

IISME

Size Exclusion Chromatography and Enzyme Assay Activity

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Objectives-Students Will be Able To...

- Explain how molecules are separated by chromatography
- Identify the relative size of various biomolecules
- Explain the relationship between an enzyme and its substrate
- Describe the role of a catalyst

Standards Addressed (Science, Grades 9-12)

Chemistry

- Students know how molecules in a solution are separated or purified by the methods of chromatography and distillation.
- Students know the rate of reaction is the decrease in concentration of reactants or the increase in concentration of products with time.
- Students know the role a catalyst plays in increasing the reaction rate.

Biology

- Students know enzymes are proteins that catalyze biochemical reactions without altering the reaction equilibrium and the activities of enzymes depend on the temperature, ionic conditions, and the pH of the surroundings.

Investigation and Experimentation:

- Select and use appropriate tools and technology (such as computer-linked probes, spreadsheets, and graphing calculators) to perform tests, collect data, analyze relationships, and display data.
- Analyze situations and solve problems that require combining and applying concepts from more than one area of science.

Instructional Time Needed (need 2 hour block periods)

3 days

Day 1 (20 minutes)	Students prepare and pour gel filtration columns. While columns settle instructor presents information on column chromatography.
Day 2 (2 hours)	Students add "unknown" sample to column and collect fractions.
Day 3 (2 hours)	Students add enzyme to column, assay fractions

Required Materials:

Material	Suggested Vendor
Micropipette (P20, P200)-one per group or can be shared	
Micropipette tips	
12x75 test tubes -approximately 30 per group	
Gel filtration column 1 cm diameter, 18" height with stop cock- 1 per group, Tygon tubing (inner diameter 1/32" outer diameter 3/32" wall 1/32"	Columns-Sigma C3919 \$95.20 for 10 Tubing adaptor Sigma A7427 \$13.50 for 10 Stop cock Sigma S7396 \$23.70 for 10
Test tube rack (1 per group)	

96 well Microplate-can be shared (for assay)		
"Unknown" mixture	Vitamin B12 (375 µl)	Sigma V2876 (250 mg \$14.95)-(makes 25 class sets -per class 10mg/ml)
	Blue Dextran (750 µl)	Sigma D5751 (1 g \$21.15)-(makes 40 class sets -per class 25 mg/ml)
	Bovine Hemoglobin (750 µl)	Sigma H2500 (1 g \$10.50)-(makes 40 class sets -per class 25 mg/ml)
Sephacryl S-300 (resin(gel) for column) need about 14 ml of resin for 1.0cm x 18 cm column		Sigma S-300-HR (100 ml \$60.75)
Enzyme: Wheat Germ Acid Phosphatase		Sigma P_3627 (1 g \$19.00-lasts forever)
Substrate: PNPP		Sigma 104-0 (1 g = \$21.00 makes 500 ml @ 5mM)
NaAcetate Buffer		Sigma S-8625 (500 mg for \$18.70)

Timeline

Day	Student Activity	Time	Teacher Activity	Time
0	NONE		Prepare resin, buffer, and student supplies	45 minutes
1	Pour gel filtration column, take notes on column chromatography PowerPoint Prepare fraction collection tubes for Day 2	Complete at beginning of class, should take no more than 20 min.	Prepare unknown solution Store in refrig.	30 minutes
2	Run unknown through column Flush column with buffer Prepare fraction collection tubes for Day 3	Column will run for about 1 hour, flushing can be started at end of class (teacher will need to finish)	Prepare WGAP solution and PNPP Store in refrig.	30 minutes
3	Run enzyme on column Enzyme assay Flush column Clean column	Run column-1 hour Assay-20 min.	Store resin (can be re-used)	20 min.

** One possible way to speed up the running of the column is to increase the head pressure (increase length of tubing/height of column). This will however create less than ideal separation of the molecules.

Background Information

Column Chromatography:

<http://lsvl.la.asu.edu/resources/mamajis/chromatography/chromatography.html>

Enzyme/Substrate Reactions:

<http://www.cat.cc.md.us/~gkaiser/biotutorials/proteins/enzyme.html#enzsub>

Acid Phosphatase & pNPP reaction:

<http://www.bio.mtu.edu/campbell/bl482/lectures/lec2/482ex2a.htm>

Introductory Activity (if desired)

A possible introductory activity is the "toothpickase" activity. In this activity the student's fingers represent the active site on the enzyme. The toothpick represents the substrate and broken toothpicks represent product. Students can do a variety of activities modeling differences in enzyme concentration, substrate concentration, inhibitors (twist ties work well as competitive inhibitors to the toothpicks), etc.

Procedure

- See Student Procedure Handout
- See Teacher Procedure Handout

Slide 1

Chromatography

Davis-Biology

Slide 2

What is chromatography?

- A method to separate compounds in a mixture that are not easily separated by another method
- There are many types of chromatography but all operate on the same principle
 - One substance is **stationary** and the other is **moving**
 - The mixture to be separated is distributed between the stationary and moving substances and eluted at different rates

Slide 3

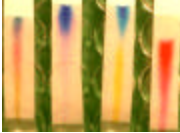
How does chromatography work?

- 3 examples of chromatography
 - Paper chromatography (Lab 5-Photosynthesis)
 - Gel Filtration (this lab)
 - Ion exchange

Slide 4

How does chromatography work?

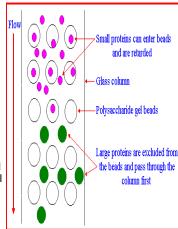
- Paper chromatography (Lab 5-Photosynthesis)
 - Sample is applied to paper close to edge (stationary)
 - Bottom of paper is put into solvent (moving)
 - Solvent moves up the paper and separates mixture



Slide 5

How does chromatography work?

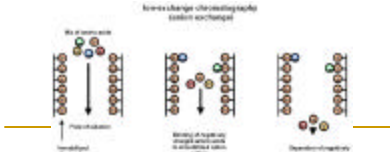
- Gel Filtration (this lab)
 - Separates molecules according to size
 - Gel particles (like beads with holes-stationary) with solvent (moving)
 - Sample is added to gel
 - if molecule is large → it will not enter the gel beads and will flow through the column quickly
 - if small → molecules will enter the gel pores, and due to this they will move more slowly through the gel bed
 - Molecules leave the column in order of decreasing size (largest → smallest)



Slide 6

How does chromatography work?

- Ion exchange
 - Molecules are separated based on charge
 - Molecules will bind to the column (stationary) when they have a specific charge (cation exchanger, (-)charge binds cations(+), anion exchanger (+)charge binds anions(-))
 - Molecular charges change when the pH of a solution changes, by changing the pH of the solvent (moving) molecules can either bind to the column or be eluted.



Procedure: Teacher Guide

Preparation of Solutions and Sample Student Data

1. Sodium Acetate Buffer (pH 5.2)

Dissolve 30.2 grams NaAcetate in 2 L H₂O

Makes 2 liters (combine with H₂O); adjust pH at room temperature.

(This buffer is used to run the columns, and mix with the unknown solution materials)

A Phosphate Buffer CANNOT be used because it acts as a competitive inhibitor to the enzyme.

2. “Unknown” solution (mixture of Vitamin B12, Blue Dextran and Bovine Hemoglobin):

Hemoglobin solution should be at a concentration of about 10 mg/ml

1. Mix 50 mg of Blue Dextran with 2 ml of NaAcetate Buffer (to get 25 mg/ml)
2. Mix 10 mg of Vit. B12 with 1 ml NaAcetate buffer (to get 10 mg/ml)
3. Vortex both so that all solute is in solution.

Combine 750 μ l Blue Dextran solution, 750 μ l ml Hemoglobin solution, and 375 μ l of Vitamin B12 solution (makes enough for 8 columns using 175 μ l each); vortex to mix

Store in refrigerator-can be made several days ahead

3. KOH (1.0 M)

Volume needed- 50 ml per group

Dissolve 28.05 grams of KOH to a final volume of 500 ml with H₂O

The reaction is exothermic so wait for the solution to cool before storing in a plastic container (don't prep it in a plastic container, or if you use plastic, place in ice bath). Store at room temperature, but place in a secondary container, such as a plastic beaker (in case of any spills or leakage)

4. p-nitrophenylphosphate (PNPP) WGAP substrate (5 mM)

Combine 92.8 mg of PNPP to 50 ml of Na-acetate buffer.

The solid pNPP is light sensitive; store in dark, refrigerate, powder must be stored DRY (desiccant). If the powder is yellow in color that it has been hydrolyzed and cannot be used. It should be a white/light yellow color.

Be sure substrate is at room temperature before using.

The solution needs to be made within a day of use (good for 1-2 days).

Store in refrigerator.

5. WGAP

2.5 ml NaAcetate buffer + 50 mg of WGAP (20 mg/ml solution) (each student group uses 175 μ l)

Mix well; if any particulate material remains, centrifuge to remove it.

Gel Filtration-

Resin should be stored mixed with NaAcetate buffer. It will need to be mixed well before using. Be sure the resin is at room temperature before using. Aliquot resin slurry (mixed with buffer) into ~ 20 ml fractions for student use. Slurry should be 75% resin and 25% buffer by volume. As the resin is settling in the columns be sure to keep it hydrated, do this by carefully adding buffer to the top of the column. If using 20 cm columns with 1 cm diameter students will need about 14 ml of packed resin.

Cleaning Columns:

After columns have been used, add buffer and run buffer through the columns until you are sure that no sample solution remains in the resin. Resin can then be removed from the column with a pipet and stored with buffer in the refrigerator. It can continue to be re-used.

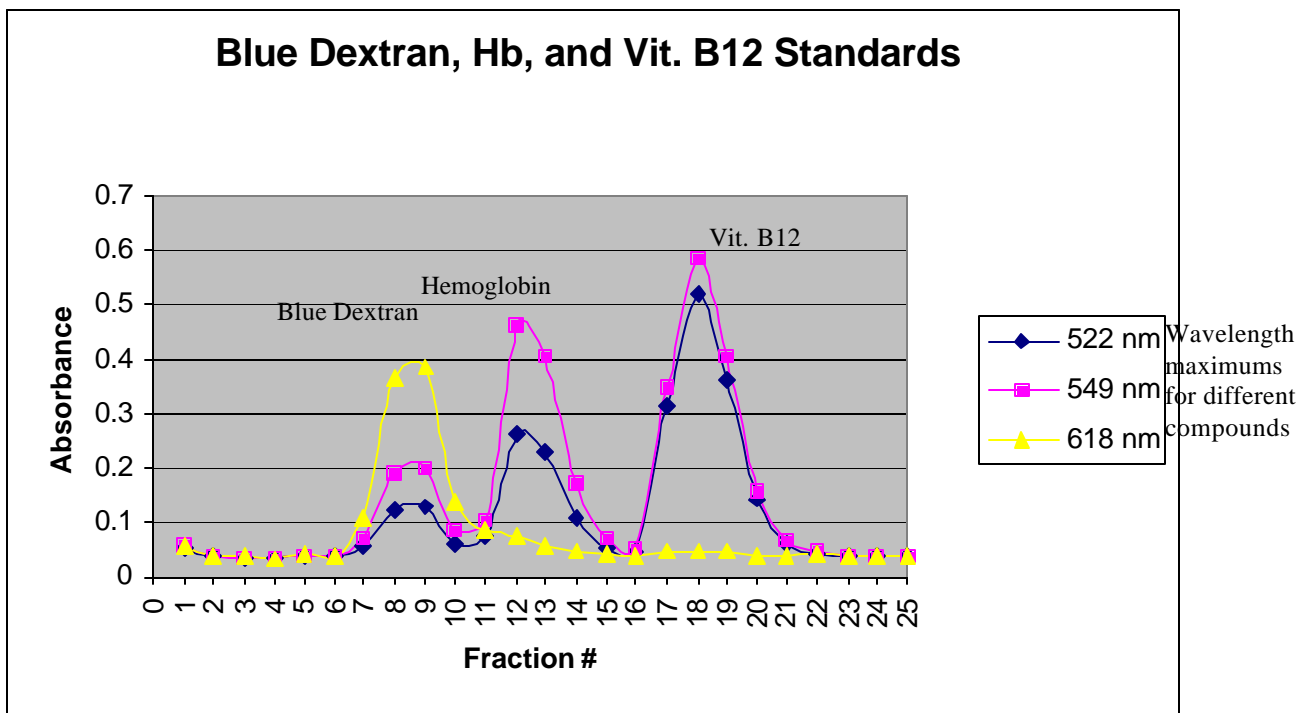
Storing Resin:

Allow the resin mixed with buffer to settle, pour off the majority of the buffer and then add 10-20% ethanol solution (in water) to the resin to prevent anything from growing. The resin can then be stored until next use in refrigerator. When using again, allow resin to settle and pour off ethanol/water. Then add water, settle resin and pour off excess, finally repeat with the buffer you will be using (to equilibrate).

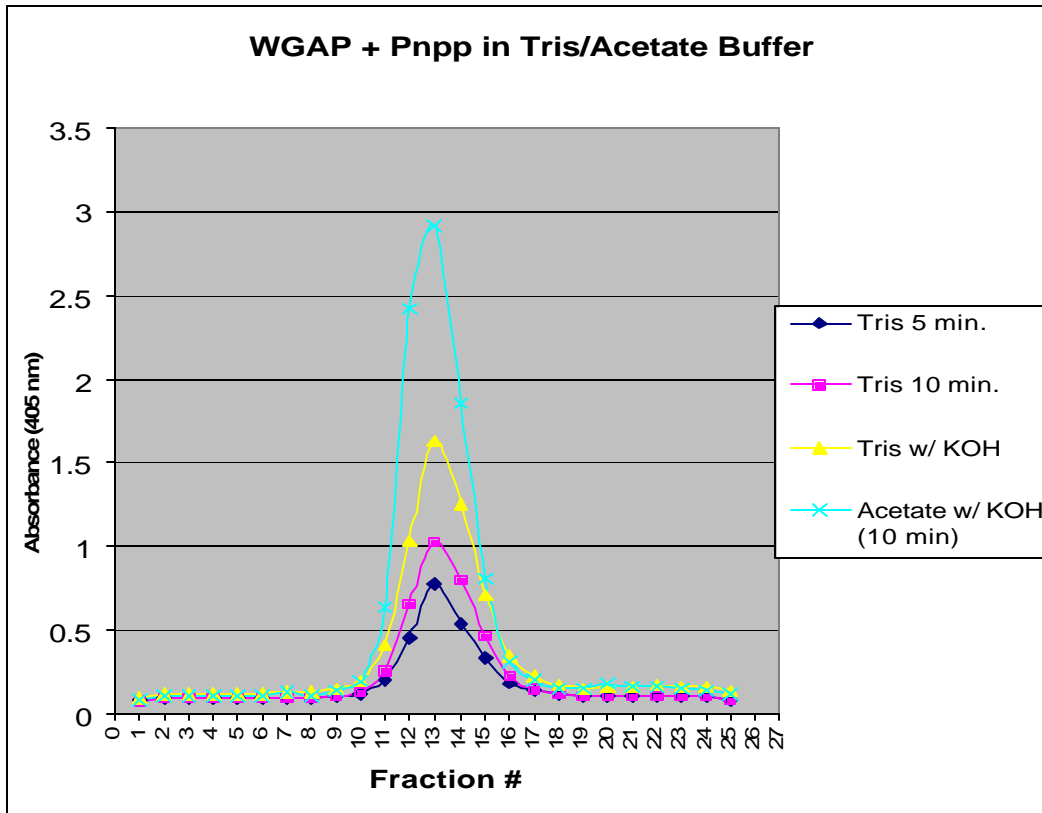
Sample Data

The following is absorbance data taken using a microplate reader (similar to a spectrophotometer). This shows where in the fractions collection one would expect to find each molecule (these fractions were about 0.8 ml, slightly smaller than the student fractions)

The 1st curve represents the Blue Dextran, the 2nd curve the Hemoglobin, and the 3rd curve is the Vitamin B12. Blue Dextran has a molecular weight of approximately 2,000,000, Hemoglobin 66,000 and Vitamin B12 1,350. The Blue Dextran should appear very blue in color, the hemoglobin a brownish-red, and the Vitamin B12 hot pink.



The second graph shows where the highest concentration of the enzyme Wheat Germ Acid Phosphatase should be located relative to the graph above. You can see that the Hemoglobin is found in highest concentrations in Fraction 12, while the WGAP is most concentrated in Fraction 13. This is because the two molecules are VERY close in size, Hemoglobin is slightly larger at 66,000 molecular weight while the WGAP is about 55,000 molecular weight.



The graph above shows several curves because the enzyme reactions operates at several pHs but the more acidic pH is optimum. The more enzyme activity, the more yellow color, hence the higher peak in the Acetate buffer.

Tris 5 min: pNPP and WGAP were prepared and the column was run in Tris buffer (pH 7.4). The reaction ran for 5 minutes and then the color was read with a plate reader, slight yellow appearance.

Tris 10 min: after 10 minutes the yellow color becomes more intense.

Tris w/ KOH: the reaction was stopped with the base KOH and the yellow color where the enzyme is present becomes even more yellow.

Acetate w/ KOH (10 min): in the acetate buffer one will not see the yellow color of the reaction until the KOH is added, but the color will be the most INTENSE using this lower pH (optimum for enzyme activity).

Suggested Variations:

- Longer columns (to give better separation)
- Other standards (myoglobin (~16,000) cytochrome (14,000), etc.
- Run a time course of the WGAP reaction (similar to that shown above in the Tris buffer) –can be done with acetate buffer-run reaction for different time period, then stop with KOH and observe differences in color.

- Measure color of enzyme reaction with plate reader if available
- Alter temperature of WGAP reaction (use heating block)
- Alter pH of pNPP buffer (could be a Tris buffer-to make Tris buffer dissolve 31.02 g Tris HCL + .388 g Tris Base in H₂O- makes 4 liters, pH 7.0 at room temp.)

Name: _____

Date: _____ Period: _____

Column Chromatography and Enzyme Assay

Objectives: Students will be able to...

- Explain how molecules are separated by chromatography
- Identify the number of molecules in a mystery solution
- Identify the relative size of various biomolecules
- Explain the relationship between an enzyme and its substrate
- Describe the role of a catalyst

Materials needed:

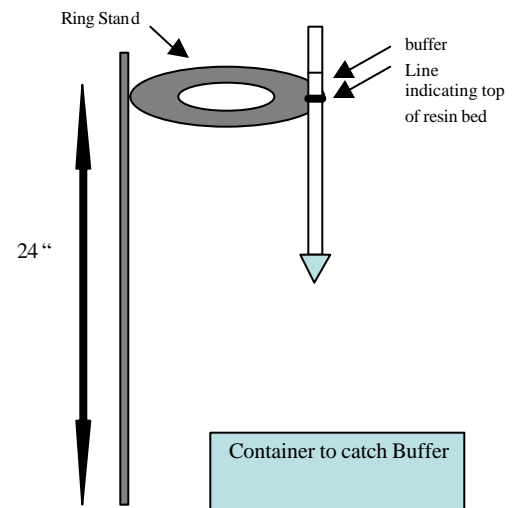
Ring Stand
 1 1x20 cm gel filtration column
 Stop cock, tubing & tubing adaptor
 Sodium-Acetate Buffer
 30 small test tubes
 test tube rack
 S-300 resin
 P-200 Micropipette and tips
 KOH

SAFETY PRECAUTIONS: Be sure to wear eye-protection and gloves when completing this lab. Some of the materials used can be hazardous.

Student procedure:

Day One: Pouring the Column

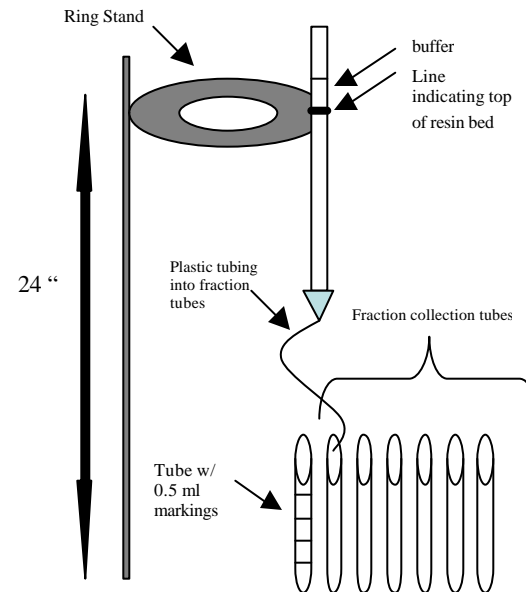
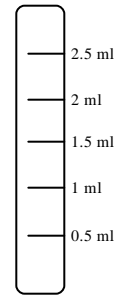
1. Obtain a 1 cm x 20 cm gel filtration column.
2. Secure the column to a ring stand so that the top of the column is approximately 2 feet above your lab station. Make sure the column is level.
3. Obtain about 20 ml of resin (S300). Make sure that the resin is thoroughly mixed before taking your sample.
4. Put about 5 ml of buffer into the column and let it drain. This will ensure that there are no bubbles trapped in the column. Do NOT let the column run dry.
5. Be sure that the stop cock is closed at the bottom of the column.
6. Fill the column with the mixed resin slowly so that you do not create bubbles.
7. Wait and allow the resin to settle (approximately 30 minutes). The resin should appear white and opaque, the remaining buffer at the top of the column should appear clear.
8. Place a small piece of laboratory tape length-wise on the column next to the top of the resin. Draw a small line indicating where the top of the resin is, using a Sharpie.



9. Cover the column with Parafilm to ensure that it will not run dry. Leave column set up for tomorrow's laboratory.

Day Two: Running the Column

1. Set up approximately 30 fraction collection tubes in a test tube rack. Number each tube.
2. Create your own graduated tube by filling it with 500 μ l of water at a time. Mark with a Sharpie the location of 0.5 milliliter. You will use this to measure the amount of liquid that you have collected in your fraction tubes.
3. Add a small piece of tubing to the bottom of the stop cock. Position the tubing so that the other end extends into your collection tube.
4. Check to make sure that your column has remained moist overnight.
5. Carefully remove the excess buffer from the top of the column until there is only a VERY SMALL amount covering the resin.
6. SLOWLY pipette 175 μ l of the "unknown" mixture onto the top of the resin (you can pipette it into the small amount of buffer on top). Pipette slowly enough that you do not upset the resin bed. Be sure to evenly place the liquid mixture on top of the resin (not lopsided).
7. Wait until you know that the mixture has entered the resin (it has moved past the line on the tape that you marked yesterday).
8. VERY SLOWLY add buffer to the top of the column using a small transfer pipette. Again, add slowly so that the buffer does not upset the resin bed. It is helpful to pipette against the side of the column to create a gently flow of buffer rather than squirting against the resin.
9. Continue to add buffer until the reservoir at the top of the column is filled $\frac{3}{4}$ full (once you have a substantial amount of buffer on top of the column you may add the buffer more quickly).
10. Open the stop cock at the bottom of the column and begin collecting drops in your collection tubes.
11. When you have collected 1 ml in each tube, move to the next fraction tube.
12. Continue to add buffer slowly to the top of the column as it runs. You want the reservoir to remain about $\frac{3}{4}$ full the entire time.
13. The column will take approximately 1 hour and 30 minutes to run completely. You are finished when there is no color remaining in your fractions. At this time you may close the stop cock on your column and begin analysis of your fraction tubes.
14. If a microplate is available, place approximately 200 μ l of each fraction into a microplate for analysis. Viewing the samples in this way above a white piece of paper will increase your ability to distinguish colors.



Fraction Tube Analysis:

1. Use Excel to create a chart similar to the one below or create your own on paper. Describe the colors that you see in each fraction of your sample. Also describe the intensity of the color (ex: yellow+, yellow ++).

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
clear																												

- Using your fraction tubes and color observations, determine the number of biological molecules that were present in your “unknown” sample. Indicate where you think each molecule was most concentrated on your spreadsheet.
- Indicate the relative sizes of the various biological molecules on your spreadsheet (for example, which molecule was largest? Smallest? How do you know?)

When you are finished, be sure the reservoir is filled with buffer. Allow the column to drip into the container that you have below the column to be sure that the sample is completely out of the resin so that we can use it again tomorrow. Do not allow the column to run dry.

Day Three: Enzyme Assay

- Prepare the column for loading the sample in the same manner as you did during Day Two’s experiment.
- This time load 175 ul of the enzyme Wheat Germ Acid Phosphatase (WGAP). Again collect 1 ml fractions as the sample runs through the column. It is unlikely that you will see any color coming through in the fractions. This is okay, we will find the enzyme using an enzyme substrate reaction.
- After collecting all of the fractions, transfer 50 ul of each fraction into the wells of a 96 well plate.
- Next, add 200 ul of PNPP to these wells (this is the substrate) every 20 seconds.
- After 10 minutes, add KOH to each well (CAUTION, THIS IS A STRONG BASE!), also every 20 seconds (this way each reaction runs for 10 minutes) Create a table (see example below) to help you time the beginning and end of the reaction. One person should be in charge of adding the PNPP, a different person should add the KOH, the third member of the group should watch the time and tell the other person when to add the materials.

EXAMPLE for first 8 fractions

Time	1	2	3	4	5	6	7	8
Start	0	0:20	0:40	1:00	1:20	1:40	2:00	2:20
Stop	10:00	10:20	10:40	11:00	11:20	11:40	12:00	12:20

- After adding the KOH look for any color change in the well plates. Be sure to place the wells over a white piece of paper so that any color change will be more obvious.
- Rate each fraction for the presence of color (0=no color, +=color is present, ++=color more intense, etc.)

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29

- Compare your results to your results from Day Two. In what fractions is the enzyme present? In what fraction is the enzyme most concentrated? How do you know?
- How does the size of the enzyme compare to the sizes of the molecules separated yesterday?
- If you were to run a column containing all of the molecules from Day 2 and the enzyme from Day 3, in what order would you expect them to appear in the fractions? Why?

CLEAN UP: Put all of the waste from the enzyme assay (includes the KOH) into a waste disposal bottle provided by the instructor.

Column Chromatography Assessment

1. What is chromatography used for?
2. What is chromatography being used for in the laboratory?
3. Describe the stationary and moving parts of chromatography and give one example of each.
4. Draw a cartoon picture of a gel-filtration column and explain how molecules are separated in this column.
5. Give an example of one other type of chromatography (besides gel filtration), and state how it works.
6. If you were to run a gel-filtration column of unknown molecules, what molecules would elute first, the largest or smallest? Why?
7. Describe the relationship between an enzyme and its substrate. Identify the enzyme and substrate in this lab activity.
8. Describe the role of temperature and pH in relation to enzyme activity.
9. Give an example of another enzyme and substrate that you have learned about during this course.

Column Chromatography Assessment-KEY

1. What is chromatography used for?

Chromatography is used to separate compounds that are not easily separated by another method.

2. What is chromatography being used for in the laboratory?

In this lab chromatography is used to separate a mixture of unknown molecules in order to determine the number of molecules in the mixture and the relative size of those molecules. It is also used to isolate an enzyme and determine the relative size of the enzyme in relation to the other molecules identified.

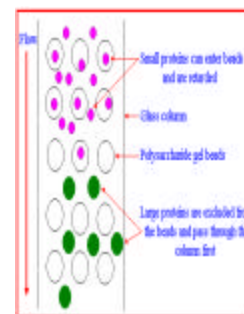
3. Describe the stationary and moving parts of chromatography and give one example of each.

In chromatography the mixture that is to be separated must be distributed between a stationary and moving substance. The stationary substance will bind to, or slow down the some molecules while others pass by (they are moved along by the moving substance). This causes different molecules to elute at different times.

4. Draw a cartoon picture of a gel-filtration column and explain how molecules are separated in this column.

The small molecules get caught in the bead holes and are slowed down.

Large molecules cannot enter the beads and therefore move through the column very quickly, eluting first.



5. Give an example of one other type of chromatography (besides gel filtration), and state how it works.

Paper chromatography-moving-solvent, stationary-paper-solvent moves up paper distributing sample

Ion-exchange-moving-buffer, stationary column with charge- charged molecules bind to the column, when pH is changed, charge changes and molecules no longer bind-come off column

6. If you were to run a gel-filtration column of unknown molecules, what molecules would elute first, the largest or smallest? Why?

Largest, because it is not slowed down by the column beads.

7. Describe the relationship between an enzyme and its substrate. Identify the enzyme and substrate in this lab activity.

An enzyme is specific to its substrate. The enzyme binds to the substrate at the enzyme's active site, the enzyme then creates an induced fit to "hold" the substrate tightly. The substrate is then converted into products. In this lab the enzyme was wheat germ acid phosphatase and the substrate was pNPP.

8. Describe the role of temperature and pH in relation to enzyme activity.

Temperature and pH affect the rate of the enzyme/substrate reaction. Each enzyme has an optimal pH and temperature at which it operates. pH of temperature outside the range of the enzyme will denature the enzyme and it can no longer act on the substrate. In this lab the enzyme was denatured with KOH.

9. Give an example of another enzyme and substrate that you have learned about during this course.

Enzyme=catalase

Substrate=Hydrogen peroxide