HiPer® Ion Exchange Chromatography Teaching Kit

Product Code: HTC001

Number of experiments that can be performed: 5

Duration of Experiment: 2 days

Day 1: Revival of strain

Day 2: Protocol: 5 - 6 hours

Storage Instructions:

- The kit is stable for 12 months from the date of manufacture
- Store the standard lysozyme at -20°C
- Store the CM-cellulose, Buffer A, Buffer B, 1M Phosphate Buffer, pH 7.0 and *Micrococcus luteus* culture at 2-8°C
- Other kit contents can be stored at room temperature (15-25°C)

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WHO GMP CERTIFIED
ISO 13485-2016 CERTIFIED

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Aim:

To purify lysozyme from chicken egg white using ion exchange chromatography and estimation of the enzymatic activity and protein concentration.

Introduction:

Chromatography is the process through which biomolecules are separated and analyzed from a complex mixture. This separation process consists of two phases: a stationary phase and a mobile phase. The mobile phase consists of the mixture to be separated which percolates through the stationary phase. Ion exchange chromatography works on the basic principle that oppositely charged particles are attracted to each other. The stationary phase consists of fixed charges on a solid support. These charges can be either negative or positive. When protein mixtures are applied to the oppositely charged, chromatographic matrix, the various proteins are bound by reversible, electrostatic interactions. The adsorbed proteins are eluted in order of least to most strongly bound molecules by increasing the ionic strength or varying the pH of the elution buffer.

Principle:

Ion exchange chromatography, also called adsorption chromatography, is based on a reversible interaction between a charged molecule in solution and an oppositely charged group on the matrix or stationary phase. These charges can be either negative or positive. Hence, there are two types of ion exchangers i.e., cation and anion exchangers. Cation exchanger contains negatively charged groups which attract positively charged molecules, e.g., Carboxymethylcellulose or CM-cellulose. Conversely, anion exchanger has positively charged groups that attract negatively charged molecules and thus separate anionic molecules. E.g. Diethylaminoethyl-cellulose. Ion-exchange chromatography separates proteins based on molecular charge. When a mixture of protein is applied to an oppositely charged chromatographic matrix various protein molecules are attracted to it by an electrostatic interaction. This is reversible binding and the adsorbed proteins are eluted in order of least to most strongly bound molecules (by increasing the ionic strength or varying the pH of the elution buffer), collected as individual chromatographic fractions, and analyzed separately. Proteins carry both negatively and positively charged groups. The net charge of the protein depends upon the pH of the solution and isoelectric point of a protein is the pH at which its net charge is zero.

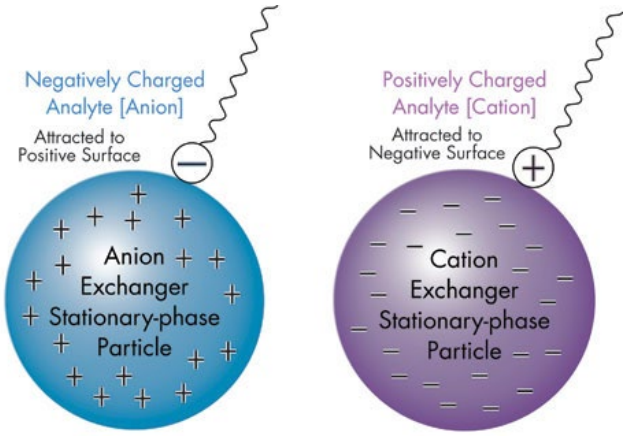


Fig 1: In ion exchange chromatography there are two types of ion exchanger: cation and anion exchanger

ionic strength or varying the pH of the elution buffer. As a result the protein mixture is separated based upon its net charge. The fractionated individual protein samples are analyzed to determine the extent of purification process, e.g. after purification of an enzyme (proteins that catalyze chemical reactions) its specific activity is determined. Specific activity is the ratio of enzyme activity to mass of protein in the sample and is expressed as units of activity per milligram of protein (U/mg). Specific activity of a particular

enzyme is increased upon purification. Therefore, the extent of purification of a certain enzyme can be determined by measuring its specific activity before and after purification.

Lysozyme is an enzyme used to break down bacterial cell wall to improve protein or nucleic acid extraction efficiency. It occurs naturally in plant and animal tissues and in secretions such as tears, saliva and mucus and is especially abundant in egg whites. Isoelectric point of lysozyme is 11 and below this pH, lysozyme has a net positive charge, resulting in its retention by a cation exchange matrix (e.g. CM-Cellulose) when most other proteins would run through due to a net negative charge. Finally, lysozyme is eluted out by increasing the concentration of cations which compete with positively charged groups of lysozyme for binding sites on the matrix. Enzyme Activity of lysozyme can be determined by using the bacterium *Micrococcus luteus*. Lysozyme lyses the bacterial cell walls and bacterial membranes break open due to osmotic shock. As a result the cloudy bacterial suspension becomes clearer and the absorbance decreases. One unit of lysozyme is defined as the amount of lysozyme that will produce a decrease in absorbance at 450 nm of 0.001 absorbance units/minute.

Kit Contents:

HiPer® Ion Exchange Chromatography Teaching Kit enables the purification of lysozyme from chicken egg white using ion exchange chromatography and subsequent estimation of the enzymatic activity of the purified lysozyme.

Table 1: Enlists the materials provided in this kit with their quantity and recommended storage

Sr. No.	Product Code	Materials Provided	Quantity	Storage
			5 expts	
1	TKC299	CM- cellulose	6 ml	2-8°C
2	TKC300	Buffer A	500 ml	2-8°C
3	TKC301	Buffer B	90 ml	2-8°C
4	TKC302	1 M Phosphate Buffer, pH 7.0	10 ml	2-8°C
5	ML008	5 M NaCl	12 ml	RT
6	TKC304	Standard Lysozyme	2 ml	-20 °C
7	TKC305	Column	1 No.	RT
8	PW143	Centrifuge Tube (50 ml)	1 No.	RT
9	M1245	Luria Bertani (LB) Broth	2.5 g	RT
10	MB053	Agar Powder, Bacteriological	1.5 g	RT

Materials Required But Not Provided:

Culture Requirement: *Micrococcus luteus*
Glass wares: Beakers, Test tubes
Reagents: Chicken Egg, Distilled water (Recommended Product Code: ML064 – Molecular Biology Grade water)
Other requirements: Column Stand, Spectrophotometer, Centrifuge, Micropipettes, Quartz cuvettes, Tips, Sterile Loops

Storage:

The HiPer® Ion Exchange Chromatography Teaching Kit is stable for 12 months from the date of manufacture without showing any reduction in performance. On receipt, store Standard Lysozyme at -20°C. CM-cellulose, Buffer A, Buffer B, 1 M Phosphate Buffer, pH 7.0 and *Micrococcus luteus* culture should be stored at 2-8 °C. Other kit contents can be stored at room temperature.

Important Instructions:

1. Before starting the experiment read the entire procedure carefully.
2. **Preparation of 0.1M Phosphate Buffer, pH 7.0:** To prepare 50 ml of 0.1M Phosphate Buffer, take 5 ml of 1M Phosphate Buffer and add 45 ml of sterile distilled water*. Mix well.
3. **Preparation of 1M NaCl:** To prepare 50 ml of 1M NaCl, take 10 ml of 5M NaCl and add 40 ml of sterile distilled water*. Mix well.
4. Mix the CM-cellulose material gently to get a uniform suspension before use.
5. The packed CM-Cellulose column can be used 2-3 times. Discard the column material after 3 uses and pack with fresh CM-Cellulose. Store at 4°C.
6. Do not let the column dry up.
7. **Preparation of Luria Bertani Broth (10 ml):** Dissolve 0.25 g of Luria Bertani broth in 10 ml of distilled water and autoclave.
8. **Preparation of LB (Luria Bertani) agar plates:** Dissolve 1.25 g of LB media and 0.75 g of agar in 50 ml of distilled water. Sterilize by autoclaving and allow the media to cool down to 40-45°C and pour on sterile Petri plates.

* **Molecular biology grade water is recommended (Product code: ML064).**

Procedure:

Day 1: Revival of Strain

1. Open the vial containing culture and resuspend the cells with 0.25 ml of LB broth.
2. Pick up a loopful of culture and streak onto LB agar plate.
3. Incubate overnight at 37^0C.

Day 2:

I. Purification of Lysozyme using CM-Cellulose:

1. Get a column stand and fix the column vertically to it. Wash the empty column with water.
2. Remove the top cap of the column first and add 2 ml of Buffer A. Let it pass through by turning on the bottom cap.
3. Turn off the bottom cap and pack the column with 3 ml of CM-Cellulose. Turn on the bottom cap and allow the buffer to flow through the column. Turn off the bottom cap to keep the column wet. Do not let the column dry up. Equilibrate the column with 25 ml of Buffer A.
4. **Preparation of Crude Egg White Extract:**
   - I. Separate the egg white from yolk of an egg by cracking the shell approximately in half and pouring the yolk back and forth between the two halves and allow the white to fall into a beaker.
   - II. To 5 ml egg white, add 35 ml of Buffer A and mix it properly.
   - III. Centrifuge the sample at 5000 rpm for 10 minutes at 4^0C.
   - IV. Collect the supernatant. Label it as **"Crude Sample"** and save 1 ml of supernatant for measurement of lysozyme activity.

5. Load 15 ml of the "Crude Sample" to equilibrate CM-Cellulose column.
6. Replace the top cap of the column and make sure to turn off the bottom cap. Incubate the column for 1 hour at room temperature with intermittent mixing.
7. After an hour, allow the column material to settle. Slowly pipette out or decant the supernatant without disturbing the column.
8. Wash the column with approximately 30-35 ml of Buffer A.
9. Elute lysozyme from the column using 15 ml of Buffer B. Collect eluates in test tubes as 2 ml fractions. Monitor OD at 280 nm and pool the fractions that show A_{280} 0.5 and above. Label this as **"Eluate"**.
10. Wash the column with 10 ml of 1M NaCl. Replace the top cap and turn off the bottom cap. Store the column at 4^0C for next use.

II. Estimation of Protein Concentration:

A. <u>Crude Sample:</u>

1. Dilute the crude sample 20 times with 0.1M Phosphate Buffer pH 7.0 (i.e. 0.1 ml of crude sample made Upto 2ml).
2. Zero the spectrophotometer against 0.1M Phosphate Buffer Blank. Measure the absorbance at A_{260} and $A_{280.}$
3. The protein concentration in crude sample can be calculated by using the following formula:
   Protein Concentration = [(1.55 x A_{280}) – (0.77 x A_{260})] x Dilution factor

   Here Dilution Factor is 20.

   Protein Concentration = ---------- mg/ml

B. <u>Eluate:</u>

1. Dilute the eluate 10 times with 0.1M Phosphate Buffer pH 7.0 (i.e. 0.1 ml of eluate upto 1 ml).
2. Zero the spectrophotometer against 0.1M Phosphate Buffer Blank. Measure the absorbance at $A_{280.}$
3. The protein concentration in Eluate can be calculated by using following formula:

$$\textbf{Protein Concentration in Test Sample} = \frac{\textbf{A}_{280} \textbf{ x Dilution factor}}{\textbf{0.06}} \quad \textbf{mg/ml}$$

Protein Concentration = ---------- mg/ml

Where Dilution factor = 10 and 0.06 is the extinction coefficient of lysozyme i.e. A_{280} of 1 mg/ml lysozyme is 0.06.

III. Estimation of Lysozyme activity:

1. Take a loopful of colonies from the revived *Micrococcus luteus* culture plate into a test tube containing 5 ml of 0.1M Phosphate Buffer, pH 7.0 such that OD_{450} is between 0.5 to 0.7.
2. Dilute required amount of standard lysozyme 1:4 i.e. (0.25 ml of standard lysozyme + 0.75 ml of Phosphate buffer to bring down the concentration to 0.25 mg/ml (from 1 mg/ml to 0.25 mg/ml)
3. Dilute the crude sample and eluate to 0.25 mg/ml based on the protein concentration estimated. E.g. If the concentration of lysozyme is 1 mg/ml; dilute it four times with phosphate buffer to bring down the concentration to 0.25 mg/ml.
4. Zero the spectrophotometer against 0.1M Phosphate Buffer Blank.
5. Take 1 ml of *Micrococcus luteus* culture in a cuvette and measure the OD_{450} against phosphate buffer blank. This is OD at '0'second.
6. To the substrate, add 100 µl of standard lysozyme (0.25 mg/ml) and note the time.
7. Mix the contents of the cuvette for 15 seconds.
8. 8 Measure the absorbance exactly after 60 seconds of addition of lysozyme.
9. Repeat the steps 5-8 for crude and eluted samples.
10. Note down the readings as in Table 2:

Table 2: OD readings of lysozyme activity

Time	**A450**		
	Standard	**Crude**	**Eluate**
0 second			
60 seconds			
Δ A450			

IV. Estimation of Specific activity of Lysozyme:

Specific activity refers to the "purity" of the sample with respect to the enzyme of interest and the total protein before and after the purification process. Specific activity is defined as the units of enzyme activity per mg total protein. Specific activity of lysozyme in crude and eluted samples is calculated by comparing the OD readings of the samples with that of standard lysozyme, whose specific activity is known.

$$\text{Activity in U/mg} = \frac{A_{450}/\text{min. of the test} \times \text{activity of the standard}}{A_{450}/\text{min. of the standard}}$$

Where A_{450} is the difference in A_{450} between 0 sec and 60 sec

Activity of standard lysozyme is ≅ 30,000 U/mg

Estimation of Fold Purification:

$$\text{Fold Purification} = \frac{\text{Specific activity of eluted sample}}{\text{Specific activity of crude sample}}$$

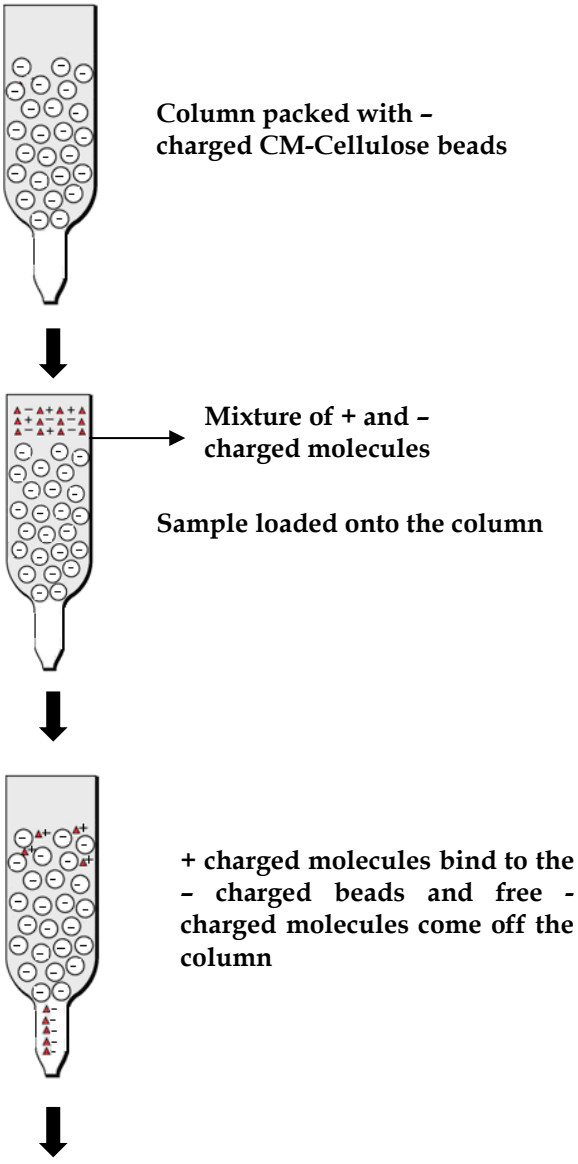
This is a measure of efficiency of purification of lysozyme using ion exchange chromatography.

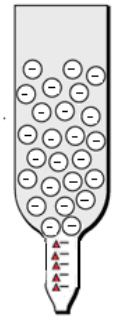
Estimation of Yield:

Yield = Protein concentration of eluate x total volume of eluate

= ---------- mg

Flowchart:





Bound molecules come out with the elution buffer

Observation and Result:

Calculate and tabulate fold purification of lysozyme as shown in table 3:

Table 3:

Fraction	A	B	C	D	E
	Total Volume	Protein Concentration (mg/ml)	Total Protein (mg) A x B	Lysozyme Specific Activity (U/mg)	Total Yield (U) C x D
Crude Sample					
Eluate					

Interpretation:

The lysozyme was effectively purified from chicken egg white proteins in a single step with reasonable yield and purity. The protein concentration was calculated and lysozyme activity was estimated by *Micrococcus luteus* assay.

Troubleshooting Guide:

Sr. No	Problem	Possible Cause	Solution
1	Column is clogged	The packing was not done properly	Mix the CM-cellulose slurry properly before packing the column. Make sure that there is no air gap
2	Improper purification of protein	The column dries up	Make sure the column never dries up. Store the column in fridge when not in use
3	Flow through of the column is very poor	Improper column packing	Use a fresh column

Technical Assistance:

At HiMedia we pride ourselves on the quality and availability of our technical support. For any kind of technical assistance mail at mb@himedialabs.com

Symbol:

	Manufacturer		Do not use if package is damaged
	Batch code		Temperature limit
	Date of manufacture (YYYY-MM)		Consult instructions for use
	Use-by date (YYYY-MM)		Catalogue number

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