Sultan Qaboos University
College of Medicine & Health Science
Instrumentation and Biomedical Techniques
BIOC 6002

Laboratory Report
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Ion exchange chromatography:
Isolation of lysozyme from egg white

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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](image)

**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 least 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>
<thead>
<tr>
<th>Types of ion exchange resin:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong anion QAE Completely ionized</td>
</tr>
<tr>
<td>Weak anion DEDE Ionization depends on pH</td>
</tr>
<tr>
<td>Strong cation SP Completely ionized</td>
</tr>
<tr>
<td>Weak cation CM Ionization depends on pH</td>
</tr>
</tbody>
</table>

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

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 $14.6 \times 10^3$ Dalton.

In this part of practical (cation exchange chromatography) was used for an easy isolation of lysozyme enzymes ($+ve$) charged with pre-swollen carboxymethyl 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 $0.5 \text{ M } \text{NaCl}$
   - 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 4 mL 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.

   \[ C_1 \times V_1 = C_2 \times V_2 \]

   \[ 0.5 \text{M} \times (?) \text{ mL} = 100 \text{ mL} \times 0.1 \text{ M} \]

   \[ \text{Then the volume needed is } (V_2) = 20 \text{ mL} \]

   *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 \( V_{\text{egg white}} = 30 \text{ mL} \).

4. We have diluted (X50) the neat sample with using 0.1 M glycine/NaOH buffer, pH = 10.

   \[ \text{X 50 dilutions done by mixing:} \]

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

   *This was our } (F_0) \text{ with } V_{F_0}=50 \text{ mL} \]

5. After that we have applied 30 mL of diluted sample into column and volume obtained was \( (V_{F_1}= 30 \text{ mL}) \) 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 \( (V_{F_2} = 75 \text{ mL}, F_2) \) 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.

   \[ \text{Volume is 100 mL then it follows that number of moles =} \]

   \[ 0.5 \text{ mol/L} \times 0.1 \text{ L} \]

   \[ = 0.05 \text{ mol} \times 58.44 \text{ g/mol} \]
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 (F₀,F₁,F₂) 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:

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

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 relationship, 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:

\[
Y = 2.4329 \times X
\]

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

Calculating calibration factor (F) for the assay:

\[
F = \frac{\text{Concentration of Standard}}{\text{Absorbance of Standard}} = 0.37 \ (0.40)
\]

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

The measurement of each samples are as follow:

<table>
<thead>
<tr>
<th>Sample order</th>
<th>Absorbance</th>
<th>Concentration (mg/mL)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00</td>
<td>0.000</td>
<td>Not Detected</td>
</tr>
<tr>
<td>2</td>
<td>0.010</td>
<td>0.004</td>
<td>Very Low Con.</td>
</tr>
<tr>
<td>3</td>
<td>0.010</td>
<td>0.004</td>
<td>Very Low Con.</td>
</tr>
<tr>
<td>4</td>
<td>0.468</td>
<td>0.187</td>
<td>High Concentration</td>
</tr>
<tr>
<td>5</td>
<td>0.673</td>
<td>0.269</td>
<td>Very High Con.</td>
</tr>
<tr>
<td>6</td>
<td>0.477</td>
<td>0.191</td>
<td>High Concentration</td>
</tr>
<tr>
<td>7</td>
<td>0.348</td>
<td>0.139</td>
<td>High Concentration</td>
</tr>
<tr>
<td>8</td>
<td>0.099</td>
<td>0.0396</td>
<td>Low Concentration</td>
</tr>
<tr>
<td>9</td>
<td>0.009</td>
<td>0.0036</td>
<td>Low Concentration</td>
</tr>
<tr>
<td>10</td>
<td>0.004</td>
<td>0.0016</td>
<td>Low Concentration</td>
</tr>
<tr>
<td>11</td>
<td>0.007</td>
<td>0.0028</td>
<td>Low Concentration</td>
</tr>
<tr>
<td>12</td>
<td>0.006</td>
<td>0.0024</td>
<td>Low Concentration</td>
</tr>
<tr>
<td>13</td>
<td>0.003</td>
<td>0.0012</td>
<td>Low Concentration</td>
</tr>
<tr>
<td>14</td>
<td>0.008</td>
<td>0.0032</td>
<td>Low Concentration</td>
</tr>
<tr>
<td>15</td>
<td>0.010</td>
<td>0.004</td>
<td>Low Concentration</td>
</tr>
<tr>
<td>16</td>
<td>0.010</td>
<td>0.004</td>
<td>Low Concentration</td>
</tr>
<tr>
<td>17</td>
<td>0.012</td>
<td>0.0048</td>
<td>Low Concentration</td>
</tr>
<tr>
<td>18</td>
<td>0.007</td>
<td>0.0028</td>
<td>Low Concentration</td>
</tr>
<tr>
<td>19</td>
<td>0.0014</td>
<td>0.00056</td>
<td>Very Low Con.</td>
</tr>
<tr>
<td>20</td>
<td>No sample</td>
<td>No Result</td>
<td>Dripping stop</td>
</tr>
</tbody>
</table>

F0: 1.248 OD<sub>595nm</sub> > 1 Repeat/Dilute
F0 (diluted X10): 0.792 0.317 Very High Con.
F1: 1.007 OD<sub>595nm</sub> > 1 Repeat/Dilute
F1 (diluted X10): 0.724 0.290 Very High Con.
F2: 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 \( X_{10} \). 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 \( (F_0) = 0.317 \text{ mg/mL} \) (was diluted by X10 and X50); we can say that the initial amount of lysozyme & other protein concentrations are equal to \( 0.317 \text{ mg/mL} \times 10 \times 50 = 158.5 \text{ mg/mL in 30 mL of initial egg white.} \ (F_0 (X50) = 3.17 \text{ mg/mL}) \)

From \( (F_1) = 0.290 \text{ mg/mL} \) (was diluted by X10); we can say that the initial amount of proteins concentration is equal to \( 0.290 \text{ mg/mL} \times 10 = 2.9 \text{ mg/mL in 30 mL.} \)

From \( (F_2) = 0.0724 \text{ mg/mL} \). 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:**


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