2 Liquid chromatography of biomolecules

Proteins, peptides, DNA, RNA, lipids, and organic cofactors have various characteristics such as electric charge, molecular weight, hydrophobicity, and surface relief. Purification is usually achieved by using methods that separate the biomolecules according to their differences in these physical characteristics, such as ion exchange (Sect. 2.1), gel filtration (Sect. 2.2), and affinity chromatography (Sect. 2.3).

2.1 Ion exchange chromatography

In ion exchange chromatography, the stationary solid phase of the chromatographic column (or sheet or other device) commonly consists of a resin with covalently attached anions or cations. When the sample, dissolved in water or another liquid, is run over the column, sample ions of the opposite charge are attracted to the stationary ions of the column by electrostatic forces and weakly bind to them. Adsorbed sample components are then eluted by application of a salt gradient which gradually desorbs the sample molecules in order of increasing electrostatic interaction with the ions of the column (Figs. 2.1 – 2.3). Because of its excellent resolving power, ion exchange chromatography is probably the most important type of chromatographic methods in many protein preparations.

The choice of ion exchange resin for the purification of a protein largely depends on the isoelectric point, pI, of the protein. At a pH value above the pI of a protein, it will have a negative net charge and adsorb to an anion exchanger. Below the pI, the protein will adsorb to a cation exchanger. For example, if the pI is 4 then in most cases it is advisable to choose a resin which binds to the protein at a pH > 4. Since at pH > 4 this protein is negatively charged, the resin has to be an anion ion exchanger, e.g., DEAE. One could also use a pH < 4 and a cation exchanger, but many proteins are not stable or aggregate under these conditions. If, in contrast, the protein we want to purify has a pI = 10, it is positively charged at usually suitable conditions for protein ion exchange chromatography, i.e., at a pH around 7. Thus, in general for this protein type we have to choose a cation ion exchange resin, e.g., CM, which is negatively charged at neutral pH.

The capacity of the resin strongly depends on the pH and the pI of the proteins to be separated (Fig. 2.4; Table 2.1), but also on the quality of the resin, the applied pressure, and the number of runs of the column (Fig. 2.5). To improve the life of the resin, it should be stored in a clean condition in the appropriate solvent and not be used outside the specified pH range and pressure limit.

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For the separation of some enzymes which may lose their activity by contact with metals in the wall of stainless steel columns, glass-packed columns may be more appropriate. The chromatographic resolution mainly depends on the type of biomolecules, type and quality of the resin, ionic strength gradient during elution, temperature, and the geometry of the column.

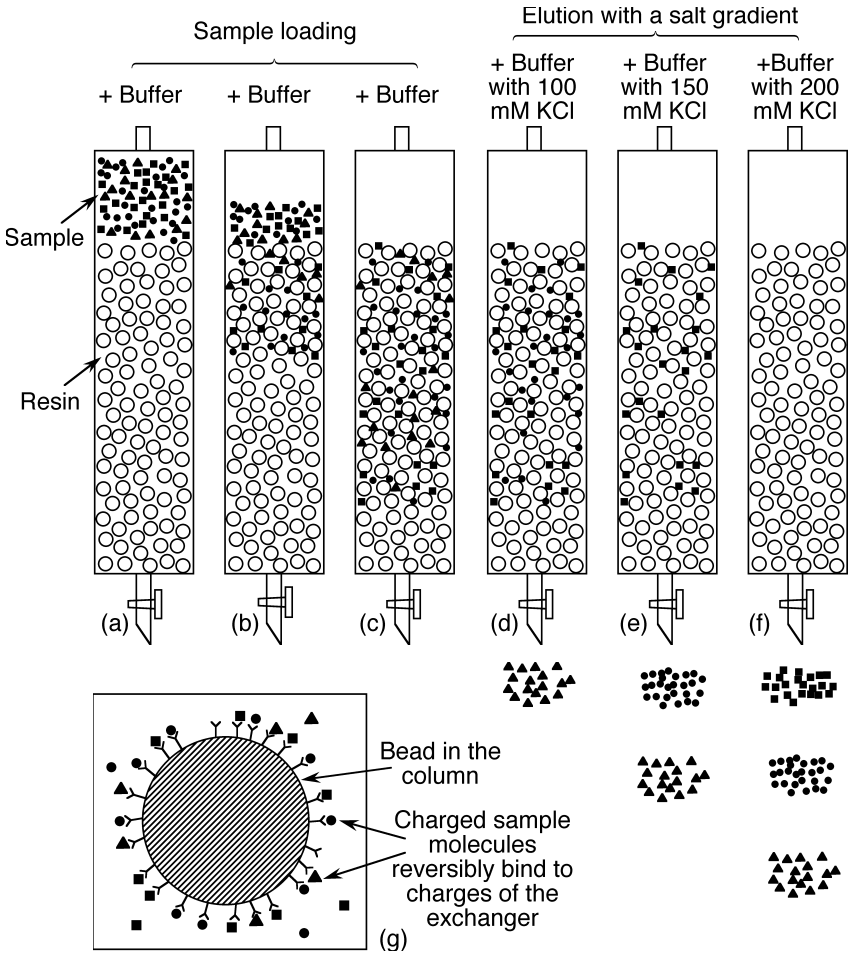

Fig. 2.1 Example of ion exchange chromatography. (**a**) – (**c**) Loading the column: mobile anions (or cations) of the sample are held near cations (or anions) that are covalently attached to the resin (stationary phase). (**d**) – (**f**) Elution of the column with a salt gradient: the salt ions weaken the electrostatic interactions between sample ions and ions of the resin; sample molecules with different electrostatic properties are eluted at different salt concentrations, typically between (0 – 2) M. (**g**) Interaction of sample molecules with ions attached to the resin: at a suitable pH and low salt concentration, most of the three types of biomolecules to be separated in this example reversibly bind to the ions of the stationary phase

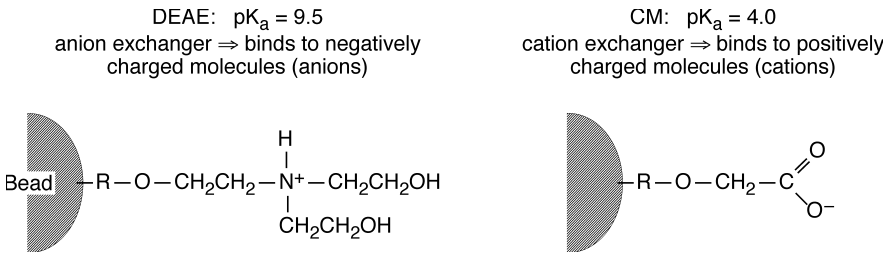

Fig. 2.2 Two ion exchangers: diethyl-amino-ethyl (DEAE) and carboxy methyl (CM). The positive charge of DEAE attracts negatively charged biomolecules. CM is suitable for purification of positively charged biomolecules

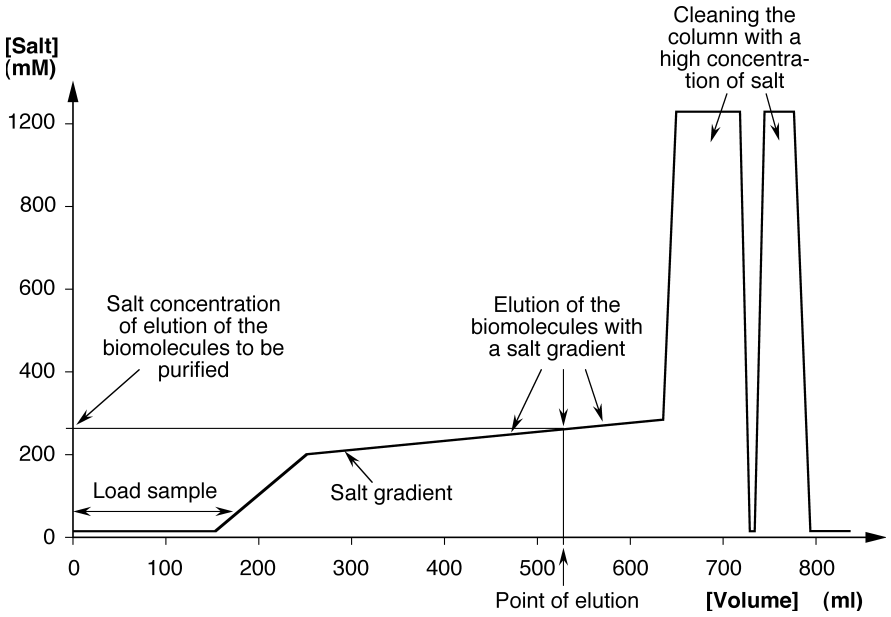

Fig. 2.3 Example for the salt concentration during adsorption of a sample to an ion exchange column, subsequent elution of the sample, and cleaning of the column. Example of a purification protocol: First the solution of biomolecules and impurities in buffer contained in a syringe is loaded onto the column. The biomolecules and some of the impurities bind to the ions attached to the resin. Loading is completed and non-binding molecules are partly rinsed through the column with some further buffer. The next step is to apply a salt gradient with a programmable pump which mixes buffer with extra salt-containing buffer. The steep salt gradient at the beginning elutes most of the weakly binding impurities. At a certain salt concentration, the biomolecules to be purified elute from the column. Elution is monitored with an absorption detector at 280 nm wavelength and the sample fraction collected. After each run the column is cleaned with (1– 2) M KCl. This removes most of the strongly binding sample impurities

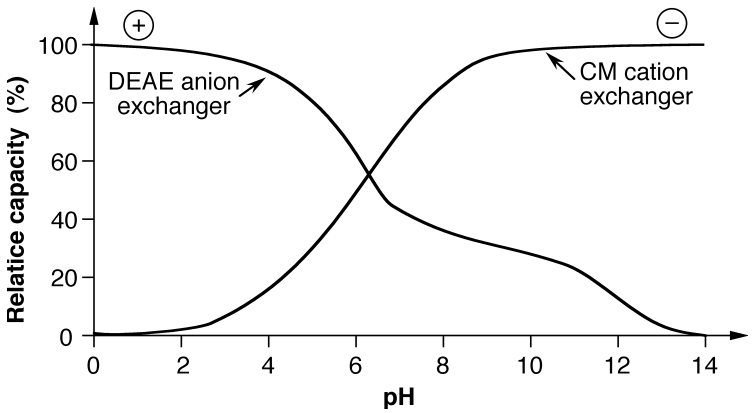

Fig. 2.4 Charge properties of anion and cation exchangers. DEAE has a significant capacity at low and medium pH; CM is highly capacious at high and medium pH

Table 2.1 Properties of some important ion exchangers

Functional group	Type of exchanger	pH range
$-N^{+}-CH_3$	Quaternary amine (strong anion)	1 – 11
$-NH_2$	Primary amine (weak anion)	1 – 8
$-NH-$	Secondary amine (weak anion)	1 – 7
$-N$	Tertiary amine (weak anion)	1 – 6
$-COO^{-}$	Carboxylic acid (weak cation)	6 – 14
$-SO_3^{-}$	Sulfonic acid (strong cation)	1 – 14

The experimental set-up (Fig. 2.6) often just consists of a bottle with buffer, a bottle with buffer with salt, a programmable FPLC or HPLC pump, the column, a detector and recorder of absorption at 280 nm, or occasionally at 220 nm, and a sample collector. If the right conditions for protein preparation are unknown, a pre-run is performed with a small fraction of the sample. Attention should be paid not to overload the column in preparative runs since this can shift peak positions and lead to substantial sample losses. In many cases of modern high expression of recombinant proteins, it is possible to obtain a protein with 99% purity with a

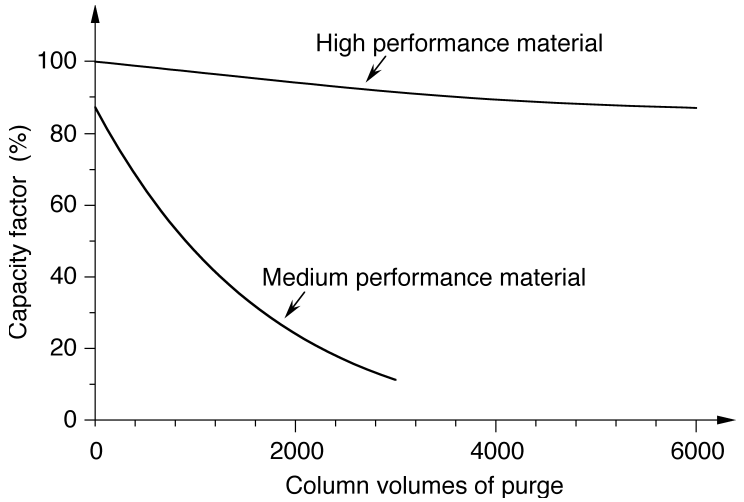

Fig. 2.5 Change of the capacity of ion exchange columns due to usage. High performance columns operated at the appropriate pressure and pH can last many 1000 runs

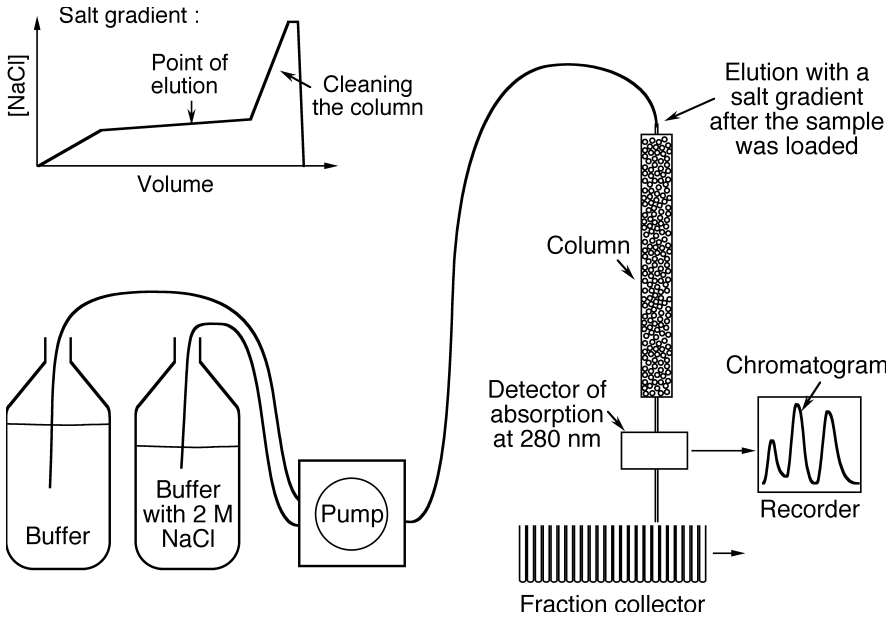

Fig. 2.6 Typical setup for chromatographic purification of proteins with ion exchange FPLC. The pump mixes the salt gradient for sample elution after the sample was loaded, e.g., with a syringe

single ion exchange chromatographic step. However, in case of comparably low expression levels and substantial sample contamination, ion exchange chromatography alone may not be sufficient. Subsequent gel filtration chromatography (Sect. 2.2) can significantly further improve the protein purity.

2.2 Gel filtration chromatography

This type of chromatography is a variant of size exclusion chromatography (molecular exclusion chromatography), and is also known as gel permeation chromatography. It lacks an attractive interaction between the stationary phase

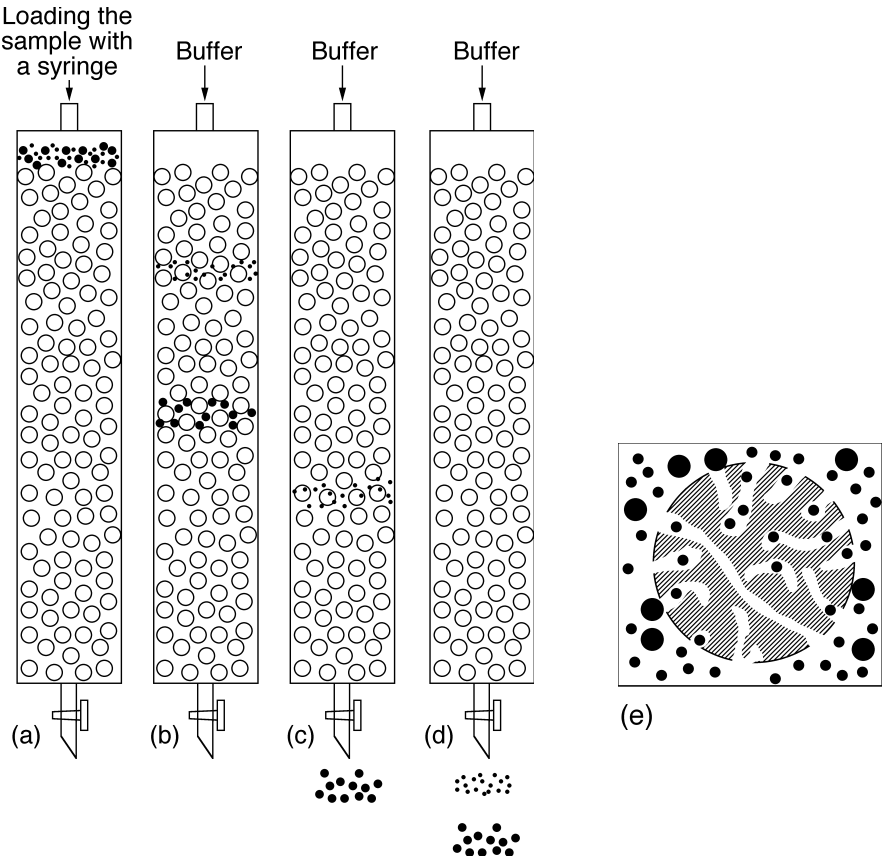

Fig. 2.7 Gel filtration chromatography. When the sample passes through the porous gel, small sample molecules can enter the pores, causing them to flow slower through the column. Large molecules which cannot enter the pores, pass through the column at a faster rate than the smaller ones. Correct pore sizes and solvents are crucial for a good separation

(gel) and solute. The sample solution passes through the porous gel separating the molecules according to their size. The smallest molecules enter the bead pores, resulting in a relatively long flow path and long retention. Large molecules cannot enter the pores and have to flow around them, resulting in a relatively short flow path (Figs. 2.7 – 2.10).

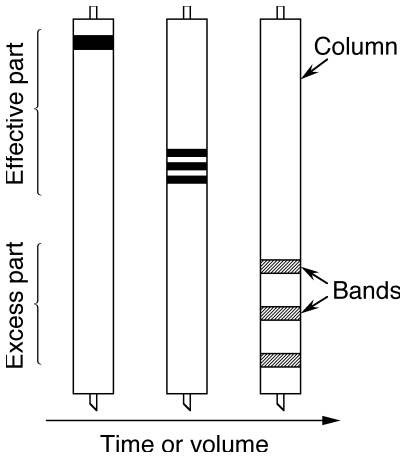

Fig. 2.8 Band broadening in a column with a longer than optimal geometry. The so-called effective part of the column is sufficient for separation. Excessively long columns do not improve purity, but just cause dilution of the sample by band broadening

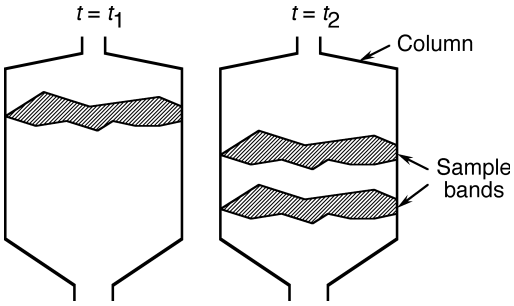

Fig. 2.9 Band broadening in a column with a larger than optimal diameter. Despite the column length is about right to separate the two bands, significant sample dilution and possibly contamination occurs due to inhomogeneous loading of the column

Gel filtration chromatography is also an auxiliary method for assessing the molecular weight of biomolecules (Fig. 2.11). Although there are more precise methods, e.g., mass spectrometry (see Chap. 3), gel filtration chromatography is important for the measurement of monomer-multimer equilibria at about μM-concentrations of biomolecules.

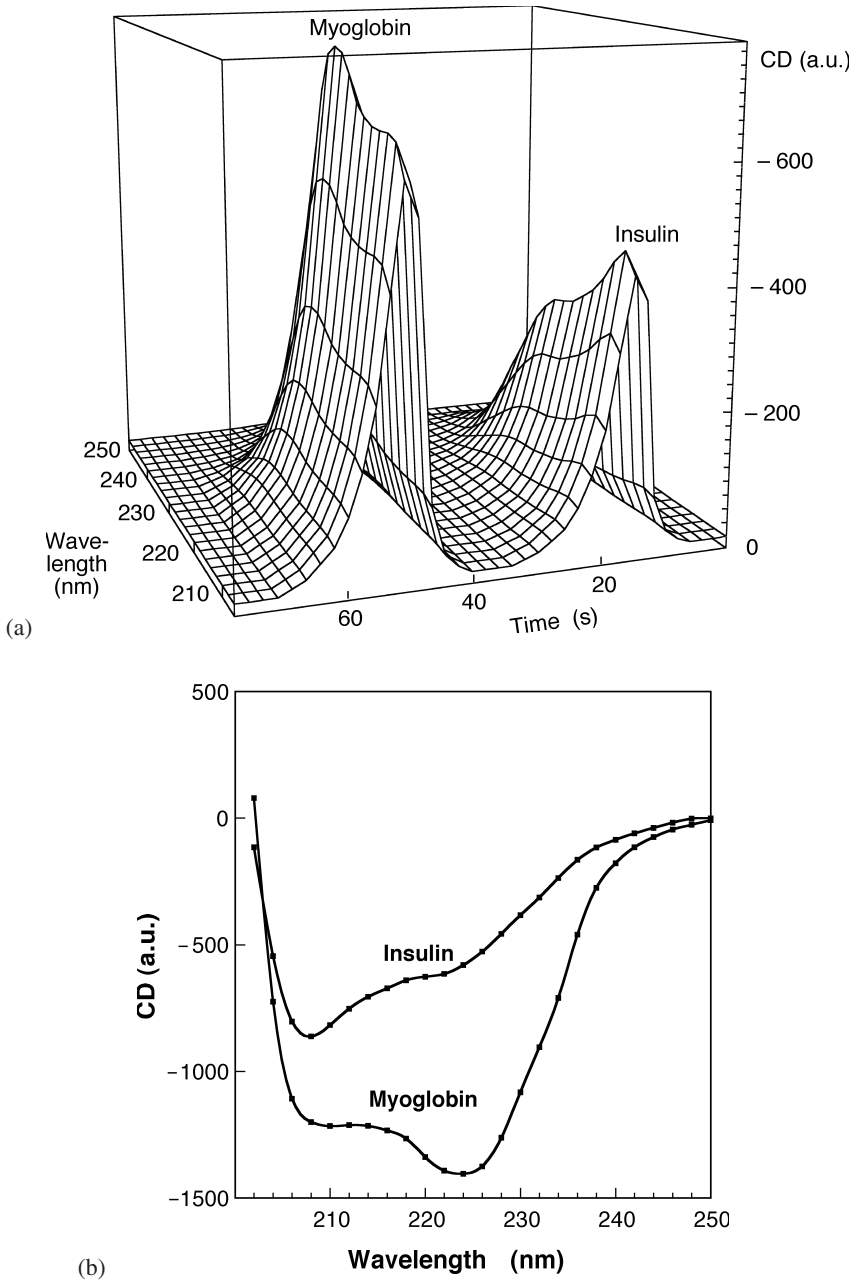

Fig. 2.10 (**a**) Chromatogram of the separation of a mixture of myoglobin and insulin with multichannel circular dichroism (CD) detection. The multiplex advantage of the multichannel detection prevents distortion of the shape of the spectra (see Nölting, 2005). (**b**) CD spectra of myoglobin and insulin for comparison

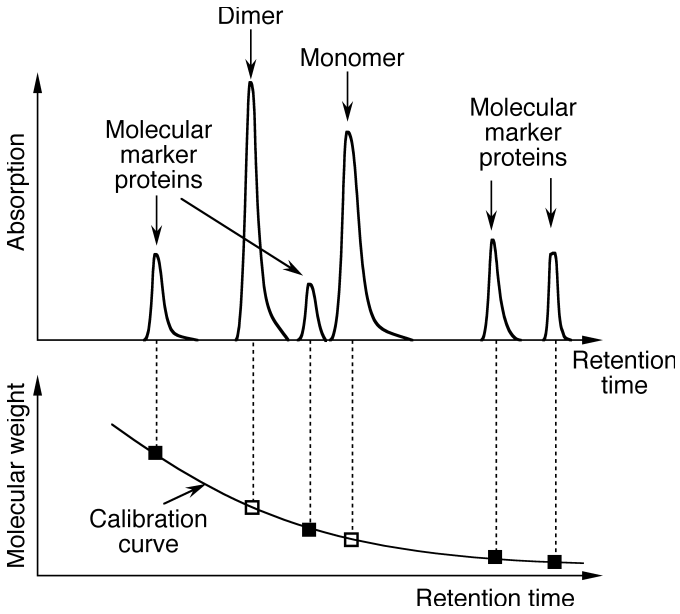

Fig. 2.11 Molecules of known molecular weight enable an estimate of the molecular weight of the unknown molecule. In this case, two peaks of the investigated molecule indicate a monomer-dimer equilibrium

2.3 Affinity chromatography

Affinity chromatography is a method enabling purification of biomolecules and other macromolecules with respect to individual structure or function. It utilizes the highly specific binding of the sample macromolecule to a second molecule which is attached to the stationary phase. The principle of operation is as follows: (a) the sample is injected into the column; (b) buffer is rinsed through the column, so that sample molecules with no affinity to the stationary phase are eluted from the column, but sample molecules with a high affinity for the stationary phase are retained in the column; (c) the retained sample molecules are eluted from the column by buffer with a high salt concentration or a different pH or a different solvent composition (Fig. 2.12). The preparation of the protein can be performed by using a number of protein tags. The tags should not cause artificial interactions and should not alter the conformation of the tagged protein. Very common are poly-histidine tags that are attached to the protein by genetic engineering (Fig. 2.13). The tag typically consists of 8 – 12 histidine residues. It binds to nickel compounds at the surface of the chromatography beads. Fig. 2.14 illustrates a somewhat different variant of affinity chromatography in which misfolded proteins are continuously refolded by chaperones and eluted with buffer.

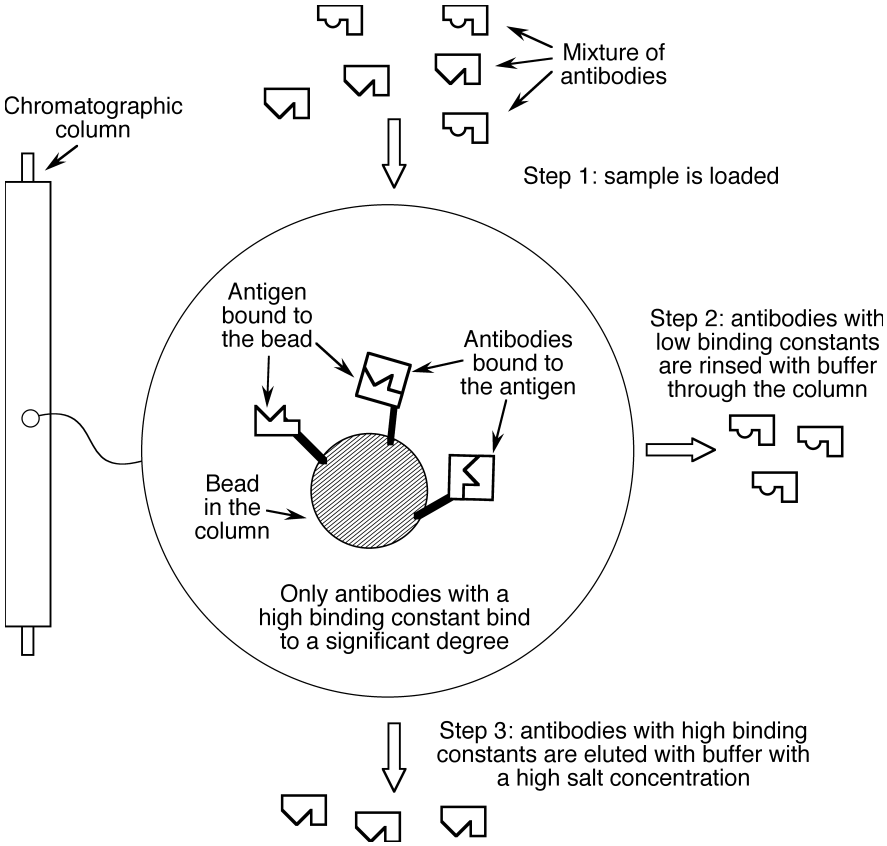

Fig. 2.12 Purification of antibodies with affinity chromatography: The antigen is chemically bound to the beads of the column and the mixture of antibodies is rinsed through the column. Antibodies with high binding constants bind to the antigen and are eluted later with a buffer with a high salt concentration

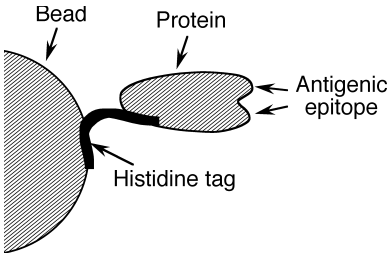

Fig. 2.13 Attachment of a protein to a bead of an affinity column with a histidine tag. About 10 histidine residues were attached to the protein by genetic engineering, e.g., by polymerase chain reaction (PCR) mutagenesis (see, e.g., Nölting, 2005). The histidine residues strongly bind to the bead made from a nickel chelate resin

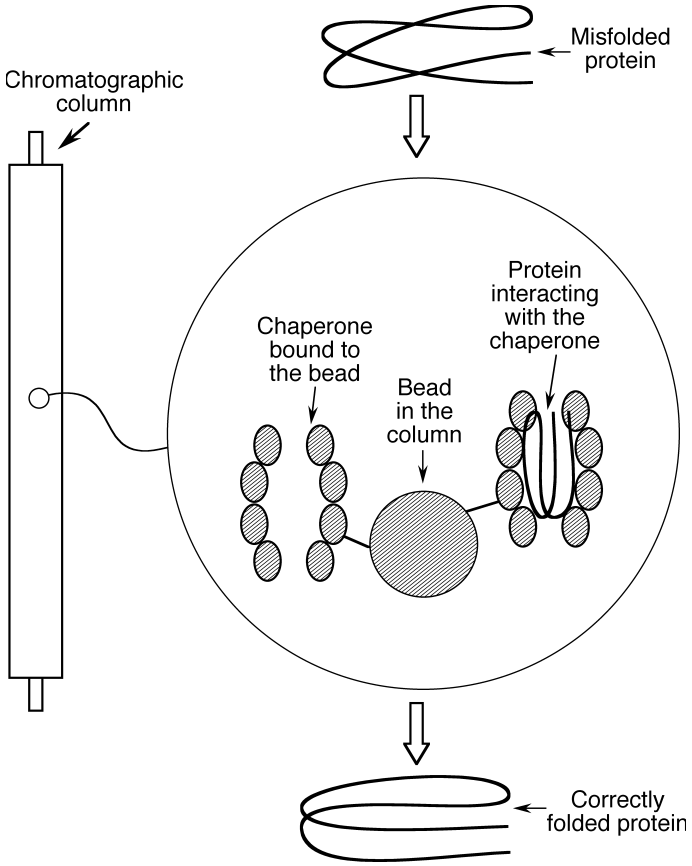

Fig. 2.14 Refolding of expensive, poorly folding proteins: Folding chaperones, also known as chaperonins, are attached to the beads and the unfolded or misfolded protein is rinsed through the column. The chaperone interacts with the sample protein and catalyses its folding into the correct conformation

2.4 Counter-current chromatography and ultrafiltration

A relatively old method of chromatography is the Craig counter-current distribution apparatus (Fig. 2.15). Nowadays it serves for the large-scale purification of some chemicals for which other chromatographic methods are too expensive. As in other types of counter-current chromatography, both stationary and mobile phase are liquids and separation is based on sample partition between the two liquids. It may, e.g., function as follows (Fig. 2.15): (a) A certain biochemical has a higher solubility in phase A than impurities of the biochemical, but has a lower solubility in phase B than the impurities. (b) Phase B with a high concentration

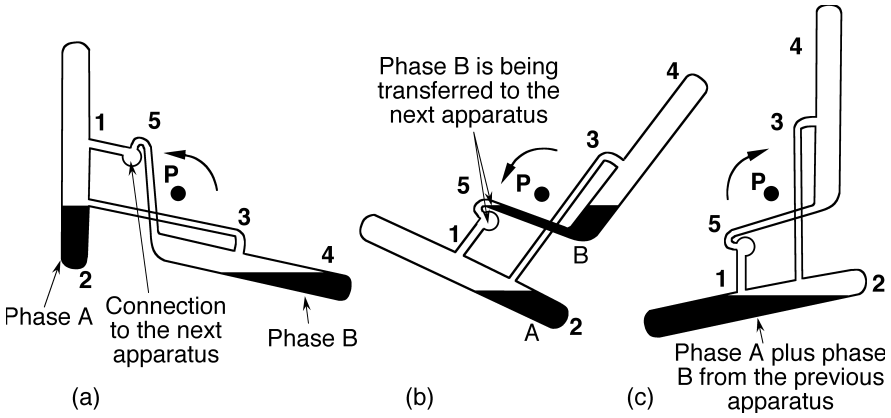

Fig. 2.15 Craig counter-current distribution apparatus: both stationary and mobile phases are liquids. Sample separation is based on its partition between the two liquid phases (see text)

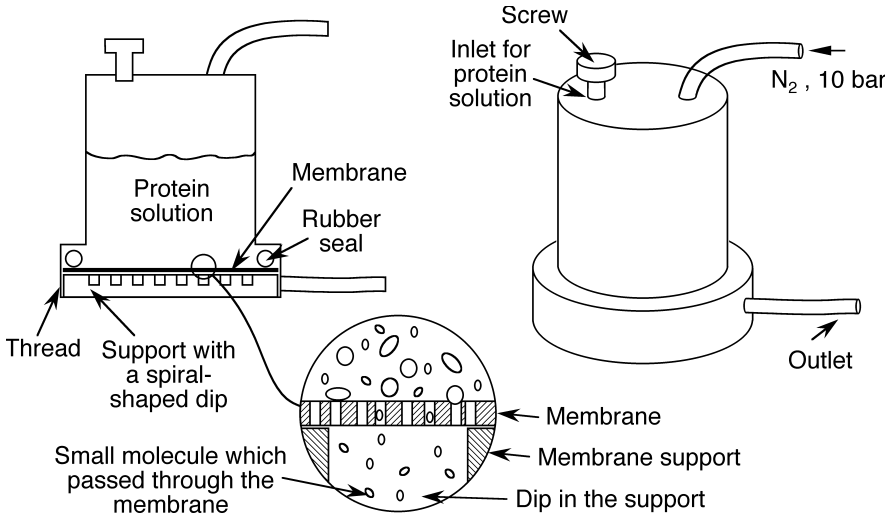

Fig. 2.16 Ultrafiltration device (supplied, e.g., by Amicon Inc., Beverly, MA). Pressurized nitrogen from a nitrogen flask presses the protein solution against the membrane. Small molecules pass the membrane and are collectable at the outlet. Large molecules stay in the ultrafiltration vessel

of impurities is transferred to the next apparatus and fresh phase B is transferred from the previous apparatus to the shown apparatus. (c) Phases A and B are mixed and separated again, and the process continues with step (a). During suc-

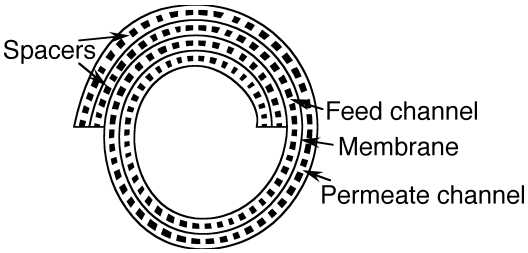

Fig. 2.17 Side view of a spiral cartridge concentrator (e.g., Millipore Corporation, Bedford, MA). Pressure is applied by centrifuging the concentrator. Similarly to the pervious ultrafiltration device (Fig. 2.16), small molecules pass the membrane and large molecules are retained

cessive cycles, different chemicals move through a chain of counter-current distribution apparatuses with different speeds, and are collected, e.g., at the end of the chain.

Strictly speaking, ultrafiltration (Figs. 2.16 and 2.17 – see the figure legends for explanation) is not a chromatographic method. However, it should be mentioned here since it is an extremely useful tool of sample preparation prior to chromatography and can sometimes even substitute chromatography. It is applicable for (a) protein purification, (b) buffer exchange, and (c) concentrating protein solutions. Purification of a protein with a particular molecular weight, M_w, requires two steps: (a) First, one runs the ultrafiltration apparatus with a membrane with a cut-off somewhat higher than M_w and collects the solution leaving the vessel. (b) Then, one runs the apparatus with a membrane with a cut-off somewhat lower than M_w and collects the solution remaining in the vessel.

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