BIOCHEM 385 LAB AFFINITY CHROMATOGRAPHY
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Separation of Active Form of Trypsin from Inactive form by Affinity Chromatography

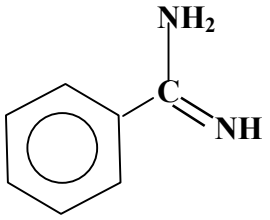
Introduction:

Affinity chromatography occupies a central role in the purification of biological materials since it is the only technique which enables purification of almost any biomolecule on the basis of its biological function. In this technique, complex formation between biospecific molecules is exploited to purify them from a mixture. For example: (1) enzymes with substrates or competitive inhibitors or coenzymes, (2) antigens with anybody, (3) hormones with receptors or other binding proteins, (4) nucleic acids with their binding proteins or complementary nucleic acids.

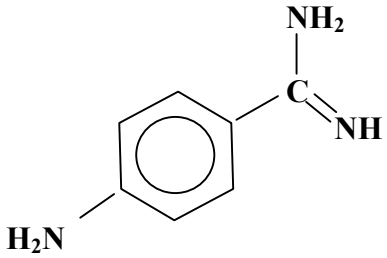
Typically it is carried out by passing a sample, containing the substance of interest, through an insoluble matrix (such as Sepharose) containing covalently bound complementary binding substance (ligand) under conditions favoring complex formation. Unbound substances are washed away and the substance in question is eluted finally from the affinity column by employing conditions that favor the dissociation of the complex formed. Although this technique is of recent origin, its application to biological systems has developed so rapidly that it is now widely used in almost every laboratory concerned with the purification of biomolecules.

In today's laboratory we will separate active trypsin from inactive trypsin. Trypsin is a proteolytic enzyme and usually cleaves the peptide bond of proteins at the carboxyl side of arginine and lysine. It also hydrolyzes several synthetic substrates. It has the tendency to undergo autodegradation during purification and storage. This leads to inactivation of some trypsin molecules. Such inactive molecules do not catalyze the hydrolysis reaction (that is why they are called inactive) because they apparently lack the correct active site geometry needed for catalysis. Hence, any mode of separation that is based on the stereochemistry of the active site will separate active enzyme from inactive protein. Affinity chromatography on p-amino-benzamidyl sepharose exactly does this function.

Benzamidine is a potent competitive inhibitor of trypsin with an inhibition constant of 2×10^{-5}M. However, the only functional group available on this molecule is required for binding at the active site of trypsin and cannot be used to immobilize this ligand on the insoluble matrix (Sepharose).



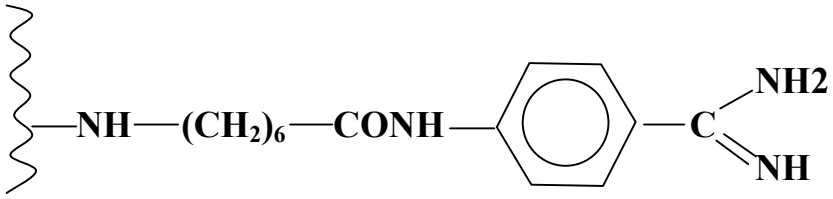
Benzamidine



P-amino-benzamidine

p-Amino benzamidine, on the other hand, has an amino group far away from the 'amidine' group that is suitable for immobilization on a matrix and still has the free 'amidine' group required for

trypsin binding. This ligand is coupled to agarose (insoluble matrix) via a side arm, or spacer, 6-amino hexanoic acid. Without the spacer arm, trypsin would not bind to the column due to steric hindrance. You will separate the active form of trypsin from the inactive form using this affinity matrix, p-amino benzamidyl-6-amino caproyl agarose.

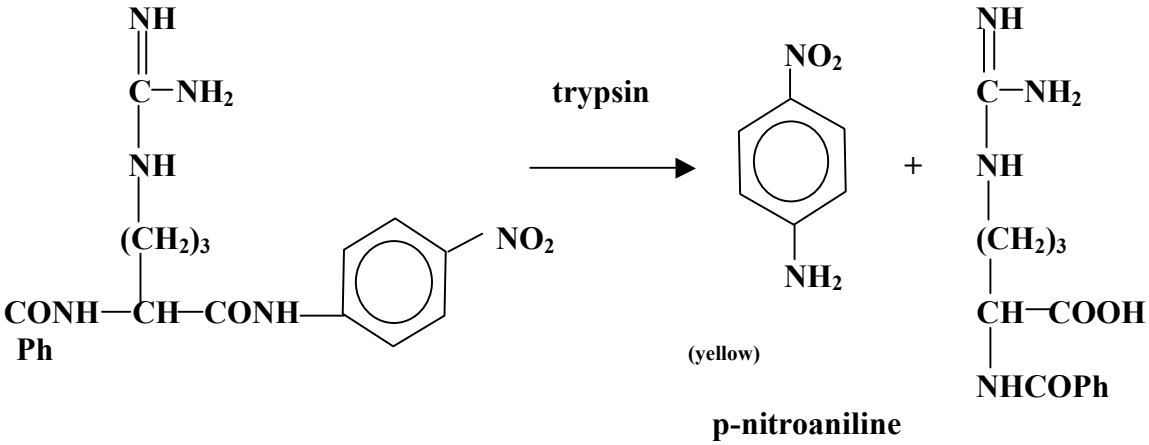


P-amino benzamidyl-6-amino caproyl agarose.

Assays:

(1)Principle of the Enzymatic Assay:

Activity of trypsin is routinely monitored with synthetic substrate rather than natural substrate because it results in color formation that can easily be monitored. We will use N- -benzoyl-DL-arginine-p-nitroanilide (BAPNA) as the substrate and monitor the enzyme activity by the release of p-nitroaniline at 410nm.



(2) Protein Assay:

One of the important and often used procedures in any biochemical laboratory is protein estimation. Several methods have been developed over the past few decades to meet this need, of which Bradford's dye binding assay is an easy, fast and convenient method (M.M.Bradford. Anal. Biochem. 72 248 (1976)). This method is based on the fact that under acidic conditions, Coomassie brilliant blue complexes with proteins forming a chromophore whose absorption can be conveniently monitored at 595nm in a common colorimeter. This reagent does not react with amino acids or small peptides and is free from interference from ammonium ions, mercaptoethanol, EDTA and reducing sugars, which are often used in protein purification procedures. Another method for protein estimation is via absorbance at 280nm.

We will use the Biorad protein assay (Bradford method) to determine the protein content of fractions eluted from the column that might contain active trypsin or inactive protein (inactive trypsin). This data will be used to determine the % recovery of active trypsin from the affinity column.

Materials:

Chromatography columns packed with p-amino benzamidyl-6-amino caproyl agarose.
Binding buffer: 0.05M TRIS with 0.5 M NaCl, pH 8.0
Elution buffer: 0.01 M HCl with 0.5 M NaCl
Reaction buffer: 0.05 M TRIS with 0.02M CaCl2 pH 8.5
Trypsin, 5.0 mg in 0.5 ml 0.05 M TRIS with 0.5 M NaCl, pH 8.0 , kept on ice
BAPNA, 0.2 mM
Biorad Protein assay reagent (Bradford method) prepared fresh as follows:
10 ml concentrated dye reagent + 40 ml water. Filtered through Whatman No.1 filter Paper.
Trypsin protein stock for protein standards, 1 mg/ml in Buffer A
Spec20 spectrophotometer

Methods:

A. Purification of Active Trypsin:

1. (done by instructor) Weigh 5.0 mg of commercial trypsin into a clean, dry test tube. Add 1.0 ml Buffer A (0.05 M TRIS with 0.5 M NaCl, pH 8.0). Wait until the protein dissolves. Label the tube and ***keep it on ice***.

2. (may have been done already) Pack about 1-2 ml of p-amino-benzamidyl agarose in a given chromatographic column.

3. Equilibrate column with Binding Buffer by passing about 10 ml of Binding Buffer through the column.

4. Loading of trypsin: ***Read this entire step before proceeding!***

    a. Allow all of the Binding Buffer to sink into the column.
    b. Just as it sinks in, add the trypsin solution (5 mg/1.0 ml) to the top of the column without touching the gel or creating foam in the trypsin sample.
    c. Begin to collect the first fraction as soon as trypsin is added.
    d. Let the trypsin just sink in and wash the inside of the column with a few *drops* of Binding Buffer, so that the top of the gel has 1-2 mm of buffer on it.
    e. Close the bottom of the column with the cap or a small piece of parafilm.
    f. Allow the trypsin to bind for 5 minutes.

5. Regardless of the volume in Fraction #1, start to collect a new, 2nd 2ml fraction: Add Binding Buffer to top of gel to about 1 inch. Open the bottom of the column and collect 2 ml fractions, continuously filling the column with Binding Buffer to elute *inactive* protein. *Do not let the column run dry!*

6. Continue to collect about 5 fractions. Label fractions consecutively, f1, f2, f3, etc. This column will be slow…please be patient!

7. *Mix each fraction gently but well, and keep all fractions on ice!*

8. After collecting 5 fractions, carefully remove Binding buffer from top of column and add Elution Buffer.

9. Elute the *active* trypsin with Elution Buffer (0.01 N HCl with 0.5 M NaCl). Collect 2 ml fractions, about 5 fractions. Label fractions consecutively from where *you* left off, for example, f6, f7, f8, etc.

10. Record fraction volumes.

11. *Mix each fraction gently but well, and keep all fractions on ice!*

B. Enzymatic assay of trypsin in collected fractions

1. Pipet 100 ul aliquot of Binding Buffer into a clean spec 20 test tube. This will be your Blank for the Spec20.

2. Mix each fraction gently but well before pipetting. Pipet 100 ul aliquot from each fraction into clean spec 20 test tubes. Label according to the fraction numbers to be tested. Keep the original fractions on ice to be used for protein assay in section C.

3. Add 900 ul of Reaction Buffer (0.05 M TRIS with 0.0.2 M $CaCl_2$, pH 8.5) to each test tube, including the Blank.

4. Add 2.0 ml of substrate solution (0.2 mM BAPNA) to each test tube. Mix by inversion.

5. Incubate at Room temperature for 15 minutes.

6. Blank the Spec20 using the tube marked blank and record absorbance of each tube at 410 nm.

7. After all measurements are taken, show results to the instructor. **If the results are approved, discard the enzyme assay solutions.**

9. Plot absorbance at 410 nm vs. fraction #. Which fraction contains trypsin activity?

C. Protein assay of collected fractions

1. Preparation of Trypsin Protein Standard

a. Obtain Trypsin Protein Standards.

Tube#	A	B	C	D	E
Standard (mg/ml)	0.2	0.4	0.6	0.8	1.0

2. Protein Assay Procedure

a. Place 200 ul of ***Binding Buffer*** in a clean dry spec20 test tube labeled **Blank.**

b. Place 200 ul of each ***standard***, from A-E, in five different clean dry spec20 test tubes. Label appropriately.

c. Place 200 ul of each ***original fraction*** to be tested in different clean dry spec20 test tubes. You will test each fraction you collected from the affinity column. Label tubes appropriately.

d. Add 2.0 ml of BioRad reagent (freshly prepared as described in Materials) to each tube, including the Blank.

e. Cover test tubes tightly with Parafilm. Gently invert each tube several times to mix the contents. Avoid foaming.

f. After 10 minutes, 'blank' the spectrophotometer at 595 nm with the tube labeled Blank. Measure the absorbance of each tube, standards and samples, at 595 nm.

g. If the absorbance of a sample is greater than the absorbance of the highest standard, you must dilute the sample as follows:
   1) Pipet 200 ul of sample from the original fraction tube into a clean tube.
   2) Pipet 200 ul of buffer into the tube. Mix well. This is a **1:2** dilution.
   3) (possible time saver!) Also make a **1:4** dilution of the original fraction tube.
   4) Re-test 200 ul of the new, diluted samples according to **steps c-f** above.
   5) Read the Absorbance at 595 nm and determine the protein concentration from the standard curve as described below. *Then multiply by 2(or 4)* to correct for the dilution.

h. Standard Curve: Plot the absorbance of the standards at 595nm versus the concentration of the standards. (See SAMPLE figure).

i. From the absorbance of the samples, read the protein concentrations, in mg/ml, from the standard curve.

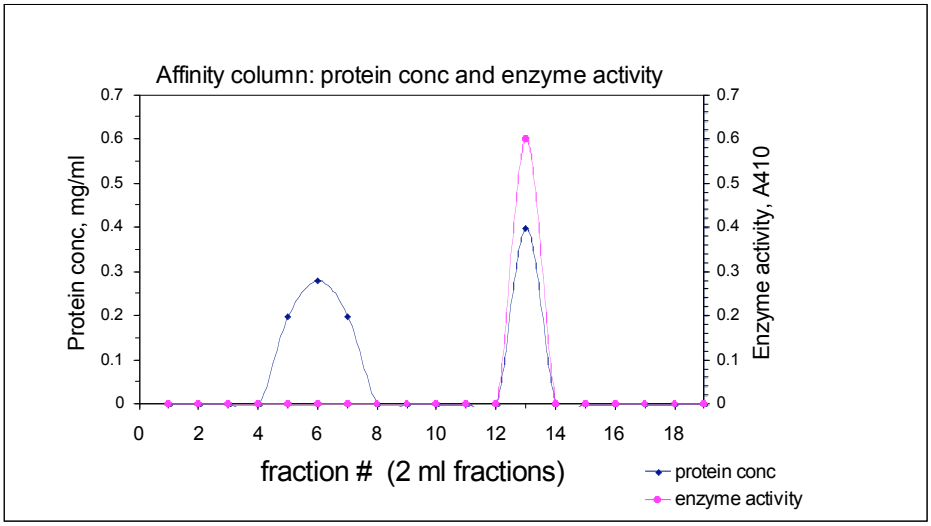
Use the linear regression for your final answers. Don't forget to check by estimation!

Graphs and Calculations:

1. Protein Standard Curve: Abs 595 nm of standards versus concentration of protein standards, mg/ml

2. Protein Concentration (y-axis, mg/ml protein) and Enzyme Activity (y-axis, A410) versus Fraction number (x-axis), as in example below:

SAMPLE GRAPH**MS Excel users*



3. Calculate the protein *content* of each fraction containing protein, in milligrams, by both linear regression and verify your answers by estimation.

4. Determine the % recovery of protein:
   (mg active + mg inactive) / 5mg * 100

5. Determine the % active trypsin recovered:
   (mg active) / (mg active + mg inactive) * 100

*MS Excel users:
Create scatter plot with one set x data (1st column), 2 sets y data (2nd, 3rd columns).
To format 2nd y-axis on right of plot:
Right click on 2nd set y data on graph
Click Format data series
Click Axis
Click plot on secondary axis. This will add y-data to right y-axis.
Right click anywhere in chart area.
Click Chart Options, then Titles
Enter data title in secondary value y-axis
Be SURE your Titles correspond to the correct axis data!!!!!!

QUESTIONS:

1. What is trypsin, and what is it's role in the body?

2. Why is it important to purify trypsin in the laboratory? Give at least 2 examples of *in vitro* use.