectra.

Procedure for Experiment 4.1a and 4.1b: This is an expanded version of the protocol given in your text.

1. Measure and record the height and diameter of the Sephadex G75 resin in your column that has been prepared for you in order to save time. This measurement is common in order to assure reproducibility in future use of the column.
2. Using a **Pasteur pipette**, carefully load the blue dextran, Hb, cyt c, and riboflavin cocktail that you will be given onto the Sephadex **G-75** column **equilibrated with 10 mM phosphate buffer at pH 6.0**. Try not to disturb the top of the bed of resin as this will drastically affect the quality of chromatographic resolution.
3. Begin resolving the material on the column with the same phosphate buffer, collecting 5 ml fractions. (*Hint: Prior to collecting fractions, determine the volume in the collection tubes that corresponds to 5 mL. Make a mark on one tube and use that as a guide for collecting all the 5 mL fractions. Alternatively, you can count the number of drops that are needed to give 5 mL*).
4. While the G-75 column is developing, pour a **1.5 x 5 cm CMC** column and begin equilibrating (i.e. allow buffer to run through the column) with **10 mM phosphate (pH 6.0)**. In order to determine if the column is equilibrated, **you must compare the pH and Conductivity** of the buffer going onto the column with the buffer coming out of the column. If pH and Conductivity are not very similar, the column is not equilibrated and WILL NOT properly bind the proteins. Allow the column

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to continue equilibrating until you are ready to load the samples from the Sephadex column (see below #7).

5. By visual inspection (i.e., eyeball) of the fractions, determine the elution profile. Record the color and its intensity for each fraction. This is most easily done by **looking directly down** on each tube rather than trying to look at the solution from the side. The two things you are looking for are (1) Color and (2) Intensity (which is related to concentration). Make a note of which tube contains the most concentrated solutions of each colored species: the void volume (V_o) corresponds to elution of the blue dextran, the total volume ($V_{total} = V_i$) corresponds to elution of the riboflavin, and the elution volume (V_e) for the two proteins. (Because the riboflavin solution may be very dilute we will also check the fluorescence of these tubes using a black light). It may be difficult to see a clear separation of the two protein bands. As an aid, use the data from (#6) to help identify these fractions.
6. Run UV-visible spectra (700 nm – 250 nm) of the two tubes corresponding to the highest concentration of the two proteins. Refer to your spectra for cyt c and metHb from Expt. 3-3 to determine the order of elution of the two proteins from the G75 column. **Note: identification of heme proteins is based on band location (i.e., wavelength) and shape (i.e., relative intensity).** A second question is the purity of the proteins, determined by the A_{Soret}/A_{280} .
7. Collect all of the fractions containing the two proteins (Hb and cyt c) into a single beaker, then load the pooled solution onto the equilibrated **1.5 x 5 cm CMC column**.
8. Once the proteins have been bound to the CMC column, begin an elution of the proteins using a **buffer gradient**. At the beginning, the buffer will be 50 mM phosphate at pH 6.0, while the upper limit of the gradient will be 200 mM phosphate at pH 8.0. Once the gradient has begun, be prepared to collect 5 ml fractions coming off the CMC column. Elution of the first protein will occur very quickly.
9. Again, run **UV-visible spectra** of the proteins eluting from the column, determining identity and purity.
10. **Please return all protein fractions coming off the CMC column to Dr. Hazzard or your TA.**

Special Cautionary Note: NEVER allow a SEC resin go dry while in the column. A SEC resin bead is like a Wiffle ball – hollow on the inside with holes on the outside. When the column goes dry, inevitably the resin beads lose their buffer interior. Air pockets inevitably will form in the column and inhibit or distort the flow through the material through the column. The only safe recourse is to empty the column and repack with a new batch of equilibrated resin. Allowing the buffer to run below the surface of an IEC column is not so critical, as long as you catch it within a relatively short period of time. If this does happen, you introduce enough buffer to completely resuspend the dried resin, then allow it to settle. However, allowing an IEC column to go dry is never a good laboratory habit.

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Procedure for Experiment 4-2: Affinity Chromatography of beta-galactosidase.

You will be given 1.5 mL of the affinity column resin that will be used to specifically bind and purify β -galactosidase from 1.5 mL of an E. coli cell lysate. The enzyme will then be eluted from the column, collecting 1 mL fractions throughout, and each fraction will then be assayed for both **enzymatic activity** as well as determining **protein concentration** using a Bradford assay. The procedure that follows is a modification of that found in NB&B:

1. Preparation of cell lysate: To the cell lysate add 480 μ L of 5 M NaCl and 13.5 μ L of β -mercaptoethanol (BME). Mix the solutions together well by gently inverting the eppi tube a few times. Remove \sim 100 μ L of this sample and set aside as Crude Cell Lysate. The remaining volume will be loaded onto your affinity column.
2. Equilibration of the affinity column is obtained by rinsing the resin well with 10 mL of Buffer B + NaCl. Use a graduated cylinder to measure the buffer and apply to column using a Pasteur pipette in order to get all the resin to the bottom of the column and packed well. Close the stop cock on your column before loading the cell lysate.
3. Gently load your cell lysate onto column without disturbing resin and open the stopcock. Begin collecting 1 mL fractions at this point and continue collecting the same volume till all the protein is eluted off the column. After the lysate has completely entered the column, shut off stopcock and add 10 mL of rinse buffer (Buffer B + NaCl) carefully so as to not disturb the resin bed. Again, continue collecting 1 mL fractions.
4. Elute the enzyme off the column using 10 mL of Elution Buffer 2 (0.1 M $\text{Na}_2\text{B}_4\text{O}_7$, pH 10). Continue collecting 1 mL fractions.

At this point you have fractions corresponding to initial addition or loading of lysate onto the column, the rinsing fractions, and finally the fractions collected during elution. In addition you also have the crude cell lysate fraction that was set aside. For each fraction you want to know two things: **how much enzyme is present** (determined by doing an enzyme activity assay) and **what is the total protein concentration** (calculated by doing a colorimetric assay, in this case a Bradford assay).

5. **Bradford Assay:** because we are using buffers containing BME, it will be necessary to generate a new standard curve using 1.5 mg/mL BSA as you have already done. Prepare the BSA standards using **Buffer B + NaCl** (instead of water as you did in Expt. 3). In order to minimize the amount of Bradford reagent used, prepare the standard curve samples using:

- 1 mL Bradford
- 86.7 μ L BSA standard

For determining your protein concentration in each fraction, including crude cell lysate use:

- 1 mL Bradford
- 50 μ L from each fraction

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Remember, you use the standard curve to determine the protein concentration for the fraction **IN THE ASSAY**. Using $c_1V_1 = c_2V_2$ you can calculate the actual concentration of protein **in the fraction**.

6. Enzyme Activity Assay:

The **purpose of every enzyme activity assay** you will ever do is quite simply: **To determine the amount of enzyme present in units of enzymatic activity.**

Units of Enzyme Activity: by convention, the amount of enzyme present is defined as *the amount of product produced per minute by the enzyme*. An enzyme Unit (notice capital U) is:

1 EU = amt of enzyme needed to produce 1 μmol Product/min

1 milliU = 1 nmol P/min

Notice, we are talking about HOW MUCH product is produced, not concentration!!!!!! Enzyme concentration is rarely given in molar, millimolar, or micromolar units, rather it almost always referred to as the Units of enzyme activity!!!!!!

Initially, we want to know which fractions contain enzyme, this is determined using a colorimetric assay using ONPG (ortho-nitrophenyl galactopyranoside) as a substrate. The enzyme hydrolyzes off the galactose yielding ONPO^- , a structural analog of PNPO^- , for which you have already determined and extinction coefficient and for which you know the color! A 96 well titer plate will be provided to initially screen your fractions to determine which ones contain β -galactosidase. Mix together:

2 mL Z-buffer

100 μL of 4 mg/mL ONPG

Add 100 μL of this to each well. After all the fractions have been collected, assay for the crude cell lysate as well as each fraction by adding 10 μL of each sample to a single well. Allow the plate to sit for 5 minutes and note which wells have turned the appropriate color. Wells which are very weak in color after 5 minutes contain only very small amount of enzyme and are not worth worrying about. Now that you know which fractions contain β -gal, we will do more quantitative enzyme assays to determine the specific amount of enzyme present.

There are two types of enzymatic assays usually performed. The first is referred to as a **fixed time assay** in which you prepare a set of test tubes containing your assay buffer and substrate, then add fraction volumes to each tube, allow them to sit for a specified amount of time, then quench (or stop) the reaction in each tube using a quenching reagent. You would next read the absorbance of each solution and thereby determine the amount of product produced in 10 minutes, from which you could determine the amount of enzyme present. There are certain dangers to fixed time assays which we shall discuss in class; however, it is a very good way of assaying a large number of fractions.

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The second method, which we shall employ, is a **continuous assay** (please see NB&B, pp. 244 – 246 for a more thorough description for the continuous assay of alkaline phosphatase which is the same method we will use in this experiment with the exception of the substrate used for the assay). In principle, you mix together buffer and substrate in a cuvette, zero the spectrophotometer at a specific wavelength to this solution, remove cuvette and add a small volume of enzyme, rapidly mix the sample by inversion, and begin recording continuously the change in absorbance at a specific wavelength as a function of time. If done properly, the absorbance change is linear, the slope of which can be converted to Units of enzyme activity (explained below).

Setting up the Cary 50:

1. Open up the **Enzyme Kinetics** module from the Varian icon window.
2. You must specify the appropriate wavelength to monitor the reaction, which should come from the PNP experiment you have already performed.
3. Set the data collection mode for no more than 1 minute, you can in fact set it for 30 seconds.
4. Mix together the assay buffer (Z-buffer) and ONPG as above, however remember you can only have ~ 1 mL in the semimicro cuvette. Place the cuvette in the sample holder and zero the instrument.
5. When you are ready to add the enzyme, have a small square of parafilm handy, then hit the Start button on the Cary. Another message will appear and say OK. Finally you will see a window with a timer. You have 2 minutes to add the enzyme, mix and get in the instrument and begin recording Abs vs time. In fact, it should take you about 10 seconds to do so. As soon as the sample compartment door is closed, hit the OK button and begin recording.
6. The change of Abs vs. time should appear linear. This plot is called a kinetic trace or an action spectrum (very old school terminology). Sometimes bumps will appear in the trace for a variety of reasons. Ultimately, you want to determine the slope of this curve and the Cary can do that for you automatically. In fact, it can convert delta Absorbance/min into delta [P]/min by taking into account the extinction coefficient you determined for PNPO⁻ in Expt. 2. This will be discussed in more detail in class.
7. What we want to know for each fraction is how many Units of enzyme activity are present in the fraction, where again, 1 EU = 1 umol P produced/min. In order to convert [ONPO⁻]/min to umol ONPO⁻/min you must take into consideration the assay volume and then apply the $c_1V_1 = c_2V_2$ equation to calculate the activity in the fraction.
8. What is the significance of this knowledge? For reasons that will be discussed later in the semester, when an enzyme activity assay is done, there is a great excess of substrate present, so that [substrate] does not become kinetically limiting in anyway, i.e. V_{max} conditions. Under V_{max} conditions, the initial velocity of the observed reaction, v_o, is directly proportional to the [enzyme]. The slope of Abs vs. time is converted to change of [P] per minute, which is the velocity of the reaction. The velocity is then converted to the Units of enzyme activity. The greater the enzyme concentration or Units of enzyme present, the greater the velocity. It's that simple!!!!!!!
8. For each fraction that turned yellow within 5 minutes in the micro-well plate, as well as the crude cell lysate, perform a continuous activity assay and determine the number of Units for that fraction. ***We will collect the crude cell lysate and the SINGLE***

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fraction containing the greatest amount of activity to be used next week!!!!!! Do Not Discard!!!!!!

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Data Analysis for b-gal activity assay:

First, ALL fractions should have been assayed for total protein concentration using the Bradford assay. This will give you the $[\text{Protein}]_{\text{total}}$ in mg/mL concentration **in the fraction** (and cell lysate), not the concentration in the assay itself.

Second, for the cell lysate and the fractions showing the highest amount of enzyme activity on the 96 well plate after 5 minutes, you performed an activity assay. So, what does this assay tell you? To begin with, activity assays are always done under the condition of saturating substrate, so the initial velocity, v_0 , that you measure corresponds to what is called maximal velocity, V_{max} , which corresponds to the enzyme "turning over" as fast as it can (we will look at enzyme kinetics in much greater detail later in the semester). By definition, $V_{\text{max}} = k_{\text{cat}}[\text{E}]_{\text{total}}$. Therefore you are directly measuring either the concentration of enzyme from which you can determine the **AMOUNT** of enzyme commonly defined as enzyme **UNITS**. One enzyme UNIT is the amount of enzyme necessary to convert 1 μmol of substrate to product (in this experiment the ONPO^-) per minute. Thus, activity assays are performed to determine **HOW MUCH** enzyme is present in **each** fraction or sample. Enzymologists rarely speak in terms of molar concentrations of enzymes; rather they express how many UNITS of enzyme activity are present.

For the calculation: you measured $\Delta A/\text{min}$ for the cell lysate and specific fractions. Now:

$$\frac{\Delta A}{\text{min} \times 0.0175 \text{ uM}^{-1} \text{ cm}^{-1}} = \frac{\text{uM Product}}{\text{min}}$$

where $0.0175 \text{ uM}^{-1} \text{ cm}^{-1}$ corresponds to the extinction coefficient for ONPO^- (the same as that for PNPO^-).

Now taking into account the volume of the assay:

$$\frac{\text{uM Product}}{\text{min}} \times \text{Volume assay (L)} = \frac{\text{umol Product}}{\text{min}} = \text{Units of activity in assay}$$

To determine the number of units you have in the **fraction** or cell lysate:

$$\frac{\text{Units} \times \text{Vol fraction or lysate (mL)}}{\text{Vol E for assay (mL)}} = \text{Total Units}$$

Now superimpose a plot of the data for $[\text{Protein}]_{\text{total}}$ in mg/mL (on the left hand y-axis vs. Fraction number (x-axis) upon a plot of Activity in $\mu\text{mol P}/\text{min}$ (right hand y-axis) vs. Fraction Number. For the Bradford data, draw a smooth line through your data points, while for the activity data, just use a different symbol, it is not necessary to draw a line. Most of you will see a lot of protein eluting off the column prior to the addition of the Elution buffer. What does this correspond to? For the fractions showing the greatest amount of activity do you also see an increase in $[\text{Protein}]_{\text{total}}$? How much does the

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activity drop off between the fraction showing the greatest amount and the very next fraction?

Data Write-Up for Experiment 4-1:

For this Lab Report, we will back off the previous rigorous format and merely ask you to prepare the appropriate plots and answer the associated questions.

1. For elution of the proteins from the G75 make a plot of color intensity (left-hand y axis) vs. fraction number for each fraction collected. On the plot, label the color of material coming off the column.
2. Based on the plot from (1), the volume of each 5 mL fraction, and using the following molecular weights, construct a plot of K_{av} vs. $\log MW$ for all of the species (Blue dextran, 2×10^6 ; Hemoglobin, 64000; cyt c, 13000; riboflavin, 376. If Hb and cyt c were not well resolved on the G75 column suggest how you might improve resolution of the two bands (i.e. change the column dimensions, use a different resin, etc.). Hint: consult Fig. 5-5 (p. 126), Table 5-1 (p. 128), and the accompanying text in NB&B).
3. Taking into consideration the actual protein elution profile from the G75 column, what purpose was served by the CMC column in the second step of the purification procedure? Elution of these two proteins takes into account two buffer parameters: first, **the ionic strength** and second, **the pH**. Using the information you gained from the ExPASy assignment, does the order of elution of the proteins from the CMC column agree with the pI 's for the two heme proteins? Did the purity ratio for Hb change when it was loaded onto, then eluted from the ion exchange column? In fact, there was a "contaminating" protein in the original "cocktail". Based on your results from the G75 and CMC columns, you should be able to determine two parameters about this protein: its relative molecular weight and its pI .
4. Suppose you had hemoglobin and cyt c in a mixture. Describe how you would purify Hb using DEAE. Clearly state what the ionic strength and pH (relative to the pI of Hb) of the loading buffer would have to be in order to get Hb to bind to the DEAE column. Would you expect cyt c to bind to the DEAE column? Finally, how would you elute the Hb from the DEAE column in as small a volume as possible?

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Write-up for Experiment 4.2:

1. Describe the chemical reactions of the activity assay used to detect the presence of β -galactosidase. Include in your discussion the pH dependence of the assay and how this is related to the pKa for para-nitrophenol that you determined in Expt. 2.
2. Plot Units of Enzyme Activity (left-hand Y axis) vs. fraction number for the loading, rinsing, and eluting fractions. Superimpose on this plot, **using the right-hand Y-axis**, the **concentration** of protein in mg/mL for the fractions determined by colorimetric assay (**not** the concentration in the assay for that fraction). Include on your plots at Fraction 0, the values obtained for the crude extract.
3. Every buffer that was used in this experiment contained BME! What influence does that have on the choice of the Bradford method as a means to determine protein concentration? Why not use the Lowry or BCA methods?
4. An important consideration about this experiment is the method in which the enzyme is first bound to the column and then subsequently eluted. The equilibration buffer is a Tris buffer at pH 7.0. Elution is accomplished using a borate buffer at pH 10.0, instead of using free product (galactose) as is often done on affinity columns. It is also worth noting that at pH 9.2, the enzyme elutes from the affinity column very poorly. Two questions arise from these facts. First, taking into consideration the enzyme and its substrate binding site, the galactosyl portion of the affinity resin and the pH dependency of the elution of the protein from the column what is the likely mechanism for release of the enzyme from the column? Consult your Lehninger text and determine which amino acid has a pKa in the pH range where the enzyme is released from the affinity column. What type of bonding interactions would the side chain of this type of amino acid have with the galactosyl moiety on the resin? Why would pH titration of this R-group result in enzyme elution? Second, taking into account the activity assay you performed on each fraction (you need to consider the substrate!), why would you **NOT** want to use free galactose to elute the enzyme from the column?
5. According to the manufacturer, each ml of resin can bind 12 mg of protein. Assuming that equilibration of the column prepared the resin to optimally bind β -galactosidase, was there enough resin present to bind all of the enzyme present in the volume of crude extract loaded onto the column? From the above plot, calculate how many mg of protein were purified by the column. Next, determine the amount of protein that may or may not have passed through the column during the loading phase. (Hint: for the fractions where you can compare the A_{400} and the mg of protein in that fraction, devise a conversion factor that can be used for your fractions collected prior to elution). Finally, using the stated binding capacity of the resin, the volume of resin you were given, and the amount of protein that passed through the column plus the amount of protein eluted from the column, how effective was the column in purifying the enzyme from the crude extract.
6. Suppose you want to prepare an affinity column resin for a protein that binds a ligand with a $K_d = 10^{-12}$ M. You must decide whether to cross-link the ligand itself or a ligand

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analog that binds to the protein with a $K_d = 10^{-8}$ M. Which species would you use to prepare your affinity resin and why?