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The rAmylase Project by Ellyn Daugherty

Using Ion Exchange Chromatography to Separate Proteins

(Lab 9c)

(Cat. # BTNM-9C)

Developed in partnership with

The rAmylase Project by Ellyn Daugherty

Using Ion Exchange Chromatography to Separate Proteins (Lab 9c)

Teacher's Guide

The following laboratory activity is adapted from "Laboratory 9c: Using Ion-Exchange Chromatography to Separate Proteins" from *Biotechnology: Laboratory Manual* by Ellyn Daugherty. For more information about the program, please visit www.emcp.com/biotechnology.

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About Ellyn Daugherty: Ellyn Daugherty is a veteran biotechnology educator and recipient of the Biotechnology Institute's National Biotechnology Teacher-Leader Award. She is the founder of the San Mateo Biotechnology Career Pathway (SMBCP). Started in 1993, SMBCP has instructed more than 7,000 high school and adult students. Annually, 30-40 SMBCP students complete internships with mentors at local biotechnology facilities.

About G-Biosciences: In addition to the Biotechnology by Ellyn Daugherty laboratory kit line and recognizing the significance and challenges of life sciences education, G-Biosciences has initiated the BioScience Excellence™ program. The program features hands-on teaching kits based on inquiry and curiosity that explore the fundamentals of life sciences and relate the techniques to the real world around us. The BioScience Excellence™ teaching tools will capture the imagination of young minds and deepen their understanding of various principles and techniques in biotechnology and improve their understanding of various social and ethical issues.

The rAmylase Project by Ellyn Daugherty

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Upon receipt, store the materials as directed in the package literature.

MATERIALS INCLUDED

This kit has enough materials and reagents for 8 lab groups (32 students in groups of 4).

- 8 tubes of 10X Equilibration Buffer, pH 7.35 (2ml)
- 8 tubes 1X Elution Buffer, pH 7.35 (5ml)
- 1 tube of 1.5mg/ml α-Amylase from *Bacillus subtilis* (250µl)
- 1 tubes of 0.5mg/ml Lysozyme (250µl)
- 6 tubes of Amylase/Lysozyme Mixture (450µl)
- Eight 2ml DEAE Sepharose Chromatography columns
- 70 Microcentrifuge Tubes (2ml)
- 1 bottle Bradford Reagent [5X] (40ml)
- 8 Transfer Pipettes

ADDITIONAL EQUIPMENT & MATERIALS REQUIRED

The following standard lab equipment should be available for each group.

- Small beakers (i.e. 50ml)
- Deionized water
- Clamps and stands to secure column
- P-1000 Micropipets and tips
- Spectrophotometer cuvettes (i.e. 13 X 100mm test tubes for a Spectronic 20D+)
- Spectrophotometer (i.e. Spectronic 20D+)
- Gloves and goggles
- Paper towels

SPECIAL HANDLING INSTRUCTIONS

- Store the amylase and lysozyme tubes frozen at -20°C.
- Store the DEAE Sepharose Chromatography Columns at 4°C.
- All other reagents can be stored at room temperature

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GENERAL SAFETY PRECAUTIONS

- The reagents and components supplied in the *The rAmylase Project*™ kits are considered non-toxic and are safe to handle (unless otherwise noted), however good laboratory procedures should be used at all times. This includes wearing lab coats, gloves and safety goggles.
- The teacher should 1) be familiar with safety practices and regulations in his/her school (district and state) and 2) know what needs to be treated as hazardous waste and how to properly dispose of non-hazardous chemicals or biological material.
- Students should know where all emergency equipment (safety shower, eyewash station, fire extinguisher, fire blanket, first aid kit etc.) is located and be versed in general lab safety.
- Remind students to read all instructions including Safety Data Sheets (SDSs) before starting the lab activities. A link for SDSs for chemicals in this kit is posted at www.gbiosciences.com
- At the end of the lab**,** all laboratory bench tops should be wiped down with a 10% bleach solution or disinfectant to ensure cleanliness.
- Remind students to wash their hands thoroughly with soap and water before leaving the laboratory.

TEACHER'S PRE EXPERIMENT SET UP

1. Air bubbles may be introduced into the resin of columns during shipping and these may result in poor or inhibited buffer flow. To remove bubbles from the resin bed, vortex (shake) vigorously to resuspend resin. Allow the column resin to resettle for 5-10 minutes, in a vertical (column running) position. Check that bubbles are no longer present.

2. Label 8 small beakers, "1X Bradford Reagent." In a 250ml beaker, dilute the Bradford Reagent to 1X by adding 40ml of the 5X Bradford Concentrate to 160ml of deionized water. Mix thoroughly. Aliquot 25ml of 1X Bradford Reagent into the 8 labeled beakers.

3. Distribute the following reagents to each lab group:

    - 1 tube of 10X Equilibration Buffer, pH 7.35 (2ml)
    - 1 tube 1X Elution Buffer, pH 7.35 (4ml)
    - 1 tube of either 1.5mg/ml α-Amylase from *Bacillus subtilis* (250µl) or 0.5mg/ml Lysozyme (250µl) or 450µl of Amylase/Lysozyme Mixture
    - A 2ml DEAE Sepharose Chromatography column
    - 7 Microcentrifuge Tubes (2ml)
    - 1 tube of CB Protein Assay Reagent (8ml)
    - 1 Small beaker (i.e. 50ml)
    - 1 beaker 1X Bradford Reagent
    - A clamp and stand to hang the chromatography column in a vertical position
    - 1 transfer pipette

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TIME REQUIRED

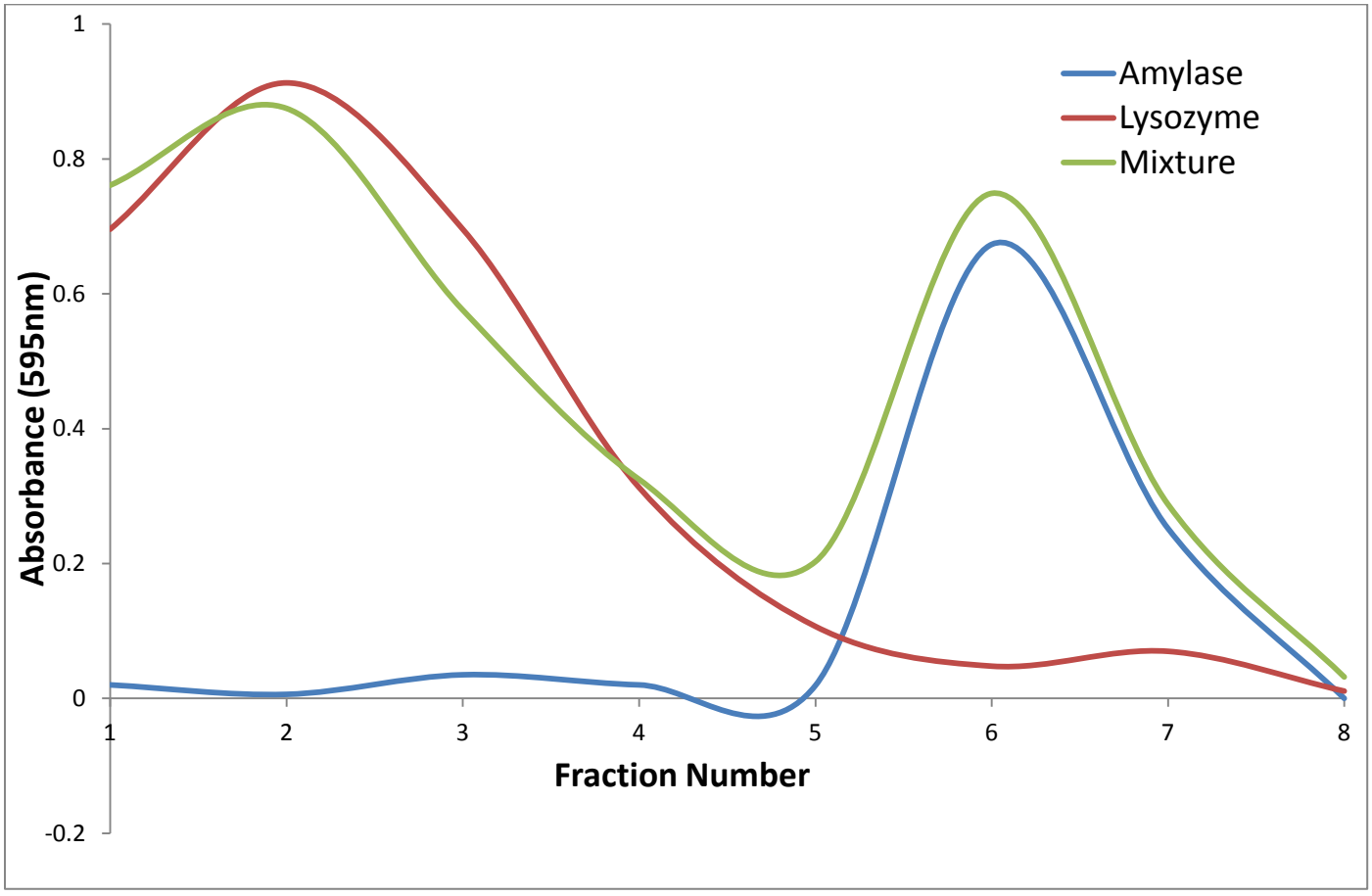
- 1 hour pre-lab preparation of buffers and column set up
- Two hour lab periods to calibrate and conduct the column chromatography (collect fractions) and test samples using the indicator and spectrophotometer
- 1 hour post-lab to graph and analyze results

NEXT GENERATION SCIENCE STANDARDS ADDRESSED

- HS-LS1: From Molecules to Organisms: Structures and Processes
- LS1.A: Structure and Function

For more information about Next Generation Science Standards, visit: http://www.nextgenscience.org/

EXPECTED RESULTS



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ANSWERS TO ADDITIONAL QUESTIONS

1. A technician checks the column bed volume on a column that is supposed to be 2ml. If the bed volume is not 2ml, is this a problem? Why or why not?
   Answer: A bed volume that is larger is only a problem because it takes longer to run and more volume to wash each sample through a larger column. A smaller bed volume means that there are not as many charged beads to bind the negatively charged protein, and some sample will be lost into wash fractions.
2. The column drips very slowly. Suggest a method to increase the rate of flow through the column. How can you check to ensure that this method does not compromise the separation?
   Answers will vary. If a column runs too slowly, putting gentle pressure from the top of the column down on the sample will increase the flow of fluids through the resin bead. An apparatus, such as a syringe (from the top) or vacuum (from the bottom) may be used to create the difference in pressure. If the pressure is too great, separation may not occur. Absorbance results would indicate this.
3. A scientist would like to know the charge of a certain protein at pH 7.2. How could this column be used to help determine the overall charge of the protein at pH 7.2? What problems might occur using the proposed approach, and how might they be addressed?
   Answer: This column may be used to help determine the overall charge of the protein at pH 7.2 by running the column, substituting the protein of interest for either lysozyme or amylase. At the proper concentration, the protein of interest will either wash through (=+charged) or bind to the column (= -charged). The concentration of the sample is an issue, though. If there is too much protein in the load, protein will be in all of the fractions. To determine the column's binding capacity (if it is a –charged protein), it will be necessary to run some of the columns with serial dilutions of the protein sample.

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OBJECTIVES

Using ion-exchange chromatography, how well can lysozyme (positive [+] charge at pH 7.35) be separated from amylase (negative [-] charge at pH 7.35)?

BACKGROUND

In ion-exchange chromatography, resin beads of a certain charge (positive [+] or negative [-]) are used in the column. A mixture of proteins is added to the column, and if the pH and column capacity are correct, everything passes through the column except the protein of interest. This is because the column resin and pH are chosen to produce the "most opposite charge" of the protein of interest on the beads. If the charge on a bead is positive, it will bind and release negatively charged molecules. This technique is called anion exchange. If the beads are negatively charged, they bind and release positively charged molecules (cation exchange). A scientist picks a resin to use based on the properties of the protein of interest. Under ideal conditions, during ion-exchange chromatography, the protein binds to the oppositely charged beads. Buffers are used to flush contaminant proteins out of the column. Finally, a buffer containing an ion with a greater attraction to the bead than the protein of interest knocks (elutes) the protein off the bead (this is the ion exchange). Often, a high-salt buffer is used to elute the desired protein from the column (see Figure).

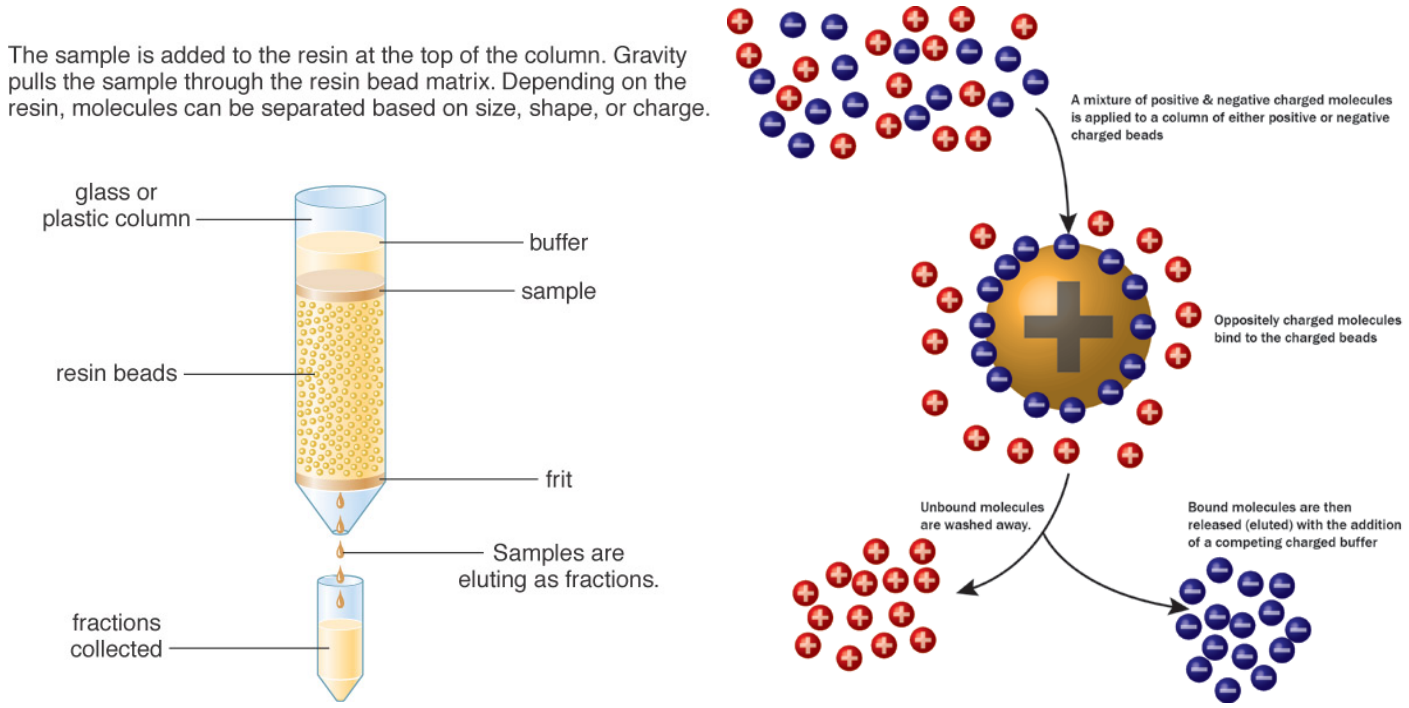

Figure: Ion Exchange Chromatography. Left panel shows the basic set up of a gravity chromatography column and the right panel shows the general principle of ion exchange chromatography

The goal of this chromatography is to separate amylase from lysozyme. The behavior of each of these proteins alone on a column indicates how each protein with run on the column when it is in a mixture. The charge of each protein, at pH 7.35, will be known by its presence in only some of the columns, by the end of the chromatography.

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MATERIALS FOR EACH GROUP

Supply each group with the following components.

- 1 tube of 10X Equilibration Buffer, pH 7.35 (2ml)
- 1 tube 1X Elution Buffer, pH 7.35 (4ml)
- 1 tube of either 1.5mg/ml α-Amylase from *Bacillus subtilis* (250µl) or 0.5mg/ml Lysozyme (250µl) or 450µl of Amylase/Lysozyme Mixture
- A 2ml DEAE Sepharose Chromatography column
- 8 Microcentrifuge Tubes (2ml)
- 1 small beaker (~25ml)
- 1 beaker of 1X Bradford Reagent
- 1 Transfer Pipette

ADDITIONAL MATERIALS FOR EACH GROUP

The following standard lab equipment should be available for each group.

- P-1000 Micropipets and tips
- A clamp and stand to hang the chromatography column in a vertical position
- Spectrophotometer cuvettes (i.e. 13 X 100mm test tubes for a Spectronic 20D+)
- Spectrophotometer (i.e. Spectronic 20D+)
- Gloves and goggles
- Paper towels

PROCEDURE

Each group will run one of the protein samples (amylase alone, lysozyme alone, or the mixture) and share results. The teacher will assign the protein your group will run on your column.

Prepare 1X Equilibration Buffer

1. Label a small beaker "1X Equilibration Buffer".
2. Add all 2ml of 10X Equilibration buffer to the beaker.
3. Add 18ml of deionized water to the beaker and mix the solution thoroughly.

Prepare the Ion Exchange Column for Chromatography

1. Have a stand and clamp ready to hold the chromatographic column. Before clamping the column in place tiny air bubbles that are introduced into the resin bead must be removed. Air bubbles may result in poor or inhibited buffer flow. To remove bubbles from the resin bed, vortex (shake) vigorously to resuspend resin.
2. Check that bubbles are no longer present, attach (clamp) the column to the stand (in a vertical position) and allow the resin to settle until there is a define top to the resin bed (5-10 minutes).

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3. Open the top cap first and then the bottom cap of the column to prevent air entering the resin. Allow the preservative buffer to drain out of the column, under gravity, to a waste container. Make sure that the column resin evenly settles down in the column. Quickly move onto the chromatography.

Ion Exchange Chromatography

1. With the column stopper in place, gently add 2ml of equilibration buffer to wash out any residual preservative. When adding the buffer let it gently drip down the inside of the column to avoid disturbing the top of the column bed. Unplug the stopper and let the buffer pass through the column into a trash beaker. Quickly, replace the stopper on the bottom of the column as the last buffer enters the column.
2. Add an additional 2 x 4ml of the equilibration buffer to establish the charges on the resin beads in the column. Unplug the stopper and allow the buffer to flow through into a trash beaker.
3. While the equilibration buffer is flowing through the column, label eight 2ml tubes (1, 2, 3, 4, 5, 6, 7 and 8).
4. Gently add the assigned protein sample (the "load") without disturbing the top of the bed, either: 200µl lysozyme, 200µl α-amylase, or 400µl amylase/lysozyme mixture
5. Allow the sample to "load" into the column and discard the flow-through
6. Move collection tube No. 1 under the column. Add 1mL of equilibration buffer and let it pass through the column. Collect the flow-through in tube No. 1
7. Move collection tube No. 2 under the column. Add 1mL of equilibration buffer and let it pass through the column. Collect the flow-through in tube No. 2.
8. Repeat step 6, two more times, collecting fractions into tubes No. 3 and 4.
9. When the fourth wash (into tube No. 4) is complete, move tube No. 5 under the column. Add 1ml of elution (high-salt) buffer to the top of the column. Collect the flow-through into tube No. 5.
10. Repeat step 8, three more times, collecting fractions into tubes No. 6, 7 and 8.

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Bradford Protein Assay

- Make sure you are familiar with how to use the spectrophotometer in your lab. To take absorbance reading of all the samples, you must work quickly when using the spectrophotometer.
- For use in the visible spectrophotometer the samples must be transferred to appropriate tubes for mixing with the colored protein indicator (Bradford Reagent). If the spectrophotometry cuvettes are an appropriate size, they can be used for the mixing, and then for spectrophotometry. If not, then each sample and Bradford Reagent is mixed in a separate test tube and then transferred to a cuvette for analysis.

1. Label 9 spectrophotometer cuvette tubes or mixing tubes with Blank and 1-8 and transfer 500µl equilibration buffer and elute from fractions 1-8 to each.
2. Learn how to use the model of spectrophotometer in your lab. Warm up your spectrophotometer and set the spectrophotometer to a wavelength of 595 nm.
3. Using a transfer pipet and without touching the inside of each tube, add 2.5ml of 1X Bradford Reagent to the samples. Be careful to not touch the solutions in the tubes, spill or accidentally contaminate one sample with another. Gently mix thoroughly without letting the mixture bubble. Within 3 minutes use the spectrophotometer to observe (take absorbance readings) of all the samples.
4. Look at the colors of the solutions in the tubes. Are they the colors you expected? Yes or no? Why or why not? Record your observations.
5. Blank the spectrophotometer using the prepared blank form step 1..
6. In your notebook, create a data table similar to the table below to record the absorbance of fractions 1 to 8. Measure the absorbance of each tube and record the values.

    *The absorbance can be measured with a microplate reader instead of using a spectrophotometer. Transfer 250µl from each assay tube to a microtiter plate well. Add 250µl distilled water to a well as reference blank. Read the absorbance at 595nm.*
7. Collect data from two other groups so that you have absorbance data for amylase alone, lysozyme alone, and the mixture of lysozyme and amylase.

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Table: Absorbance of Ion-Exchange Fractions at 595nm.

	Fraction #							
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Lysozyme								
α-Amylase								
Both								

DATA ANALYSIS & CONCLUSION

Examine the absorbance data. Considering that the minimum absorbance reading on a spectrophotometer is normally 0.02 au, and the maximum is 2.0 au, do the numbers make sense for what was expected? Explain.

Plot your data on a three-line, line graph. Let each line represent the absorbance of ion-exchange fractions from one of the three protein samples.

In a written concluding statement discuss how the data shows that the column actually separated lysozyme from amylase? Consider the peaks and valleys of each line. Explain why the shape of each line looks the way it does. If the results are not what you expected, discuss errors that might lead to fallacious data. Explain how the ability to separate molecules, such as amylase and lysozyme, on a column is utilized at a biotechnology company or in research. If the separation of amylase and lysozyme was not complete on the column, propose a modification of the protocol that might improve the separation.

ADDITIONAL QUESTIONS

1. A technician checks the column bed volume on a column that is supposed to be 2ml. If the bed volume is not 2ml, is this a problem? Why or why not?

2. The column drips very slowly. Suggest a method to increase the rate of flow through the column. How can you check to ensure that this method does not compromise the separation?

3. A scientist would like to know the charge of a certain protein at pH 7.2. How could this column be used to help determine the overall charge of the protein at pH 7.2? What problems might occur using the proposed approach, and how might they be addressed?

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