Size Exclusion Chromatography

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Protein Purification - Why?

- For biochemical characterization.
- For use in another biological assay.
- For the synthesis of a biological material.
- To study the crystal structure and to devise novel inhibitors as drug candidates.
- For Therapeutic application.
- For Immunochemical work.

How pure you need? Be realistic.

- You may not need highly purified proteins for some work such as a coupled assay, synthesis of some biomolecules etc. In these cases, a partially purified enzyme is good enough as long as there is no interfering material is present.
- For other work, you may need highly purified preparation. These include medical application, biochemical and molecular characterizations, crystallographic work, and immunology work.

Approach -Find a unique property of the protein.

- Knowledge on size, charge, location, affinity and molecular interaction for a specific material or any other unique property is highly useful in selectively purifying a protein.

Different protein purification techniques

- Salting out (also use of organic solvents).
- Ion exchange chromatography.
- Hydrophobic chormatography.
- Size exclusion chromatography.
- Chromatofocusing.
- Isoelectric focusing.
- Affinity chromatography.
- Preparative gel electrophoresis.

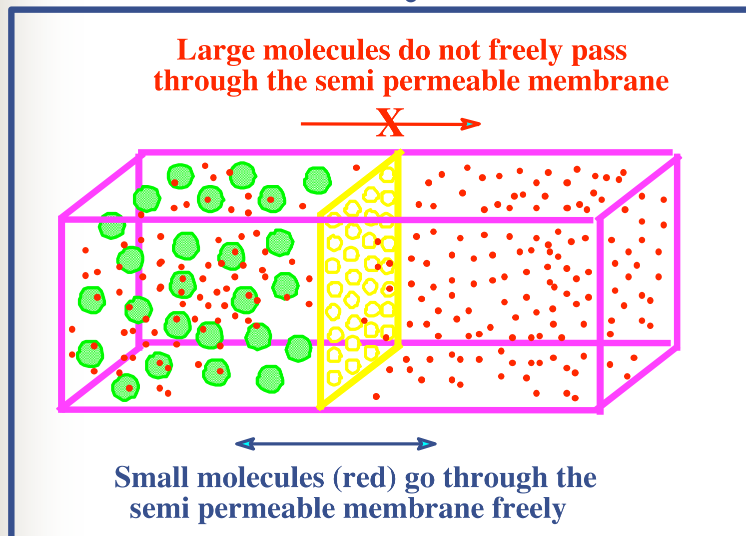
Size

- Most proteins have a molecular weight in the range of 10,000 to 100,000.
- Rarely some proteins fall out side this range.
- If it is too small you can make use of this property and purify that protein.
- If it is too big, you could use molecular sieve chromatography.
- Even proteins in 10,000 - 100,000 m.wt range can be resolved on size exclusion chromatography.

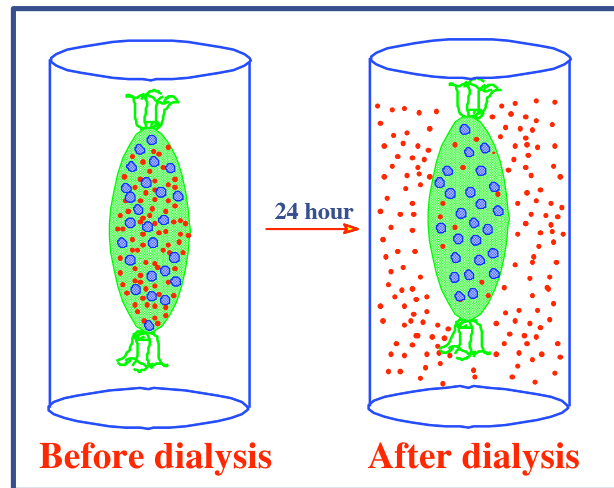
Size exclusion chromatography/ Molecular sieve chromatography

- Based on the principles of dialysis.
- A semi permeable membrane will allow a small molecule to go across the membrane but will prevent the passage of a large molecule.

Dialysis



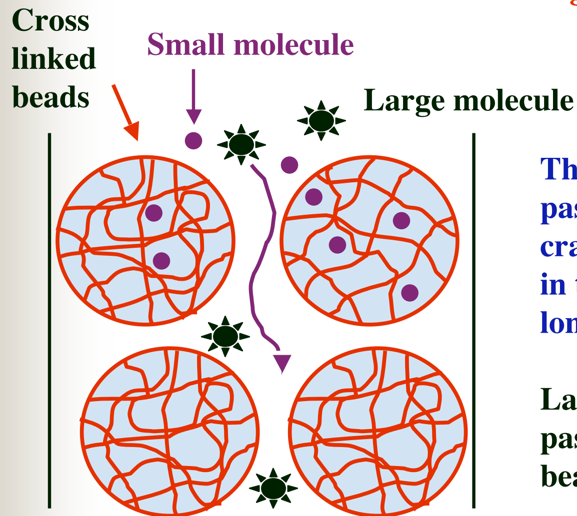
Dialysis



Porous beads made from insoluble but highly hydrated polymers such as dextran, agarose, (carbohydrate polymers) and polyacrylamide polymer are routinely used as column materials.

- Commercial polymers are: Sephadex, Sepharose, Sephacryl, Bio-gel.
- Typically they are 100 µm in size and are crosslinked.
- They will allow small molecules to go through them. Large molecules cannot enter the gel. So they go in between the space and come out fast.

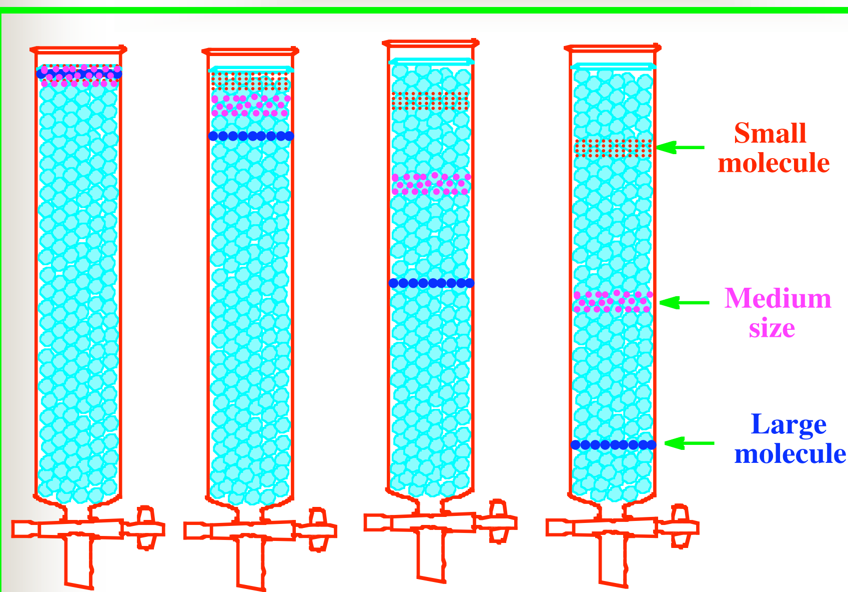
Molecular sieve (or size exclusion) chromatography



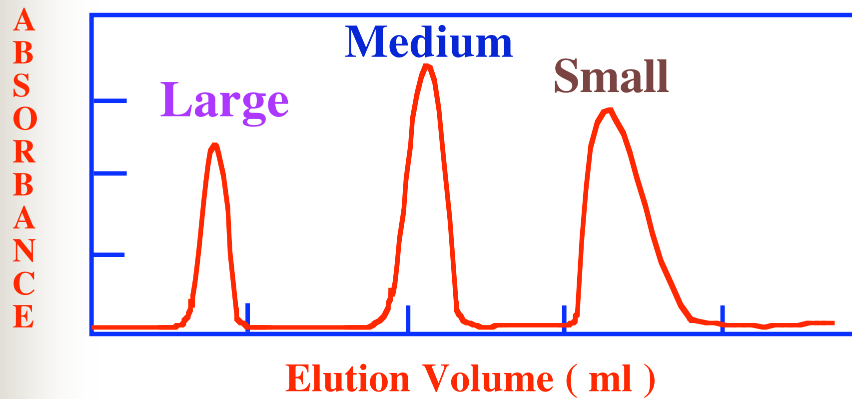
The small molecules pass through the cracks and crevices in the bead taking a longer path.

Large molecules pass in between the beads and travel fast

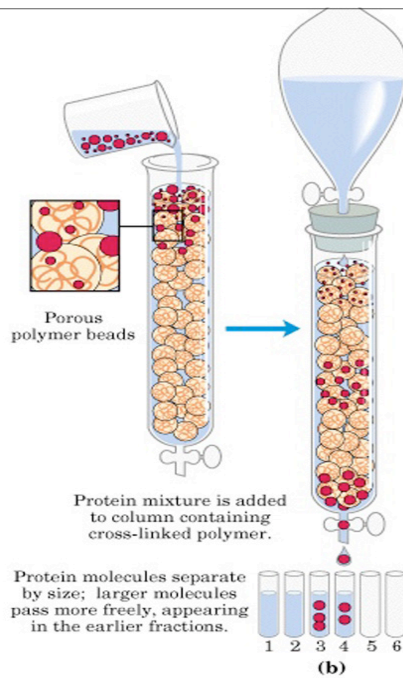
Size exclusion chromatography



Chromatographic Separation



Example:



Uses of molecular sieve chromatography

- 1. Separation of proteins based on molecular size.
- 2. Estimation of approximate molecular weight of proteins.
- 3. Establishing the binding between two molecules (and even monitoring the weak binding between ligand & macromolecule).
- 4. Desalting protein samples.

Size exclusion chromatography - Use 1

- Separation of proteins based on their molecular weight.

Size - Too small - Protease inhibitors

- Get ride of all high molecular weight stuff by size exclusion chromatography and use low molecular weight fraction for purification.
- Or, use a dialysis membrane that will allow 5000 and lower M.Wt molecules to dialyze out. high molecular weight materials are retained inside. Collect the material outside the dialysis bag for further purification.

Purification of Protease inhibitor

The purification of two low molecular weight protease inhibitors by size exclusion chromatography as the last step is shown here.
The molecular weight of Peak A is 10,000 and that of Peak B is 5,000.

Step	Total Volume (ml)	Total activity (U)	Total Protein (mg)	specific activity (U/mg)	Yield (%)	Fold purification
Crude	315	19350	2325	8.4		
HClO4	335	19698	146	134.9	100	16
Am.SO4	26	14716	73.6	200	75	24
Trypsin Sepharose	57	9206	10.2	902.5	47	107
Sephadex-G 75 A	13.6	5304	3.7	1433	27	171
Sephadex G 75 B	10	4000	1.9	2105	20	251

Size -Large -Arylphorin

- Arylphorin from insect hemolymph (=blood) has a M.Wt of 600,000.
- It is easily purified by molecular sieve chromatography.
- Location is also an advantage in this case. This protein is the major hemolymph protein at the last larval instar of most insects. So purifying from this stage is a simple one step process.
- Ref: S. J. Kramer et al., Insect Biochem. 1, 279-288 (1980).

About 90% of the proteins in blood at this this stage is arylphrin

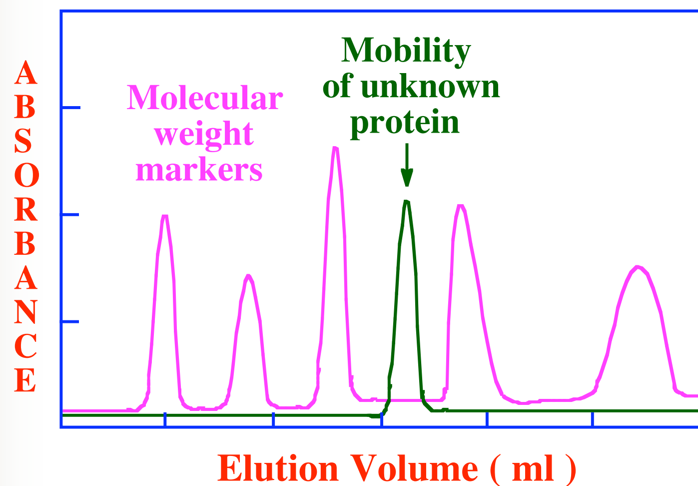
Size - Large -
Pyruvate dehydrogenase complex

- Pyruvate dehydrogenase complex of *E.coli* has a M. Wt of 2.4 million. When the cells are burst opened, they can be easily collected by centrifugation as they are practically insoluble.

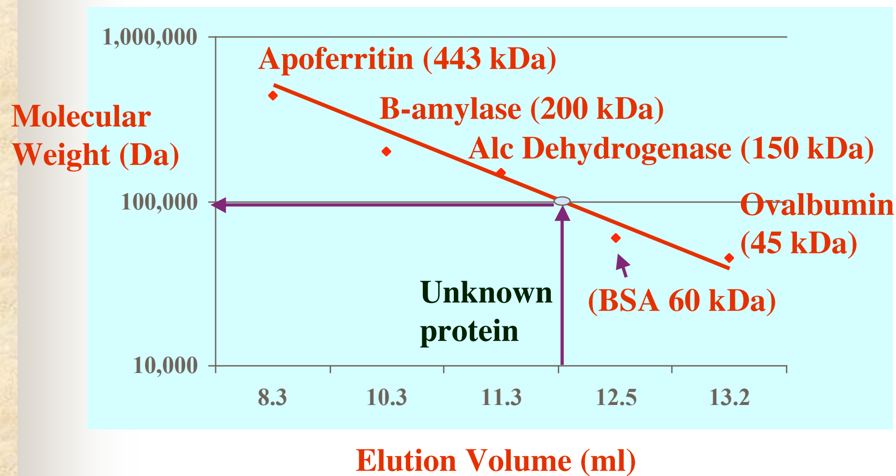
Size exclusion chromatography Use -2

- **Estimating the approximate molecular weight of proteins based on their mobility on size exclusion columns.**
- **Determine the elution profile of known molecular weight marker proteins on a size exclusion column first. Then find out the elution volume of the protein whose m.wt. needs to be determined. By comparing the mobilities, one can predict the approximate molecular weight of the unknown protein.**

Molecular weight estimation



Molecular weight estimation by size exclusion chromatography



Elution Volume (ml)

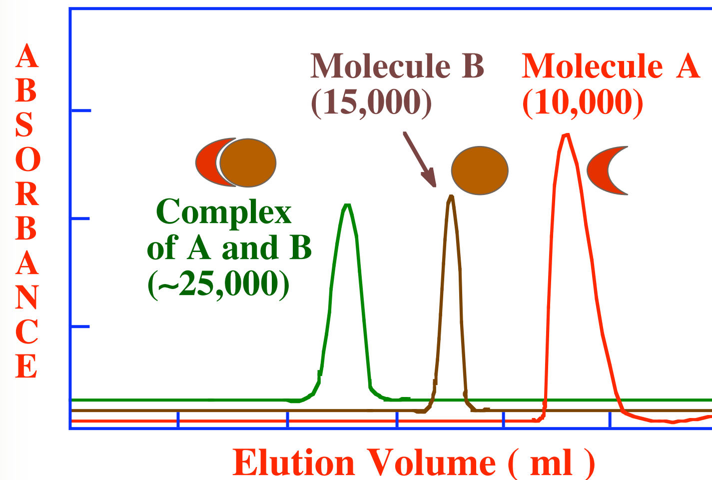
Size exclusion chromatography Use -3

- Determining the molecular interaction between two molecules (at least one has to be a macromolecule).

Size exclusion chromatography can be used to determine if two molecules are interacting.

- If a protein of 10,000 m.wt. forms a complex with a protein of 15,000 m.wt., then the complex will elute with an approximate molecular weight of 25,000.
- These three components can easily be resolved on a molecular sieve column.
- Thus under very gentle conditions, one can determine the molecular interactions.

Complex formation between two macromolecules can be monitored by molecular sieve chromatography.

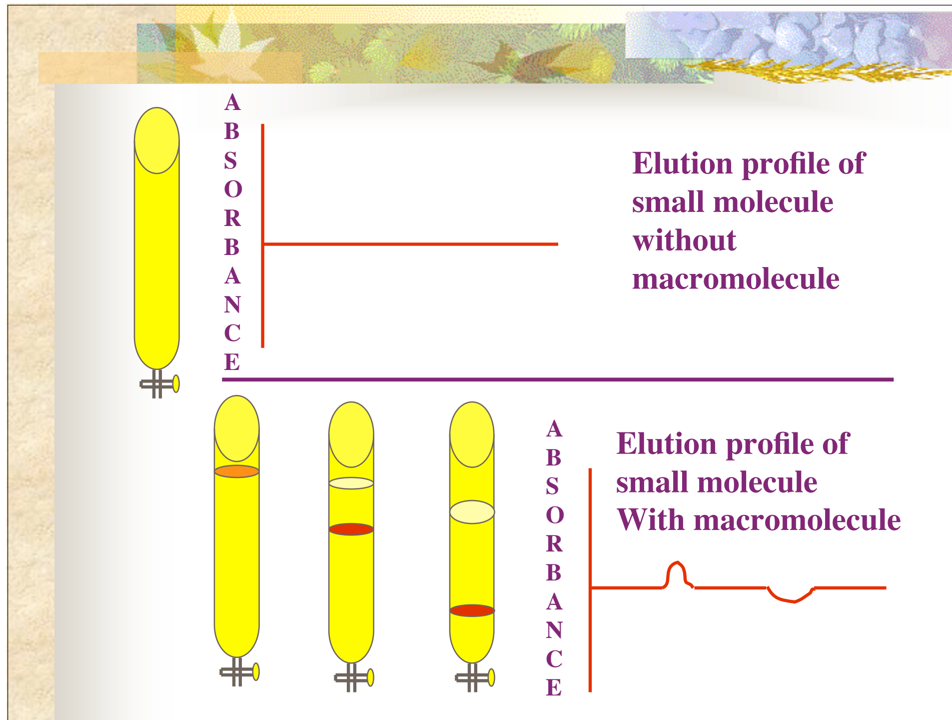


Even a molecular interaction between a small molecule and a protein can be determined on a size exclusion chromatography.

- **The complex formed between a small molecule and a protein does not have significantly different molecular weight from the protein (say 100 m.wt compound and a protein 10,000 m.wt = complex m.wt is approximately 10,100).**
- **Due to diffusion, both the protein and the complex will elute almost at the same place.**
- **However, performing size exclusion by a different mode will allow the detection of the complex.**

Complex formation between a small ligand and a large protein

- **Equilibrate the size exclusion column with buffer containing the small molecule, whose absorbance can be monitored.**
- **Chromatograph the protein in this buffer.**
- **When the protein forms complex with the small molecule, there will be a concentration drop. As the protein moves fast on the column relative to the small molecule, it will drag the bound small molecule. Hence, when the large w.wt. protein comes out of the column, there will be more of the small molecule. There will be a dip in concentration when the small molecule comes out.**



Size exclusion chromatography Use - 4

- **Desalting protein solutions.**
- **This technique is useful for the removal of excess salt in protein solution as well as buffer exchange.**

Desalting of Proteins

- Often protein solutions have salts that need to be removed.
- Desalting on size exclusion chromatography offers a convenient way to remove salts.
- The large molecular weight proteins are easily separated from small molecular weight salts by gel filtration on Sephadex columns.

Problems associated with size exclusion chromatography

- Diffusion makes it difficult to get the materials in a concentrated form. Some times it does affect even the separation.
- Proteins exhibiting binding to the matrix (or to each other) will exhibit abnormal elution profile.
- Tedious and time consuming.

separations

- Here separation of 1) hemoglobin and blue dextran and 2) riboflavin and hemoglobin are shown in two columns.

References

- 1. **Protein Purification: Principles and practice. Robert Scopes. Springer-Verlag New York., Third edition, 1994.**
- 2. **Protein purification methods: A practical approach. Edited by E.L.V. Harris and S. Angal. IRL Press, 1994**
- 3. **Protein Purification: Bench top techniques. I. M. Rosenberg, Birkhauser, Boston. 1996**