Lecture 3: Ion Exchange Chromatography-I

Ion exchange chromatography is a fast, economical and versatile technique for effective separation of ions, amino acids, peptides, nucleotide and nucleic acids etc. This technique is widely used in the pre-fractionation or purification of a target protein from crude biological samples. Before we go in details of ion-exchange chromatography of proteins, let us discuss, ionization and charge on proteins with respect to pH

Concept of charge on Protein: A typical protein contains several ionizable group (basic amino acid side chains of lysine, arginine and histidine as well as acidic side chains of glutamate and aspartate). Additionally, N-terminal amino group and C-terminal; carboxy group can also ionize. Ionization state of these amino acid side chains depends on pK (also depainds on localized environ ment of side chain) and pH which is described by the Henderson-hasselbalch equation.

$$pH = pK + \log\frac{[\text{conjugate base}]}{[\text{conjugate acid}]}$$

Let us try to understand effect of Lysine on net charge on protein. Charge due to Lysine can range between 0 (when pH >> pK) and +1 (when pH << pK_A). The side chain on lysine normally has a pK value of approx 9.0 (the precise value depends on side chain environment). When pH = pK, the concentration of the protonated and deprotonated forms is equal and the charge due to Lysine is +0.5. When pH = 11.0, the lysine is about 99% deprotonated, leading to a charge of +0.01. When pH = 7.0, the lysine is about 99% protonated, leading to a charge of +0.99.

At a given pH over all charge on protein depends on sum of charges on indivisual amino acid side chain as well as on C- and N-terminal. pH value with net zero charge is called isolelectric point. As proteins in a crude mixture vary in terms of sequence and amino acid compositions, they are likely to have different net charge at a given pH value.

Principle of Ion Exchange Chromatography: Ion exchange chromatography separates proteins or other molecules based on differences in their accessible surface charges. In ion exchange chromatography the analyte molecules are retained on the column based on coulombic (ionic) interactions. The stationary phase surface contains ionic functional groups of opposite charge that interact with analyte ions. The elution is done by increasing salt gradient. Most commonly used salt is NaCl, exists in equilibrium with Na^+ (cation) and Cl^- (anion) in aqueous solution. As the concentration of salt increases concentration of Na^+ (cation) and Cl^- (anion) also increases. The basic principle of ion exchange chromatography is the reversible exchange of analyte ions bound to solid support with similar ions generated from salt in liquid phase. Many biological molecules such as proteins, amino acids, nucleotides and other ions have ionisable groups which carries a net charge (positive or negative) dependent on their pKa and on the pH of the solution, which can be utilised in separating mixture of such molecules as explained in box. Ion exchange chromatography experiments are carried out mainly in columns packed with ion exchangers. On the basis of type of exchanger used for separation this chromatography is further subdivided into cation exchange chromatography and anion exchange chromatography.

Many biological molecules, especially proteins, are stable within a narrow pH range so the type of exchanger selected must operate within this range. Suppose if protein is most stable below its isolecteic point (pI), there will be net positive charge on the protein surface, so for separation of this protein cation exchanger should be used (experimental pH value should be between lowest pH wehere protein is stable and pI). If protein is most stable above its pI, there will be net negative charge on the protein surface and anion exchanger should be used (experimental pH value should be between highest pH where protein is stable and pI value). If protein is stable over a wide range of pH, it can be separated by either type of ion exchanger (experimental pH value may be decided considering lowest and highest pH value stability of the protein). Weak electrolyte requires very high or very low pH for ionisation so it can only be separated on strong exchanger, as they only operate over a wide pH range, whereas in case of strong electrolytes, weak exchangers are preferred.

Cation Exchange Chromatography: Commonly used cation exchange resins functional groups are following:

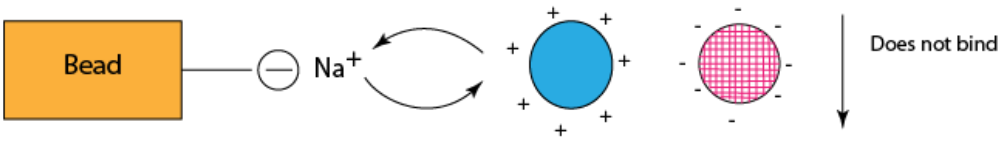
Carboxymethyl (CM)	$-O-CH_2-COO^-$
Sulphopropyl (SP)	$-O-CH_2-CH_2-CH_2SO_3^-$
Methyl sulphonate (S)	$-O-CH_2SO_3^-$

Solid support with these functional groups can be prepared with various beads. They differ in few properties like flow rate, stability, binding capacity (linked with porocity) etc. Cation exchangers based on dextran (Sephadex), agarose (Sepharose) and cross-linked cellulose (Sephacel) are the ion exchange matrices with high porosity, leading to improved flow properties and high capacities for macromolecules. Typically, cation exchange chromatography is performed using buffers at pH's between 4 and 7 and running a gradient from a solution containing just this buffer to a solution containing this buffer with 1M NaCl.

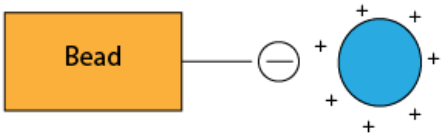
The surface charge on the molecules (proteins, nucleic acids etc), which binds to cation exchanger should be net positive. Thus, to get binding of a specific protein pH should be below the pI of that protein. Once salt concentration increases, it results in increase in Na^+ (cation) and Cl^- (anion). Beyond a certain point, positively charged protein is exchanged with cation. Thus, this type of chromatography is called cation exchange chromatography (Fig. 1 and Fig.2).

Some commen cation exchanger

Step 1 Na^+ is attached to negatively charged cation exchanger



Step 2 Positively charged Protein exchanges with Na^+ and binds to cations exchanger. Negatively charged Protein does not bind.



Step 3 A competing Na^+ (NaCl gradient) elutes the Protein. Protein with less positive charge at given pH shall be eluted at lower NaCl concetration while Protein with higher positive charge get eluted at higher concentration at NaCl.

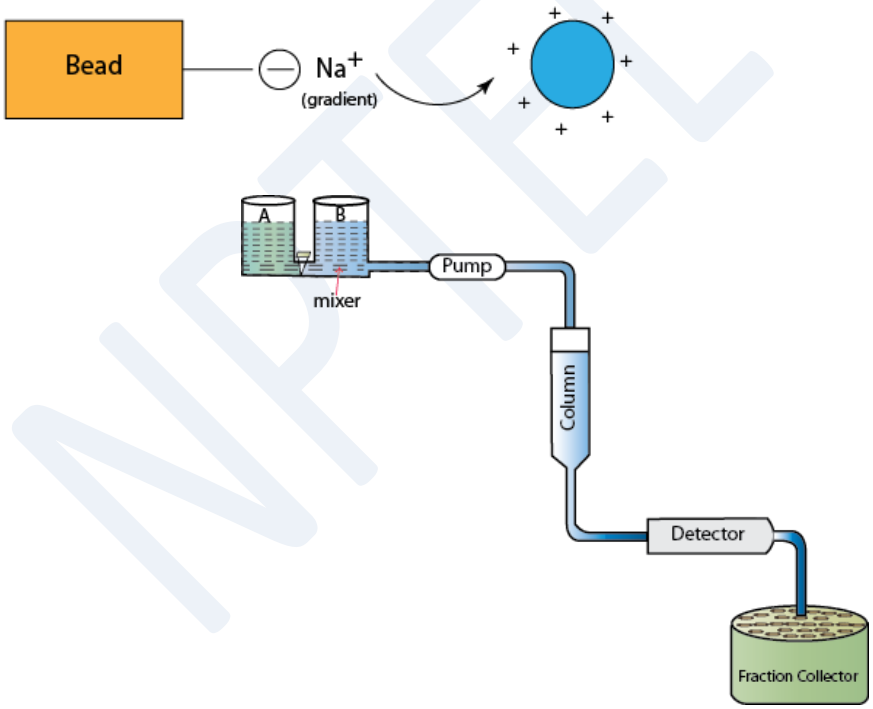


Figure 1: Various steps of cation excahnge chromatography and expermental set-up.

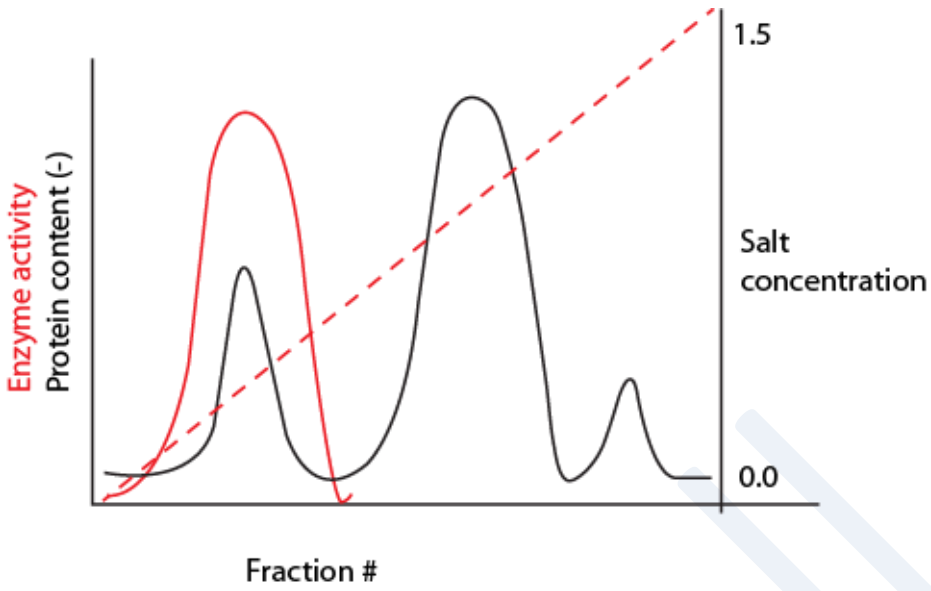


Figure 2: A typical chromatogram for an enzyme purification. First protein peak is showing enzyme activity while the second peak is not active. Thus, enzyme is purified/partially purified in the first peak. Unwanted proteins are separated in second peak. Purity of the enzyme in first peak may be checked (we shall discuss methods in coming classes). In case of partial purification, fractions covering first peak may be pooled for next step of purification.