Experiment 4

AFFINITY CHROMATOGRAPHY OF PROTEINS

Affinity chromatography is a type of adsorption chromatography in which substances are separated on the basis of differences in biological activity. In contrast to conventional chromatography which separates on the basis of gross physical and chemical differences, affinity chromatography is highly discriminating because of the highly specific nature of biological interaction between molecules. An excellent example of a very specific noncovalent interaction is that between an enzyme and its competitive inhibitor. The enzyme to be purified is passed through a column containing a cross-linked polymer to which a specific inhibitor of the enzyme has been covalently attached. All proteins without affinity to the inhibitor will pass through, whereas the protein that recognizes the inhibitor will be bound. The bound enzyme can be eluted by adding soluble competitive inhibitor or substrate or by change of ionic strength or pH.

In principle what one needs is a water insoluble matrix to which one of the components of the interacting system has been immobilized by means of a covalent bond and a method of selectively eluting the noncovalently bound component. The insoluble support should have minimal interactions with proteins; i.e., it must be porous enough to allow easy entry and exit of macromolecules. The chemical structure of the matrix should be such that attachment of a specific ligand is convenient, extensive and stable. Beaded agarose (trade name Sepharose) has been shown to be an excellent matrix. Agarose is a polymer of alternating residues of D-galactose and 3,6-anhydro L-galactose linked by 1,3 and 1,6 α-linkages. Sepharose 2-B is porous enough to allow compounds of molecular weight of 20-30 x 10^6 to pass through. There are enough hydroxyl (-OH) groups to which the ligand can be attached.

Various procedures for attaching the ligand have been devised. The first step is the activation of the agarose. The method of activation depends on the type of ligand to be attached. Cyanogen bromide-activated Sepharose allows for any ligand containing primary amino groups to be coupled.
For coupling of ligands which contain carboxylic groups, activated Sepharose is allowed to react with diamino compounds and then coupled with the ligand in the presence of carbodiimides, such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) or 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate (CMC). The scheme below shows the simpler compound dicyclohexyl carbodiimide (DCC).

The length of the alkyl chain of the diamino compound should be long enough for sufficient distance between the matrix and the ligand to avoid steric hindrance in the interaction of the protein with the ligand. This long alkyl chain is referred to as a "spacer arm".

These are just two of numerous methods available for attaching ligands to Sepharose. In the choice of method we must remember that if our ligand is an enzyme, the active site of the enzyme must not be coupled to the matrix.

The purpose of this experiment is to isolate trypsin from a pancreatic extract. The pancreas secretes numerous enzymes such as amylases, proteases, lipases and nucleases, as well as other proteins. The major proteases are trypsin, chymotrypsin and carboxypeptidases A and B. By chromatography on a column of Sepharose-chicken ovomucoid, we shall be able to isolate trypsin.

Chicken ovomucoid is a proteinaceous inhibitor specific for trypsin. The rationale of our procedure lies in taking advantage of this specific enzyme-inhibitor interaction. We shall pass a solution of pancreatic homogenate through a Sepharose-chicken ovomucoid column. The Sepharose-chicken ovomucoid column will bind trypsin while other proteins will pass through. The column is then eluted at low pH to obtain trypsin.
In enzyme purification it is essential to keep a balance sheet. The amounts of protein/mL and activity/mL are determined both on the original solution and each column fraction. From these data, the specific activity, activity recovered and fold purification are calculated.

PROCEDURE:

**Chromatography.** Remove all the liquid above the affinity matrix of the column with a Pasteur pipette. Apply 1.0 mL of pancreaticin solution. Open outlet tube. Start collecting 5 mL fractions at a rate of ~1.0 mL/min. When all the enzyme solution has just gone into the bed, wash walls of tube with small amounts of 0.1 M Tris-0.02 M CaCl$_2$ buffer, pH 8.0. Be sure not to let the column bed become dry at the top. Add buffer above the bed and place reservoir of buffer above the column. Collect 10 fractions
of 5.0 mL each. Then elute the column with 0.05 M HCl. An abrupt change in pH is best and gives the sharpest, most concentrated peak possible. Collect 10 fractions of 5 mL each.

**Protein Determination.** A rapid method for determining protein concentration is the dye-binding method. Proteins under specific conditions have been shown to complex certain organic dyes such as Orange G, Amido Black, Cochineal Red A and Coomassie Brilliant Blue G. Only one of these is in common use.

Coomassie Brilliant Blue G-250 exists in two different color forms, red and blue. Upon binding of protein to the dye the red form is converted to the blue form, leading to a shift in the absorption maximum from 465 nm to 595 nm. The dye reagent contains strong acid, so that most proteins are below their iso-electric pH and hence are positively charged and interact with an anionic dye. The dye binding process is very rapid (about 2 min) and the protein-dye complex is stable for a relatively long time (approximately 1 h). The protein-dye complex has a high extinction coefficient allowing for great sensitivity. However, different proteins give different results because of difference in number of positively charged groups. This must be kept in mind when applying this procedure as an absolute analytical method for protein determination.

On the basis of sample size and time for assay, this method is quite useful in monitoring protein concentration at different steps of purification. Interfering color was seen only in the presence of high amounts of detergents such as sodium dodecyl sulfate, Triton X-100 and commercial glassware detergents. Other interferences which limits the application of the Lowry method were not observed.

**REAGENTS:**

- **Dye Reagent:** Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 mL of 95% ethanol. To this solution 100 mL of 85% (w/v) phosphoric acid was added. The solution was diluted to a final volume of one liter. Final concentrations in the reagent were: 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol and 8.5% (w/v) phosphoric acid.

- **Standard Protein Solution:** 0.3 mg/mL of bovine serum albumin.

- **Pancreatin:** 2.25% crude pancreatin in 1 mM HCl, stirred for 15 min, then filtered through Whatman #1. (If insufficient activity is found in the pancreatin, 4 mg/mL of crystalline trypsin can be added.)
**Protein Standard Curve.** Do the following in duplicate. To test tubes add 0, 0.02, 0.04, 0.06, 0.08 or 0.10 mL of standard protein solution. Add water to each tube to give a final total volume of 0.10 mL. Add 3.0 mL of Dye Reagent. Mix several times by gentle inversion of test tube. Let stand for at least 5 min but not more than one h. Measure absorbance at 595 nm. You should use the "quantitative" mode of the Shimadzu spectrophotometer, setting it for the 6 standards and using as a blank the tube to which no protein was added (zero the spectrophotometer using this tube). Plot the data as shown in Fig. 4-1.

![Figure 4-1. Typical standard curve for the dye-binding protein assay.](image)

**Protein Determination.** Determine the protein contents of the original pancreatin extract and of all the fractions collected from the Sepharose-chicken ovomucoid column. The maximum sample volume allowable is 0.1 mL. If less than 0.1 mL is used, remember to bring the total volume to 0.1 mL by addition of necessary amount of water. An absorbance outside the range of your standard curve indicates that the protein concentration is too high. If such is the case, the protein concentration may be reduced by testing a smaller sample size or by diluting the sample (e.g., to 0.01 mL extract add 0.99 mL of water. This is equivalent to a 100-fold dilution. Check with your instructor.). Remember to take into account the dilution factor and the sample volume when reporting protein concentration.

**Trypsin Activity Determination.** Zero the spectrophotometer against distilled water at 550 nm.

a) Crude pancreatin. Place 3.0 mL of 0.01 M TAME in 0.001 M Tris buffer, pH 8.0, and 0.1 mL of 0.02% Phenol Red in a cuvette. Mix. Set the Shimadzu spectrophotometer on "kinetic" mode, wavelength to 550 nm, with absorbance upper limit at 1.50 and lower limit at 0.00. Record absorbance at 10 sec intervals for 2 min (no lag time). Add 0.1 mL of crude pancreatin (the pancreatin might have to be diluted). Mix. Return tube to spectrophotometer immediately and press start. Determine initial
rate from the plot of absorbance against time. The first 2 min without enzyme gives the blank rate, to be subtracted from the enzyme-catalyzed rate.

b) Column fractions. Place 3.0 mL of 0.01 M TAME in 0.001 M Tris buffer, pH 8.0, and 0.1 mL of 0.04% Phenol Red in cuvette. Mix. Add 0.1 mL of enzyme aliquot. Mix. Immediately return cuvette to spectrophotometer and press start. For those tubes collected after addition of 0.05 M HCl for elution, follow procedure as above but remember to neutralize them first by adding equivalent volumes of 0.05 M NaOH to the tube. Record absorbance at 10 second intervals for 2 min. Determine initial rate from the plots of absorbance against time. Subtract the blank values, with no enzyme, from the values with enzyme present.

![Figure 4-2. Typical curve of absorbance at 550 nm vs. time for trypsin activity.](image)

One unit of trypsin activity is defined as that amount which will produce a change in absorbance of 0.001 in one min.
EXPERIMENT 4

Affinity Chromatography of Proteins

Laboratory Report

Name: __________________________ Date: __________________________

1. Record all experimental details including sample size, etc.

2. For the standard protein curve, submit a graph of absorbance versus protein content (see Fig. 4.1).

3. Plot the change in absorbance versus time for each of the activity assays, as shown in Fig. 4-2. Determine the initial velocity.

4. Plot the data on one graph as follows: protein content/mL versus tube number in blue, and trypsin activity in units/mL versus tube number in green. Indicate the fraction at which the eluting buffer was changed. You may use different colors or different symbols.

5. Prepare a table of purification similar to below.

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<th>Volume (mL)</th>
<th>Activity (units/mL)</th>
<th>Total units</th>
<th>Protein (mg/mL)</th>
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<th>Specific Activity (units/mg)</th>
<th>Yield (%)</th>
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6. Submit answers to questions:
   a) What is the Beer-Lambert law?
   b) How much purer is the most active trypsin fraction from the column as compared with the original pancreatin supernatant?
   c) How would you prove that the most active fraction of trypsin is pure as a protein? Pure as a catalyst?
EXPERIMENT 4, Demo

DETERMINATION OF THE PURITY OF TRYPSIN AS A PROTEIN BY ELECTROPHORESIS

The movement of molecules under the influence of an electrical field is called electrophoresis. Electrophoresis may be performed in the absence (free boundary or moving boundary method) or in the presence (zone method) of a stabilizing medium. Zone electrophoresis is the most commonly used method. The stabilizing medium may be cellulose, cellulose acetate strips, agar, starch or polyacrylamide gel. Polyacrylamide gels are used most frequently because of versatility in changing the pore size and because of toughness and clarity of gels. At low polyacrylamide concentration (3-6%), pore size of the gel is large and hence separation is largely on the basis of charge difference. At high polyacrylamide concentration (10-30%), pore size of the gel is small and hence separation is on the basis of difference in molecular weight.

Polyacrylamide gel electrophoresis in the presence of the detergent, sodium dodecyl sulfate (Formula 4-1) was first introduced in 1967 (Shapiro et al.). Weber and Osborn (1969) showed that the electrophoretic mobility of proteins (molecular weight range from 15,000 to 200,000) on SDS gels is proportional to their molecular weights. Thus, the molecular weight of an unknown protein can be determined by comparing its electrophoretic mobility on SDS gels to mobilities of marker proteins with known molecular weights. It may also be used to determine the purity of a presumably pure protein. Purity is a relative term. A single protein band may result also because the impurities are at too low a concentration to be detectable. There could, in fact, be many impurities in the sample, each present at a low level but in sum, amounting to substantial contamination.

In the presence of 1% SDS and 0.1 M mercaptoethanol in neutral pH, denatured proteins bind SDS maximally. The secondary structure is lost, disulfide bridges are broken by the mercaptoethanol and the SDS-protein complex assumes a random coil configuration. In the case of multichain proteins, the proteins dissociate into sub-units in random coil configuration. The amount of SDS bound per unit weight of protein is essentially constant (1.4 gm of SDS per gm of protein). The charge of the protein is then determined by the bound SDS and not by the intrinsic charge of the amino acids. Since the amount of SDS bound is constant, the effective mobility of the protein-SDS complex is related only to its molecular weight.
**Formula 4-1.** Sodium Dodecyl Sulfate (SDS).

PROCEDURE: (written for tube gels – we will use slab gels)

Do not use mouth for pipeting.

The lower 5% SDS gel has been prepared for you. Remove the layer of buffer on top of lower gel. To 6 mL of stacking gel, add 0.06 mL of ammonium persulfate. Mix. Fill tube to the mark with stacking gel solution. Layer water over the surface of the stacking gel (without disrupting the gel solution; this will be demonstrated). Let stand for 30 min to allow gel to polymerize.

**Sample Preparation.** Dialyze the following samples against distilled water: crude pancreatin homogenate, peak tube of void volume and fraction with highest specific activity of trypsin from your affinity column (Experiment 4, part A).

Into each of 3 test tubes: pipette 10 µl of 0.025% Bromophenol Blue, 20 µl of 3% SDS, 20-100 µl of protein sample (equivalent of 0.2 mg of crude protein or 0.08 mg of pure protein) and 2 crystals of sucrose. Place in boiling water bath. Boil for 2 min. If sample dries up, add 10-20 µl of water.

**Electrophoresis.** Remove water from top of stacking gel. Place tubes or slab in electrophoresis set-up. Pour reservoir buffer into the lower tray. Remove air bubbles between gel and reservoir buffer. Layer buffer on top of stacking gel. Pour reservoir buffer into the upper tray. Remove air bubbles between stacking gel and reservoir buffer. Introduce sample (20-40 µl) by carefully layering on top of stacking gel. (This will be demonstrated.) Connect anode to the bottom terminal and cathode to top terminal. During stacking, initially run at 1 mA/gel and then at 2-3 mA per tube. When tracking dye reaches bottom of gel (~ 1 h), turn current off.

To remove gels from the glass tubing, insert a thin wire to rim the gel, place a pipette bulb over the top end and press the bulb to release the gel. Avoid pulling the gels as they are quite fragile. For removing a slab gel from between plates, try to slide one plate; prying with a spatula will generally break the glass.
Quick-stain Procedure: Leave gels in test tubes containing 0.04% Coomassie Brilliant Blue G-250 in 3.5% perchloric acid for ~ 90 min or until protein bands are visible. Remove gels from dye and store in solution of 5% (v/v) acetic acid.

Traditional staining: Gels are placed in a dish with Coomassie Brilliant Blue R dissolved in methanol/acetic acid/water for several hours until the entire gel is a nearly opaque blue-black. The gel is transferred to destaining solution, which is the same solvent as used for the staining, but without the dye. The gel is incubated with gentle mixing overnight, or until the background is colorless.

References: