Country:

PRACTICAL EXAMINATION # 1: MOLECULAR LABORATORY

This practical examination is composed of two 45-minute Tasks:

Task 1: Measurement of enzyme activity **(20 points)**

Task 2: Separation of proteins by chromatography and electrophoresis **(20 points)**

After 45 minutes, competitors will swap tasks.

Competitors are requested to follow instructions when swapping from one task to another.

There is to be no discussion or exchange of any materials between competitors when changing tasks.

Competitors must not commence the new task until instructed.

THESE INSTRUCTIONS ARE FOR TASK 2

GENERAL INSTRUCTIONS

Competitors are advised to read the examination before commencing.

It is recommended that Competitors proportion their time according to the allotted points for each task and question.

IMPORTANT

All answers must be recorded on the answer sheets provided.

Ensure that your 3 digit code number is written and coded on the top of each page of the answer sheets.

Using the pencil provided, fill in the appropriate circle on the answer sheet.

TASK 2

SEPARATION OF PROTEINS BY CHROMATOGRAPHY AND ELECTROPHORESIS

IMPORTANT : There are two Parts to this Task.

Please read Part A so that you can plan your time before commencing.

PART A

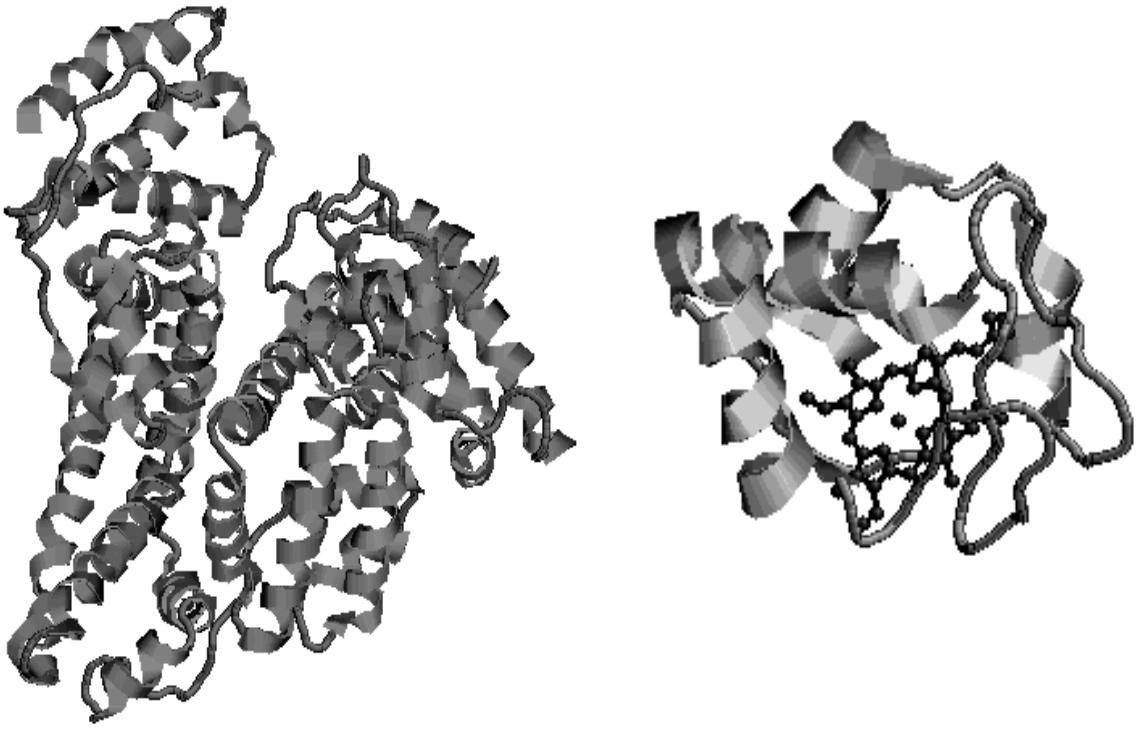
ION EXCHANGE CHROMATOGRAPHY

INTRODUCTION

Ion exchange chromatography is a technique used to separate proteins on the basis of overall electric charge. Such a separation depends on the acid-base properties of the relevant proteins and the charge on the chromatographic matrix used to separate them. Since the net charge on a protein is dependent on pH, ion exchange chromatography is only effective under conditions of defined pH. At pH 8.0, cation exchange matrices, such as the Hi-Trap "SP" to be used in this task, carry a negative charge and therefore bind positively charged proteins. Ions in the solution with a like charge to the proteins will compete with those proteins for binding to the matrix. Competing ions in excess prevent or reverse binding of proteins, thereby causing them to be *eluted* (washed from the column) from the matrix.

In this experiment you will be provided with a protein sample containing two proteins, albumin and cytochrome c. Albumin, a major protein of blood plasma, has a molecular mass of 68,000 Daltons (Da) and consists of a single chain of amino acids. Cytochrome c functions in electron transport in mitochondria and consists of a single chain of amino acids that is bound to an iron-containing heme group that absorbs visible light at a wavelength of 410 nm. Cytochrome c has a total molecular

mass of 12,400 Da. So called “Ribbon” structures of these two proteins are shown below: Albumin (left), cytochrome c (right).



In this practical session you will use ion exchange chromatography to separate albumin and cytochrome c.

MATERIALS AND REAGENTS PROVIDED PER STUDENT

Chemicals

Protein sample: 4 mg/mL albumin and 4 mg/mL cytochrome c

Buffer 1: 50mM Tris-HCl, pH 8.0 buffer.

Buffer 2: 50mM Tris-HCl, pH 8.0 buffer containing 0.5M NaCl

Protein Assay Reagent

Equipment

- Column containing cation exchange matrix (Hi-Trap SP)
- Clamp to hold column
- 2 x 5mL disposable syringe (labelled "Buffer 1" and "Buffer 2")
- 1 x 1mL disposable syringe (labelled "Protein Sample")
- Adjustable pipettes plus tips
- Yellow "sharps bin" for tip disposal
- 96 well microtitre plate
- Plastic beaker (labelled "Liquid waste")
- Safety glasses
- Disposable gloves
- Marker pen
- Blue card (to attract attention of demonstrator)
- Answer sheet with pencil and eraser

PROCEDURE

1. Label the microtitre plate (on the attached tape) with your blue Bench Card Number AND Competitor Number. (For example, if your Bench Card Number is "5" and your Competitor Number is "14-3", label the plate: 5/14-3).
2. The ion-exchange column is equilibrated with Buffer 1 (50mM Tris-HCl, pH 8.0) and ready for immediate use.
3. Draw up 5mL of Buffer 1 into the 5-mL "Buffer 1" plastic syringe.
4. Remove the black screw-on cap from the outlet plug at the bottom of the column.
5. Attach the syringe to the top of the column by firmly pushing into the black adapter
6. Load 1mL of buffer onto the column by gently and evenly depressing the syringe plunger. Run the waste into the plastic beaker.
7. Now draw up 0.2mL of the protein sample into a 1mL plastic syringe.
8. Load the protein sample onto the column by gently and evenly depressing the syringe plunger. While loading, start to collect fractions of 4 drops each into each well of **Row A** of the 96 well microtitre plate.
9. Once the sample has been loaded, replace the syringe with the 5mL syringe containing Buffer 1.
10. Continue to collect 4-drop fractions into each well of **Row A**.
11. When **Row A** is complete (Fractions 1-12), replace the screw-on plug to the outlet of the column and remove the syringe from the column.
12. Fill a new 5mL syringe with Buffer 2 (50mM Tris-HCl, pH 8.0 buffer containing 0.5 M NaCl).
13. Attach the new syringe to the column, remove the screw-on plug and continue to collect 4-drop fractions into **Row C** of the 96 well microtitre plate.
14. When Row C is complete (Fractions 13-24), replace the screw-on plug to the column.

15. Using an adjustable pipette, transfer 20μL from each well in **Row A** (Fractions 1-12) into the corresponding well in **Row B**.
16. Similarly, transfer 20μL from each well in **Row C** (Fractions 13-24) into the corresponding well in **Row D**.
17. Using an adjustable pipette, add 200μL of the Protein Assay Reagent to each well of **Rows B and D**. This reagent reacts with protein to yield a blue colour that can be measured by spectrophotometry at 595 nm using a plate reader.
18. Check for and eliminate air bubbles in the wells of your 96-well microtitre plate (do this carefully, using a clean yellow tip).
19. **HOLD UP THE BLUE CARD PROVIDED TO ATTRACT THE ATTENTION OF THE DEMONSTRATOR** to note that your 96-well microtitre plate is ready to be analyzed. The plate reader will measure the absorbance of the fractions at both 595nm and 410nm. A printout of the results will be returned to you by the demonstrator.

IMPORTANT: MOVE ON TO PART B WHILE YOU ARE WAITING FOR THESE RESULTS

Questions **ENTER YOUR ANSWERS ON THE ANSWER SHEET**

Q1. Which fraction (1-24) contained the first eluted A_{595} peak **(Task P1.T2.1) (1 point)**

Q2. Which fraction (1-24) contained the second eluted A_{595} peak **(Task P1.T2.2) (1 point)**

Q3. Which fraction (1-24) contained the A_{410} peak **(Task P1.T2.3) (1 point)**

Q4. Subtract the fraction number obtained in answer T2.1 from that obtained in answer T2.2 and enter the value. **(Task P1.T2.4) (1 point)**

Q5. In which fraction (1-24) did the peak of cytochrome c elute? **(Task P1.T2.5) (4 points)**

Q6. In your experiment you would have noted that one protein eluted directly from the column but the second protein needed added salt to be eluted. Consider these statements:

1. The salt neutralised the ionic interaction between the matrix and the second protein eluted
2. The protein that eluted first was more positively charged than the protein eluted with salt
3. The protein that eluted first was more negatively charged than the protein eluted with salt
4. The protein that eluted first did so because it was larger than the protein eluted with salt
5. The protein that eluted first did so because it was smaller than the protein eluted with salt

Which combination of statements is correct?

A. 1, 2

B. 1, 3

C. 2, 3, 4

D. 1, 3, 4

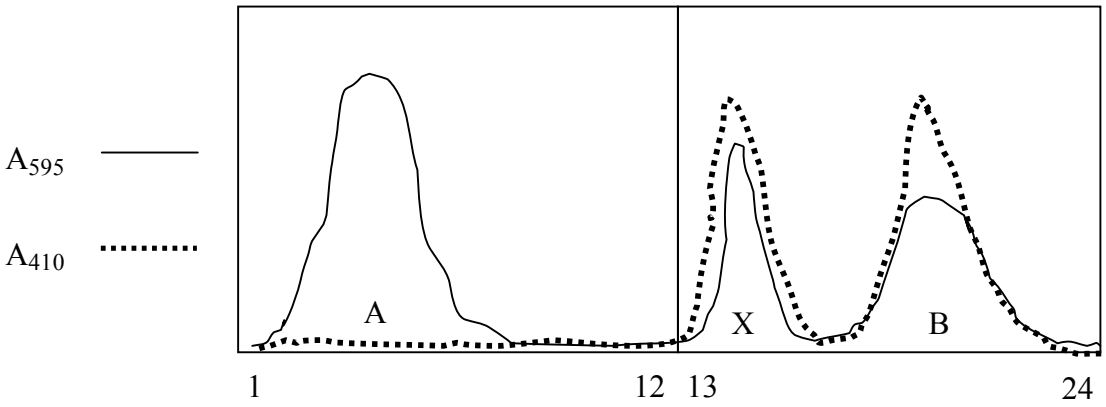
E. 2, 3, 4

F. 1, 3, 5

G. 2, 3, 5

ENTER YOUR ANSWER ON THE ANSWER SHEET (Task P1.T2.6) (2 points)

Q7. In another experiment you added a third protein (Protein X) to the protein sample containing albumin and cytochrome c and repeated the ion-exchange chromatography separation and detection exactly as before. The collated results obtained from the plate reader are shown below: The elution peak corresponding to Protein X is labelled. For convenience, the other two proteins are arbitrarily labelled "A" and "B".



Consider the statements below regarding these results:

1. Protein X has a less positive charge overall than protein B
2. Protein X has a more positive charge overall than protein B
3. Protein X contains a non-polypeptide component
4. Protein X is 100% polypeptide
5. Protein X eluted after protein A because it was larger
6. Protein X eluted after protein A because it was smaller

Choose which combination of statements is correct:

A. 1, 3

B. 2, 3

C. 1, 3, 5

D. 2, 3, 6

E. 2, 3, 5

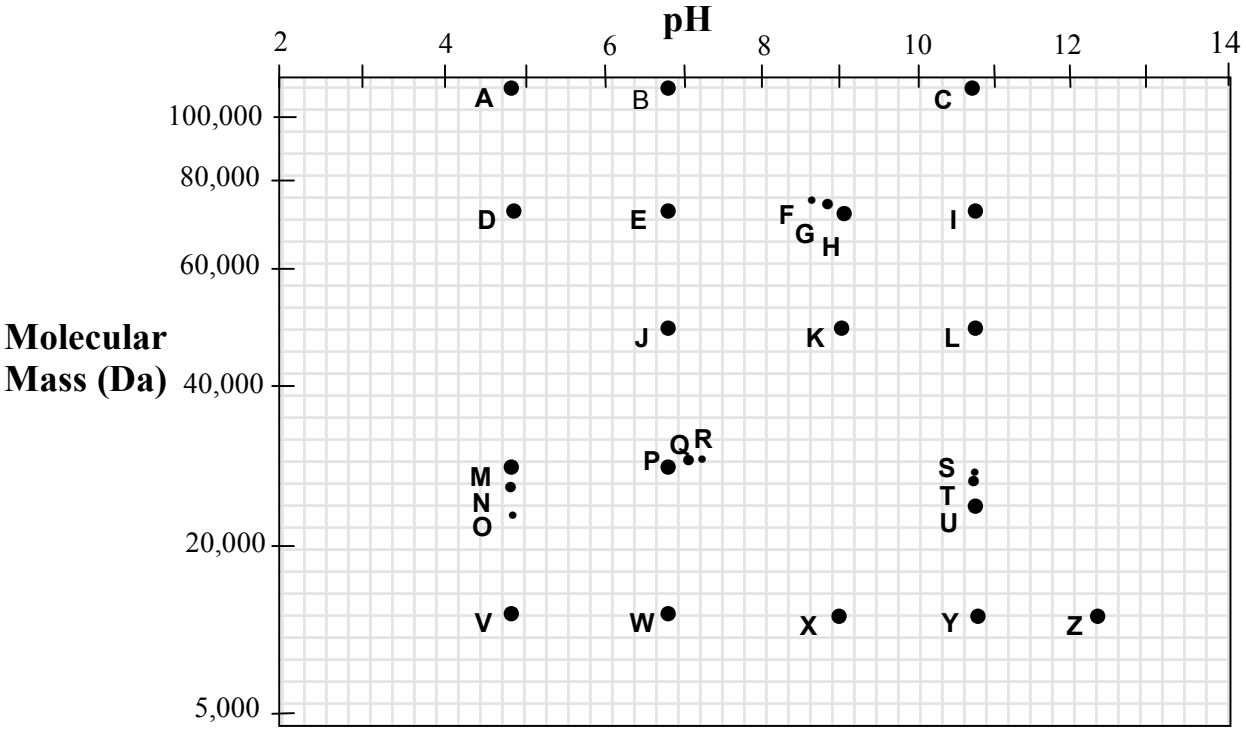
F. 1, 3, 6

ENTER YOUR ANSWER ON THE ANSWER SHEET (Task P1.T2.7) (4 points)

PART B

TWO-DIMENSIONAL GEL ELECTROPHORESIS

The diagram below shows the results of an experiment where a mixture of albumin, cytochrome c and other, unknown proteins were separated by two-dimensional gel electrophoresis. In this technique, the proteins were separated in the first dimension on the basis of their isoelectric point (pI) followed by separation in the second dimension on the basis of their molecular mass. The isoelectric point is defined as the pH at which the sum of the positive and negative charges on the protein is zero. The isoelectric point (pI) for albumin is 4.9 and for cytochrome c is 10.7. The individual proteins were subsequently detected using a protein stain. Each protein "spot" has been given an alphabetical letter identifier.



Answer the following questions: **ENTER YOUR ANSWERS ON THE ANSWER SHEET**

Q8. Which spot corresponds to albumin? **(Task P1.T2.8) (2 points)**

Q9. Which spot corresponds to cytochrome c? **(Task P1.T2.9) (2 points)**

Q10. Phosphorylation is a relatively common modification of proteins that occurs after they have been synthesised. The proteins affected can have a variable number of attached negatively charged phosphate groups; this also leads to a slight increase in their molecular mass.

From the data presented in the figure above, choose which group of proteins best represents the situation where a "parent" protein has been modified to generate a number of phosphorylated species that are less abundant. List the proteins in this group in order, starting with the "parent" protein through to the most phosphorylated protein. **(Task P1.T2.10) (2 points)**

END OF TASK 2

PLACE YOUR ANSWER SHEET ON TOP OF THE REST OF YOUR PAPERWORK AT YOUR WORKSTATION