Chromatography

Lecture-3

- 3 Hydrophobic Interaction Chromatography (HIC)
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3– Hydrophobic Interaction Chromatography (HIC)

Hydrophobic interaction chromatography is a useful tool for the **purification of proteins,** providing a powerful complementary separation step to **ion-exchange and gel filtration chromatography.** It has also shown much promise as **a simultaneous protein purification and refolding step**. **As hydrophobic interactions are strongest at high ionic strengths**, this approach is often used fairly early in downstream bioprocessing**, directly after precipitation steps when salt levels are high, or in combination with gel filtration or ion-exchange chromatography.** Although hydrophobic interaction chromatography is an adsorptive process, the **interactions are generally weaker than in affinity and ion-exchange chromatography**, thus resulting in minimal structural damage and loss of activity to the target protein. Hydrophobic interaction chromatography is also very useful in the removal of DNA impurities and for purifying DNA-based products.

4– Gel Filtration Chromatography (*Gel filtration GF)*

Gel filtration chromatography does not involve bind–elute chromatography but rather separates proteins on the basis of size through porous beads. Large molecules are not able to enter the pores and elute first whereas small molecules elute later. In order for gel filtration chromatography to separate entirely on the basis of size alone, there must be no interaction between the matrix and the molecules in the mobile phase.

Gel filtration chromatography has been used for over 50 years and is primarily used as a polishing step late in downstream processing where the feed stream is relatively pure compared with earlier purification steps. Gel filtration is mainly used at the industrial scale as a final chromatography step to remove protein aggregates and small impurities and has a low loading capacity and low throughput.

Size differences between proteins can be exploited in gel filtration GF also known as molecular size-exclusion chromatography which more closely describes the separation mechanism. The size-exclusion medium consists of a range of beads with slightly differing amounts of cross-linking and therefore slightly different pore sizes.

GF is simple to use and allows separation of substances with differences in molecular size, under mild conditions. can be used for protein purification (Fig. 2.6) or for group separation in which the sample is separated in two major groups (Fig. 2.7). Group separation is mainly used for desalting and buffer exchange of samples.

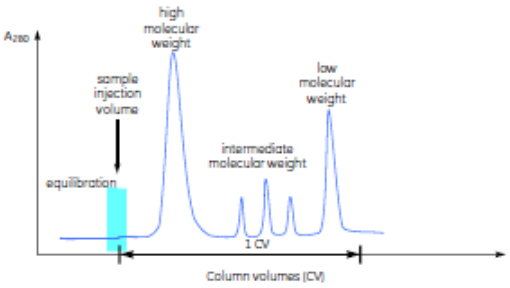

Fig 2.6. Principles of GF purification.

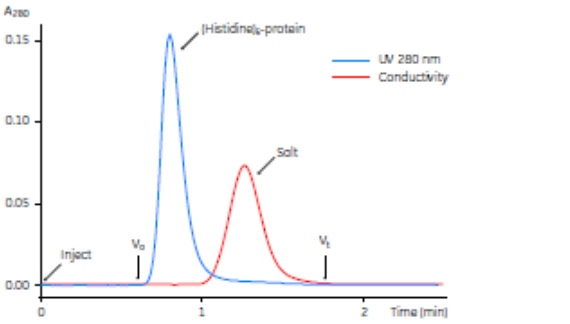

Fig 2.7. Typical example of group separation used for desalting of protein.

GF is a non-binding method (Fig 2.5), which means that no concentration of the sample components takes place. In fact, the sample zone is broadened during the passage through the column, resulting in dilution of the sample. The loaded sample volume must be kept small. In preparative GF, maximum resolution can be obtained with sample volumes of 0.5% to 2% of the total column volume; however, up to 5% may give acceptable separation. Even larger samples volumes can be appropriate if the resolution between target protein and the impurities to be removed is high. To increase capacity, the sample can be concentrated before GF. Avoid concentrations above 70 mg/ml, because

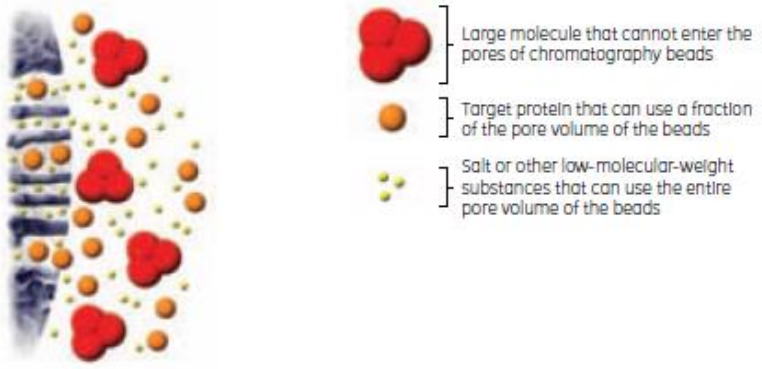

Fig 2.5. Schematic depicting GF.

viscosity effects may cause severe band broadening (so-called viscous fingering) that reduces the resolution.

Sample components are eluted isocratically (single buffer, no gradient). Separation can be performed within a broad pH, ionic strength, and temperature range, and the medium accepts a variety of additives: co-factor, protein stabilizers, detergents, urea, and guanidine hydrochloride. The buffer composition does not usually affect resolution, although including a low concentration of salt, for example, 25 to 150 mM NaCl, is recommended to eliminate any weak electrostatic interactions between proteins and the GF matrix. Buffer conditions are selected to suit the sample type and to maintain target protein activity, because the proteins are transferred to the buffer used for equilibration of the column. Equilibration buffer can thus be selected according to conditions required for further purification, analysis, storage, or use. The selection of chromatography medium is the key parameter for optimization of resolution in GF.

- The loading sample volume is the most important factor for high resolution.
- Select conditions that maintain target protein stability and that are suitable for subsequent work.
- Capacity can be increased by concentrating the sample.
- Resolution can be increased by lowering the flow rate.
- Avoid sample volumes larger than 4% of the total column volume when separating proteins by GF. Group separations allow sample volumes of up to 30% of the column volume.
- Avoid protein concentrations above may severely affect resolution.

GF is a powerful method for purification of proteins that have passed one or several initial purification steps. After those steps, the material has been concentrated and bulk impurities have been removed. GF can now be used to remove remaining impurities; it will also remove oligomers or aggregates of the target protein. The purified target protein obtained after GF will thus also be

homogeneous in size. GF is rarely used as a first purification step, but can be useful for small samples with moderate complexity.

The separation process depends on the different abilities of the various proteins to adsorb to some, all or none of the cavities on the beads, which relates the retention time on the resin to the size of the protein. The method has limited resolving power, but can be used to obtain a separation between large and small protein molecules and therefore be useful when the protein of interest is either particularly large or particularly small.

- A major advantage is that this method is very gentle on proteins and is often used as a final stage in preparations destined for protein crystallography and other applications that require functional protein.
- Size-exclusion chromatography can also be used to determine the relative molecular mass of a protein.
- Furthermore, owing to the large size difference between inorganic ions and proteins, it is frequently used for desalting of protein solutions.

An important parameter to consider for size-exclusion chromatography is the concentration of the analyte; it should be as high as practicably possible. The higher the concentration the better the resolution due to reduced diffusion. Large proteins or protein complexes will pass through the medium and elute first, therefore it is very important to thoroughly equilibrate the column before use. Some amount of material will be lost in a column through dilution and surface interactions; therefore, it is important to select the appropriate column size.

Example 5.2 **ESTIMATION OF RELATIVE MOLECULAR MASS**

Question The molecular mass of a protein was investigated by exclusion chromatography using a Sephacryl S300 column and using aldolase, catalase, ferritin, thyroglobulin and Blue Dextran as standards. The following elution data were obtained:

Protein	Molecular mass M (kDa)	Retention volume V (cm^3)
Aldolase	158	22.5
Catalase	210	21.4
Ferritin	444	18.2
Thyroglobulin	669	16.4
Blue Dextran	2000	13.6
Unknown		19.5

What is the approximate molecular mass of the unknown protein?

Answer A plot of the logarithm of the molecular mass of individual proteins versus their retention volume has a linear section from which it can be estimated that the unknown protein with a retention volume of 19.5 cm^3 has a molecular mass of 330 kDa.