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BIOCHEMISTRY

faculty of medicine - JU2017

Sheet ○ Slides

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A QUICK RECAP

Isoelectric point: is the pH at which a molecule(charged) carries no **net** charge or is electrically neutral. It can be a used as detection of the protonation state of any group. if the pH is higher than pH(I) then the group is unprotonated and vice versa.

Protein analysis

There are series of processes intended to isolate one or a few proteins from a complex mixture, usually cells after destruction. In order to be further studied for the characterization of the function, structure and interactions of the protein of interest, those processes may involve separation of protein and non-protein parts of the mixture and then separate the desired protein (protein of interest) from all other proteins. Proteins can be purified on the basis (separation steps depend on):

- **Solubility**
- **Charge**
- **Size**
- **Specific binding affinity**

Salting in / salting out

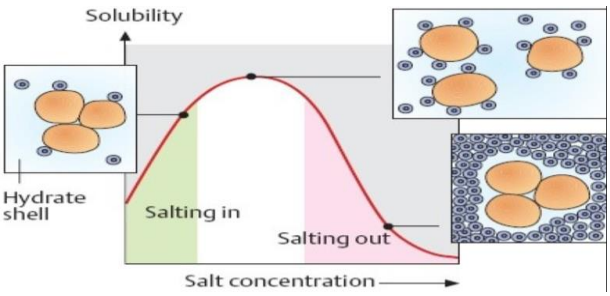
This technique allows to separate proteins **NON-selectively**.

Each protein is composed of a various sequence of amino acids, so the solubility in water differs depending on the hydrophobic and hydrophilic properties of the surface, however in general proteins are considered to be poorly soluble in pure water.

In a solution of water and proteins the solubility of proteins is low since majority of water molecules tend to interact with each other via hydrogen bonds, the rest can also bind with protein molecules through H-BONDS. So as **electrostatic forces** are stronger than **Dipole-Dipole interactions** proteins tend to aggregate. That's why proteins are poorly soluble in water.

Salting in: a process by which we can increase the solubility of a certain protein by adding salt to the solution in a low salt-concentrated environment.

seems confusing and contradictory!!!
it's simple --- the solution must be low salt-concentrated in order for the previous concept (**salting in**) to apply. Otherwise if the solution has a high Conc. Of salt, adding more salt leads to precipitation of proteins.



Salt molecules stabilize protein molecules by decreasing the **electrostatic energy** between the protein molecules which increase the solubility.

in **salting in** method, we keep on adding salt until we reach a point (the peak in the previous figure) after which adding more salt won't be able to dissolve more proteins, proteins will start to aggregate and precipitate instead. **Why!!**
Because the ions of salt compete with the protein molecules in binding with water. In this case, the protein molecules tend to associate with each other because protein-protein interaction becomes energetically more favorable than protein-solvent interactions.

that leads us to the process which is called **salting out,** a purification method that relies on the basis of protein solubility and can be used to fractionate proteins.

NOTES

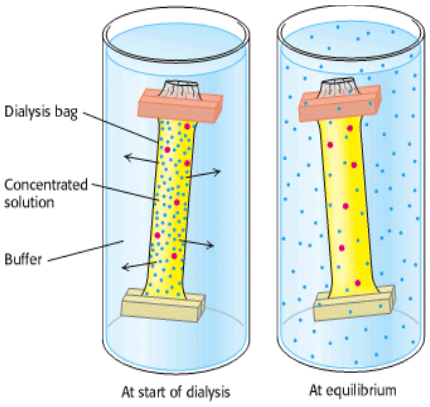
1) **proteins have different solubilities and therefore have different Conc. of salt needed for precipitation.**
2) **all proteins are soluble at very low concentration of salt.**
3) **dirty technique: when precipitation occurs, bulky groups of proteins are present and we can't get a purified protein → crude extract.**
4) **Assays and experiments are more likely to be affected because of the presence of a high salt Conc.**

DIALYSIS

A separation **technique** that facilitates the removal of small, unwanted compounds from macromolecules in solution by diffusion through a semi-permeable membrane.
Very simple, we put a high-concentrated solution containing the proteins of interest in a closed bag,
And put the bag in a container filled with a low salt conc. solution. By diffusion small molecules (have a diameter less than pore diameter) move toward outside.
disadvantages:

- **Large number of larger proteins will still exist.**
- **Smaller proteins of significance are lost.**

So, this technique leads also to dirty(crude) results.



Chromatography

Separation of molecules present in a liquid or gaseous environment (mobile phase) via passing through a column (long tube) that contains an immobile phase (stationary phase). The proteins are separated into **fractions**.

At present, there are a number of chromatographic methods that can be utilized in purifying protein samples. These include the following:

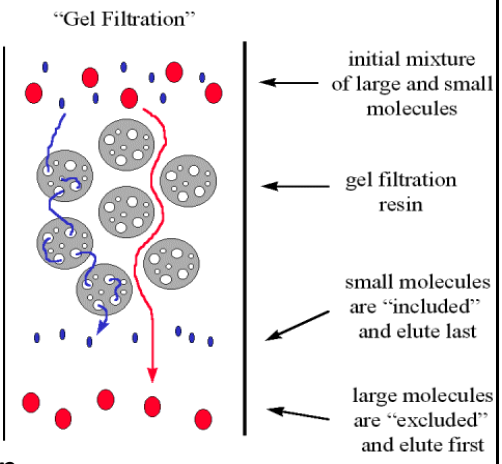
- Gel filtration chromatography.
- Ion-Exchange chromatography.
- Affinity chromatography.

Each method of the above separate proteins based on specific characteristics.

Chromatography is based on the principle where molecules in mixture applied onto the surface of a stationary phase (stable phase) are separating from each other while moving with the aid of a mobile phase. The factors that affect this separation process include molecular characteristics related to adsorption (liquid-solid), partition (liquid-solid), and affinity or differences among their molecular weights.

1) Gel filtration (size-exclusion chromatography):

The basic principle of this method is to separate macromolecules based on their differences in molecular **sizes**.

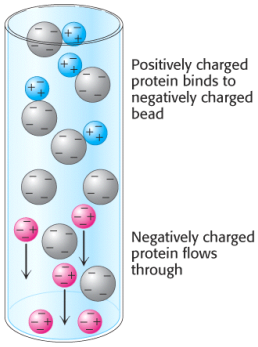


the stationary phase is porous beads, the pores are continuously connected to each other making a continuous track. So, when a molecule leaves the first bead it faces another pore in the adjacent bead and so on. This mostly happens with smaller molecules, large molecule flow more rapidly and come out first. (they don't enter the beads because they don't fit in the pores, rather they slide around the beads) **the smaller the molecule is the longer it takes to get out of the column.**

2) Ion-Exchange chromatography (IEX):

In this method, different types of proteins are separated based on their net charge. As such, columns can either be prepared to facilitate both anion exchange and cation exchange.

in this process we have 2 types of exchangers:



- **Cationic-exchange chromatography [separate +ve]**

  - the beads are negatively charged, Proteins with -ve charges are **washed out** as they don't bind with beads. proteins that have a net positive charge will tend to emerge first, followed by those having a higher positive charge density.
  - A positively charged protein bound to such a column can then be **Eluted** (released) by adding increasing concentrations of a salt like sodium chloride as sodium ions compete with positively charged groups on the protein for binding to the column.

When you increase the conc. Of the salt the protein with the least # of +ve (lowest isoelectric point relative to the group of proteins) will be eluted first followed by the one with slightly more +ve and so on. The last protein to be eluted is the one with the highest isoelectric point (most number of +ve).

Can you think of other ways of elution!!

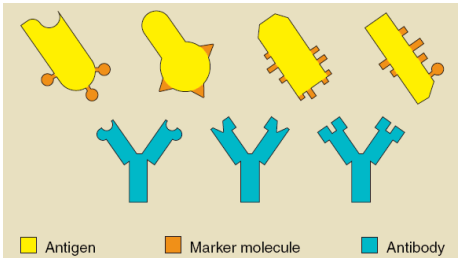
- **Anionic-exchange chromatography [separate – ve]**
  opposite to the previous one no need to clarify.
  ***Diethylaminoethyl-cellulose column* is an example [mentioned in slides]**

  ****washing out: removing the proteins that are not bound.**
  ****Elution: removing the proteins that are bound.**

See slide #15, an important problem waiting you. For soln. watch LEC 27 [28:00]

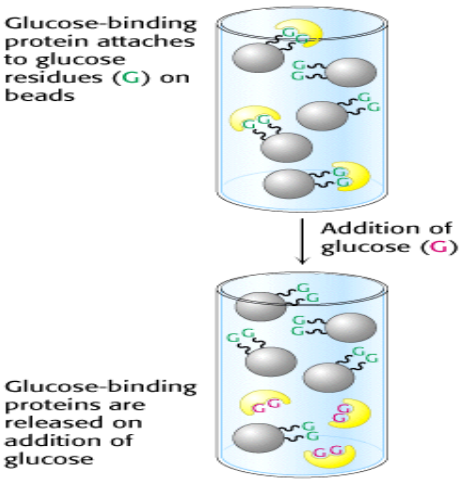
3) Affinity chromatography:

Considered to be the most selective chromatography technique, affinity chromatography is known to give the purest results as it depends on specific protein-protein interactions as well as using antibodies which are highly specific. Therefore it's used in completing the protein purification process. Since different types of proteins exhibit highly specific interactions with particular ligands under favorable conditions, the target protein can then be adsorbed from the extract as it passes through the column while the other substances will simply be washed out. **Affinity chromatography** is most effective when the interaction of the protein and the molecule that is used as the bait (trap), an anti-body, is highly specific.



Example

- The plant protein concanavalin A, which binds to glucose with high affinity, can be purified by passing a protein mixture through a column of beads attached to glucose residues.
- Concanavalin A, but not other proteins, binds to the beads.
- The bound concanavalin A can then be released by adding a concentrated solution of glucose.
  (the interactions between conA and glucose are noncovalent) the same with proteins and antibodies so these interactions can be disrupted by changing the ph.



Gel Electrophoresis

A molecule with a net charge moves in an electric field. this phenomenon, termed electrophoresis, offers a powerful means of separating proteins. In gel electrophoresis, proteins are separated as they move through a gel, which serves as a molecular sieve.

TO BE CONTINUED... つづく

TEST YOUR KNOWLEDGE

1) To elute target proteins from an affinity chromatography matrix, which of the following conditions would be the most appropriate?

A) Low salt concentration
B) Low salt concentrations
C) Adding a soluble ligand which competes with the affinity tagged protein for binding to the column
D) Just keep washing buffer through the column, isocratic elution

2) What is the starting point for selection of a suitable IEX matrix for purification of a recombinant protein?

A) Prediction of isoelectric point (pI) from the amino acid sequence
B) Test protein binding to an IEX matrix at a range of pHs and salt concentrations
C) Test protein binding to a selection of anion and cation exchange matrices
D) Pass your sample through a preparative column and elute with a salt gradient

3) For the study of a protein in details, an effort is usually made to first:

A) Conjugate the protein to a known molecule
B) Determine its amino acid sequence
C) Determine its molecular weight
D) Purify the protein

4) In a mixture of the four proteins listed below, which should elute second in size-exclusion(gel-filtration) chromatography?

A) Cytochrome C [Mw = 13,000]
B) immunoglobulin G [Mw = 145,000]
C) ribonuclease A [Mw = 13,700]
D) RNA polymerase [Mw = 450,00]

5) which one of the following is incorrect:

A) The separation in gel-filtration chromo. Is based on size, shape and net charge of a protein
B) In ion exchange chromo. , the bound proteins are eluted using NaCl solution
C) In affinity chromo. , the binding of a protein to a ligand is by specific non-covalent interaction
D) All of the above

6) Mobile phase can be:

A) ONLY SOLID
B) ONLY GAS
C) SOLID OR LIQUID
D) LIQUID OR GAS

7) Which of the following statements about column chromatography is correct?

A) Resolution increases as the length of the column increases
B) Mobile phase is a porous solid material with appropriate chemical properties held in the column
C) Stationary phase is a buffered solution that percolates through mobile phase
D) Large proteins emerge from the column sooner than small ones

8) Your "assignment" is to purify the enzyme "BC-1ase". It is an enzyme found in the cytosol of yeast cells. Its molecular weight is 25,000 Da; its pI = 5.1. we added 2.5 M of $(NH_4)_2SO_4$ for a fractional precipitation [salting out].

I. The precipitate from previous step is dissolved in buffer, pH=7.0. The high salt concentration is removed by passing the solution through a gel filtration column, the protein is expected to:

A) elute from the column after the residual salt
B) elute before the residual salt.
C) stick to the column
D) Any of the above choices are possible.

II. The desalted protein solution from the gel filtration column is next applied to an ion exchange column. The best results are expected from a column that contains:

A) anion exchange resin, pH 7.0
B) anion exchange resin, pH 3.0
C) cation exchange resin, pH 7.0.
D) cation exchange resin, pH 10.0

Answers: [seek explanations from UR 8a6a3a friend]

1	2	3	4	5	6	7	8	
							I.	II.
C	A	D	B	A	D	A	B	A

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