Sultan Qaboos University

College of Medicine & Health Science

Instrumentation and Biomedical Techniques

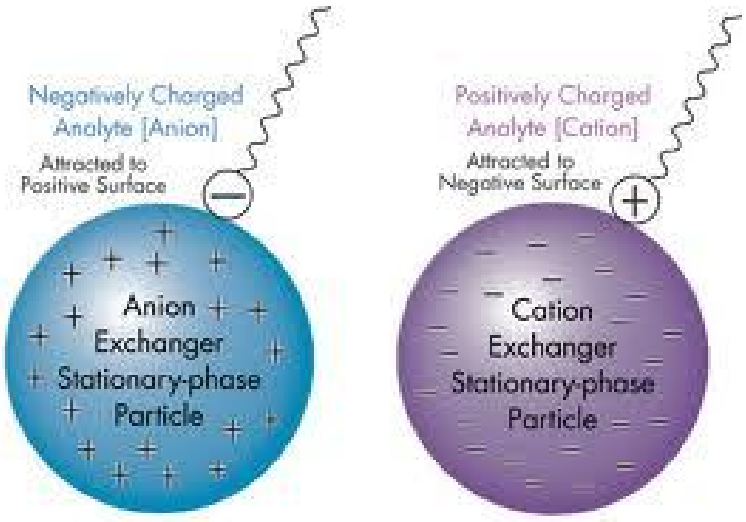
BIOC 6002

Laboratory Report

Practical No. (2): 02/10/2011

Ion exchange chromatography:

Isolation of lysozyme from egg white



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MSc. ID: M020944

Ion exchange chromatography separates molecules on the basis of their charged groups, which cause the molecules to interact electrostatically with opposite charges on a stationary-phase matrix. Therefore, the procedure is limited to purification of ionizable molecules.

Charged molecules to be purified compete with *counterions* for binding to the charged groups on the stationary phase. There are two types of ion exchange chromatography:

1. **Anion exchange chromatography:** when stationary phase bears a positive charge and exchangeable ion is an anion (-). See diagram below
2. **Cation exchange chromatography:** when stationary phase bears a negative charge and exchangeable ion is a cation (+)

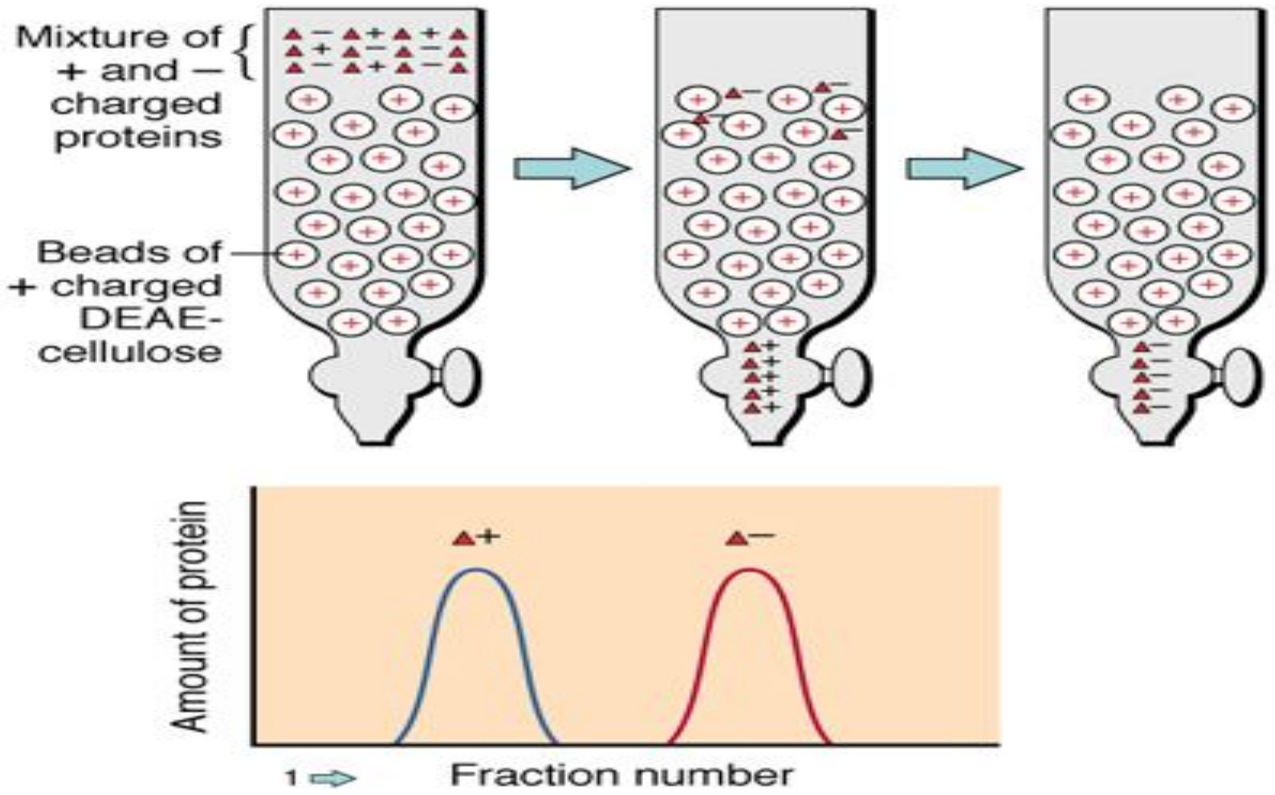


Diagram: (1) Fractionation with anion exchange chromatography

An ionizable group's charged depends on the *pH of the solvent* and *pKa of the ionizable group*. The pH at which a protein is uncharged is called the **isoelectric point (pI).** The pI of most proteins is in the range of ***5-9***. Ion exchange chromatography is usually performed at leasts *1 pH unit away from the pI of the protein* of interest to assure that it is charged.

When pH of chromatography < pI of molecule	**Molecule will be +Ve**	**Use Cation exchange resin**
When pH of chromatography > pI of molecule	Molecule will be -Ve	Use Anion exchange resin

Table: (1) pI of lysozyme = 11 & pH of buffer used = 10

Types of ion exchange resin:

Strong anion	QAE	Completely ionized
Weak anion	DEDE	Ionization depends on pH
Strong cation	SP	Completely ionized
Weak cation	**CM**	**Ionization depends on pH**

Table: (2)

- **Aim:**

The aim of this practical session is to do purification of hen egg white lysozyme, which makes up approximately **10%** of the total egg white protein. Lysozyme enzyme has high **(pI) pH = 11.0** and low molecular weight of **14.6×10^3 Dalton**.

In this part of practical (cation exchange chromatography) was used for an easy isolation of lysozyme enzymes **(+ve) charged** with pre-swollen **carboxymethly CM resin (-ve) charged** and then eluting it with a counterions (**NaCl**).

Finally, we are going to used *Bio-Rad Protein Assay* based on the *Bradford dye-binding* procedure which is a simple colorimetric assay at wavelength of **595 nm**. For measuring total protein concentration in mg/mL.

- **Materials:**

1. *Purification step:*
   - An egg of local hen
   - Muslin to separate egg white from the yolk
   - Ion exchange chromatography column with CM-Cellulose resins
   - 0.1 M Glycine/NaOH buffer, pH=10 (prepared in previous practical)
   - 0.1 M Glycine/NaOH buffer,pH= 10, containing **0.5 M NaCl**
   - Test tubes, pump, fraction collector, pipettes, paraffin, and seizer
   - Glassware and plasticware, column holder, column locker
2. *Bio-Rad Protein Assay:*
   - BSA standards (Bovine Serum Albumin)
   - Plastic cuvettes (2.5 mL)
   - Spectrophotometer

❖ **Methodology:**

1. We have poured 4mL of pre-swollen CM-cellulose resin into 20 mL chromatography column

2. Then 12 mL of 0.1 M glycine/NaOH buffer, pH=10 was added into the column resins for equilibration. 0.1 M glycine/NaOH buffer was prepared from 0.5 M glycine/NaOH buffer from our previous practical session

$$C_{1\,x}\,V_1 = C_{2\,x}\,V_2$$

0.5 M x (?) mL = 100 mL x 0.1 M

Then the volume needed is (V_2) = ***20 mL***

The pH value been recheck for confirmation and adjusted by NaOH

3. We have opened an egg and separated the white egg from the yolk by using muslin and the total volume of egg white obtained is **($V_{egg\ white}$ = 30 mL)**
4. We have diluted **(X50)** the neat sample with using 0.1 M glycine/NaOH buffer, pH = 10.

X 50 *dilutions done by mixing:*

1 mL *from egg white is mixed with* ***49 mL*** *of 0.1 M glycine/NaOH buffer*

This was our ***(F_0)*** *with* ***V_{F0}=50 mL***

5. After that we have applied **30 mL** of diluted sample into column and volume obtained was **(V_{F1}= 30 mL)** and from that 5 mL was saved for later analysis **(=F_1)**
6. The column was washed by 75 mL of 0.1 M glycine/NaOH buffer, pH = 10, the volume obtained is **(V_{F2} = 75 mL, F_2)** and 5 mL from was saved for later analysis.
7. We have eluted the column with **20 mL** of 0.1 M glycine/NaOH buffer, pH = 10 containing **0.5 M NaCl** and **1 mL fraction** was collected for the total amount. Molecular weight of NaCl is 58.44 g/mol

Volume is 100 mL then it follows that number of moles =

0.5 mol/L x 0.1 L

= 0.05 mol x 58.44 g/mol

*The grams needed to prepare this (NaCl) solution is **2.922 g** in 100 mL of 0.1 M glycine/NaOH buffer, pH = 10*

***Note:** we could not get 20 mL back from column and only 19 fractions were possible to obtained*

8. All samples and aliquots were labeled and store at +4 °C

9. Bio-Rad dye have been prepared by mixing 30 mL of dye in 120 mL of distilled water to make 1 in 4 dilution of original stock (5 folds)

10. 1 mL of Bio-Rad dye was mixed with 50 uL of each sample (F_0,F_1,F_2) and 19 eluted fractions and all put on plastic cuvettes ready for measurements

11. All samples were mixed well and incubated for 30 minute at room temperature

12. Spectrophotometer was opened for 30 minutes and reference **blank** (50 uL Buffer + 1 mL dye) was set-up at wavelength of 595 nm

13. We have prepared seven BSA STDs solutions including the blanks as follow; **0.00 mg/mL**, **0.05 mg/mL**, **0.1 mg/mL**, **0.2 mg/mL**. **0.4 mg/mL**, **0.8 mg/mL**, and **1.0 mg/mL** concentrations by doing dilution procedures. *See table (3)*

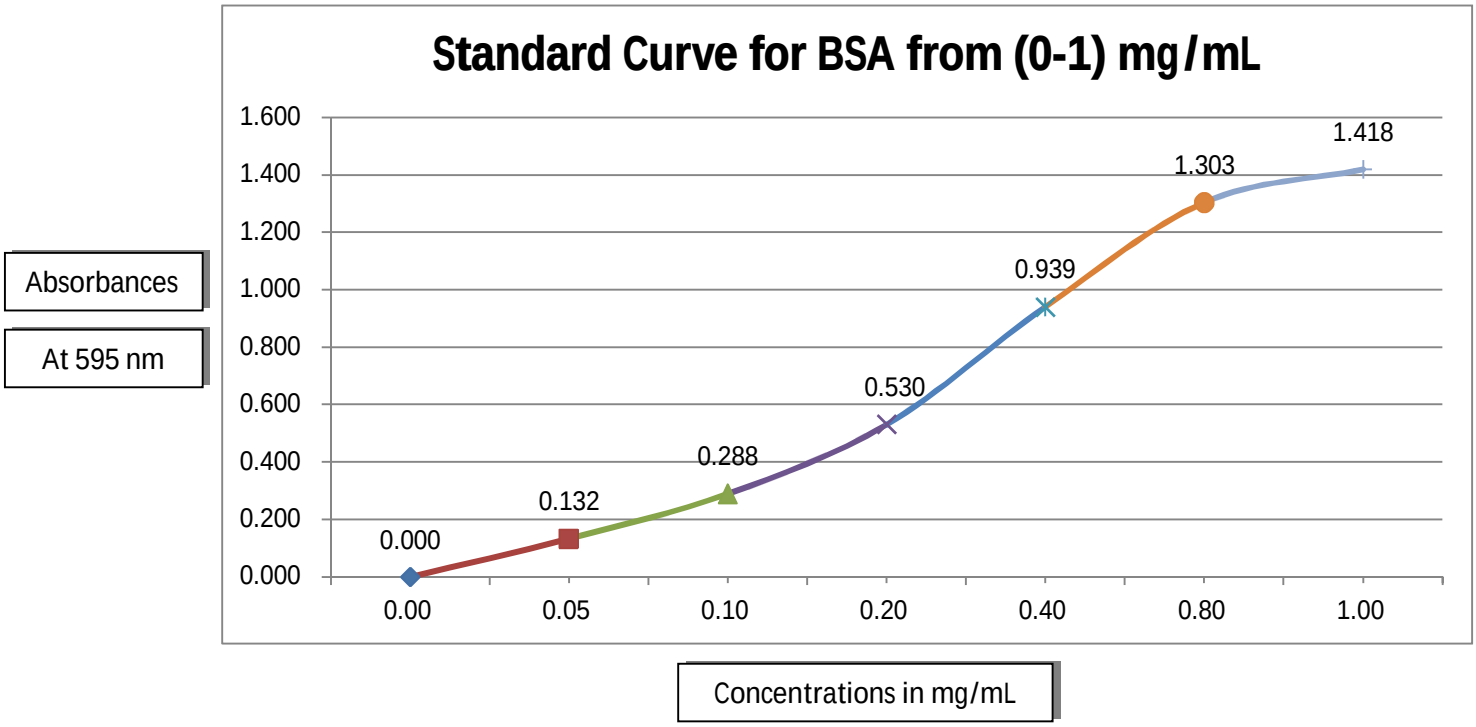
14. All absorbances been read at 595 nm using spectrophotometer starting with blanks to more concentrated solutions and results recorded and graph plotted by using *Excel version 2007 software*

❖ **Results & Interpretation:**

Serials	Concentrations	Final Volume	Volume needed	Absorbance(s)
1	0.00 mg/mL	1.00 mL	0.00 mL	**0.000**
2	0.05 mg/mL	1.00 mL	35.7 uL	**0.132**
3	0.10 mg/mL	1.00 mL	71.4 uL	**0.288**
4	0.20 mg/mL	1.00 mL	142.3 uL	**0.530**
5	0.40 mg/mL	1.00 mL	285.7 uL	**0.939**
6	0.80 mg/mL	1.00 mL	571.4 uL	**1.303**
7	1.00 mg/mL	1.00 mL	714.3 uL	**1.418**

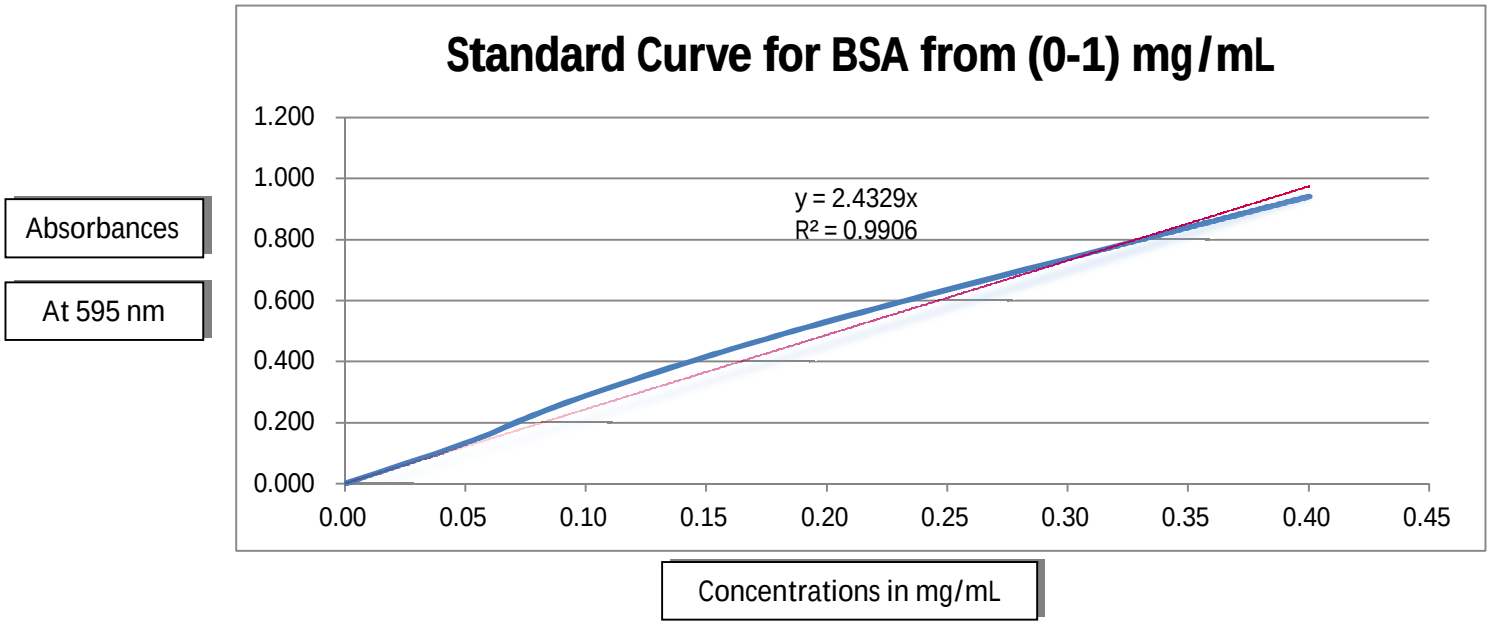
Table: (3)

Initial graph obtained as follow:



Graph: (1) Shows concentrations of BSA in mg/mL against the absorbances at 595 nm in spectrophotometer.

From the primary graph we can see that linear relationship is ended **at 0.80 mg/mL** and thus it implies that to construct the linear relation-ship, we will exclude 0.8 mg/mL and 1.0 mg/mL.



Graph: (2) Linearity cut off values for standards prepared

R value ~ 1, which means good linearity relationship. Thus our y-intercept is zero and slope is 2.4329 and linear equation is:

$$Y = 2.4329\ X$$

Another way for calculating the concentrations of protein (unknown lysozyme fractions) by:

Calculating calibration factor (F) for the assay:

$$F = \frac{Concentration\ of\ Standard}{Absorbance\ of\ Standard} = \mathbf{0.37\ (0.40)}$$

Lysozyme concentration in mg/mL = factor (F) x test absorbance

The measurement of each samples are as follow:

Sample order	Absorbance	Concentration (mg/mL)	Comments
1	0.00	0.000	Not Detected
2	0.010	0.004	Very Low Con.
3	0.010	0.004	Very Low Con.
4	0.468	0.187	High Concentration
5	**0.673**	**0.269**	**Very High Con.**
6	0.477	0.191	High Concentration
7	0.348	0.139	High Concentration
8	0.099	0.0396	Low Concentration
9	0.009	0.0036	Low Concentration
10	0.004	0.0016	Low Concentration
11	0.007	0.0028	Low Concentration
12	0.006	0.0024	Low Concentration
13	0.003	0.0012	Low Concentration
14	0.008	0.0032	Low Concentration
15	0.010	0.004	Low Concentration
16	0.010	0.004	Low Concentration
17	0.012	0.0048	Low Concentration
18	0.007	0.0028	Low Concentration
19	0.0014	0.00056	Very Low Con.
20	No sample	No Result	Dripping stop
F_0	1.248	$OD_{595nm} > 1$	Repeat/Dilute
F0 (diluted X10)	**0.792**	**0.317**	**Very High Con.**
F_1	1.007	$OD_{595nm} > 1$	Repeat/Dilute
F1 (diluted X 10)	**0.724**	**0.290**	**Very High Con.**
F_2	0.181	0.0724	High Concentration

Table (4): Show the fractions with higher total protein concentrations

❖ Observations & Conclusion:

From above table we have found that, **fraction # (4, 5, 6, and 7)** contains higher amount of total protein concentrations in mg/mL and specifically **fraction # (4)**. Moreover; **F_0>F_1>F_2** in concentrations of protein(s); while to obtain amount for F_0 and F_1 we have needed to dilute them both by **X10**. It's obvious that higher amount of lysozyme enzymes have been eluted at start point during sample fractionation due to adding of NaCl (salt) and very low fraction observed at end of fraction collection.

From **(F_0) = 0.317 mg/mL** (was diluted by X10 and X50); we can say that the initial amount of lysozyme & other protein concentrations are equal to 0.317 mg/mL x10 x 50 = **158.5 mg/mL in 30 mL of initial egg white.** *(F_0 (X50) = 3.17 mg/mL)*

From **(F_1) = 0.290 mg/mL** (was diluted by X10); we can say that the initial amount of proteins concentration is equal to 0.290 mg/mL x10 = **2.9 mg/mL in 30 mL.**

From **(F_2) = 0.0724 mg/mL**. This is obvious as its only washed elution fraction.

All other fractions been isolated and saved at + 4 °C for later analysis.

We can recover our resin solutions by eluting acid/water into it for recycling purpose.

❖ References:

I. David J. Holme, and Hazel Peck, Analytical Biochemistry. 3rd ed. Edinburgh Gate Harlow, Essex CM20 2JE, England. 1998. Pages 129-132.

II. Carl A. Burtis, Edward R. Ashwood, and David E. Bruns, Tietz textbook of clinical chemistry and molecular diagnostics. 4th ed. 11830 Westline Industrial Drive St. Louis, Missouri 63146, USA. 2006. Pages 142-143.

III. Michael L. Bishop, Janet L. Duben-Von Laufen, Edward P. Fody. Clinical chemistry-Principles, Procedures, Correlations. J.B. LIPPIN COTT COMPANY. Philadelphia. USA.1985.