Ion Exchange Chromatography

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Principle

Ion-exchange chromatography separates molecules based on their charged groups, which causes the molecules to interact electrostatically with opposite charges on the stationary phase matrix (Fig.1.) The stationary phase carries ionizable functional groups coupled to an inert matrix material. Based on the principles of electro neutrality, these immobilized charges are electrostatically associated with exchangeable counter ions from the solution. Charged molecules to be purified compete with these counter ions for binding to the charged groups on the stationary phase and are thereby retarded on the basis of their charge. Different types of molecules will bind to the matrix with affinities that depend on both the conditions used and the types and number of individual charged groups. These differences lead to resolution of various molecules by ion- exchange chromatography.

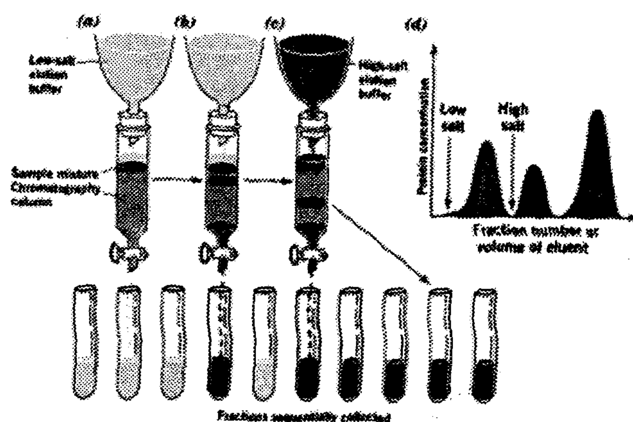


Fig. 1. Schematic of Ion Exchange Chromatography

The stationary phase has either positively or negatively charged species immobilized on its surface. Positively charged stationary phases are called anion exchangers because anions bind to them. Negatively charged stationary phases are called cation exchangers.

Strong and weak exchangers

A strong exchanger is one, which remains almost fully ionized over a wide pH range while a weak exchanger is ionized over a small pH range. Ion-exchange material exists in an ionized state (A^-) or an un-ionized state (AH).

Anion Exchangers

Anion exchangers (Table 1) can be either weak or strong. The charged group on a weak anion exchanger is a weak base, which becomes deprotonated and, therefore, loses its charge at high pH. DEAE-cellulose (Fig.2.) is an example of a weak anion exchanger, where the amino group can be positively charged below pH ~ 9 and gradually loses its charge at higher pH values. A strong anion exchanger, on the other hand, contains a strong base, which remains positively charged throughout the pH range (pH 1-14) normally used for ion exchange

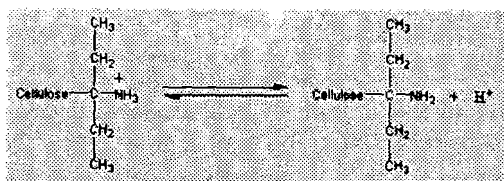


Fig. 2. Dimethylaminoethyl (DEAE) cellulose

chromatography. Likewise the charged group of a strong anion exchanger is available to bind proteins over a wider pH than a weak exchanger.

Cation Exchangers

Cation exchangers can also be classified as either weak or strong. A strong cation exchanger contains a strong acid (such as a sulphopropyl group) that remains charged from pH 1-14; whereas a weak cation exchanger contains a weak acid (such as a carboxymethyl group), which gradually loses its charge as the pH decreases below 4 or 5. A strong cation exchanger has a lower pK' a than a weak cation exchanger. This means that the charged group of a strong cation exchanger is available to bind cations over a wider range of pH's than a weak cation exchanger.

The most commonly used exchangers are the weak exchangers. Diethylaminoethyl (DEAE) exchangers are usually used as anion exchangers to purify negatively charged proteins. Carboxymethyl (CM) exchangers are most frequently used as cation exchangers. However, strong cation and anion exchangers are becoming more popular.

Charge on a Protein

The charge on the protein affects its behavior in ion exchange chromatography. Proteins contain many ionizable groups on the side chains of their amino acids as well as their amino - and carboxyl - termini. These include basic groups on the side chains of lysine, arginine and histidine and acidic groups on the side chains or glutamate, aspartate, cysteine and tyrosine. The pH of the solution, the pK of the side chain and the side chain's environment influence the charge on each side chain. The relationship between pH, pK and charge for individual amino acids can be described by the Henderson-Hasselbalch equation:

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

Table 1. Ion exchange groups used in protein purification

Strong anion		
$-CH_2N^+(CH_3)_3$	-	Triethylaminomethyl
$-C_2H_4N^+(C_2H_5)_3$	-	Triethylaminoethyl
$-C_2H_4N^+(C_2H5)_2CH_2CH(OH)CH_3$	-	Diethyl-2-hydroxypropylaminoethyl
Weak anion		
$-C_2H_4N^+H_3$	-	Aminoethyl
$-C_2H_4NH(C_2H_5)_2$	-	Diethylaminoethyl
Strong cation		
$-SO^3$	-	Sulpho
CH_2SO^{3-}	-	Sulphomethyl
$C_3H_6SO^{3-}$	-	Sulphopropyl
Weak cation		
$-COO^-$	-	Carboxy
$-CH_2COO^-$	-	Carboxymethyl

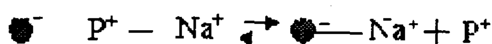
In general terms, as the pH of a solution increases, deprotonation of the acidic and basic groups on proteins occur, so that carboxyl groups are converted to carboxylate anions ($R-COOH$ to $R-COO^-$) and ammonium groups are converted to amino groups ($R-NH3^+$ to $R-NH2$). In proteins the isoelectric point (pI) is defined as the pH at which a protein has no net charge. When the pH > pI, a protein has a

net negative charge and when the pH < pI, a protein has a net positive charge. The pI varies for different proteins.

Practical aspects

Ion exchange involves the binding of target molecules to a charged material and elution. Ion exchange, like any ionic process is an equilibrium process. The opposing charges of the exchanger and the target ion draw the protein to charged site on the exchanger. But the bond is not a covalent linkage.

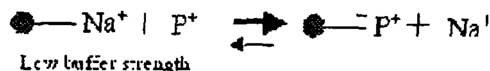
And the target ion can be readily replaced by another ion or "counter ion".



Two factors will contribute to the effectiveness of the counter ion in eluting the target protein

1. concentration of the counter ions
2. the strength of the bond

If the concentration of counter ions is low (for example, if the buffer strength is low) and the concentration of the target ion is high, then the target ion will tend to bind to the column. This principle is used in binding of ions such as proteins to the column. For example, in the ion exchange purification of proteins, a low buffer concentration must be used during binding. Otherwise the buffer components will compete with the protein for the exchange sites.



If the concentration of the counter ion is high, then the counter ion will tend to bind to the ionic sites on the column and any bound target will tend to elute:

For the elution of proteins bound to the ionic sites of an ion exchange column, the ionic bond between the protein and the column is also manipulated. This is achieved by manipulating the charge of the protein.

The charge of a protein is determined by its pI and the buffer pH. If the pH is greater than the pI, the protein will have a negative charge. The greater the difference between the pH and the pI, the more *negative will be charge of the protein. If the* pH is equal to the pI of the protein, the protein will have a no charge and therefore will not bind at all to either an anion exchange or cation exchange column. If the pH is less than the pI, then the protein will have a positive charge. For example, consider a protein with a pI of 7:

	pH < 7	pH = 7	pH >7
Charge	+	0	-
Binds to a cation exchange column?	√	X	X
Binds to an anion exchange column	X	X	√

Thus to bind a protein with a pI of 7 to an anion exchange column, the binding buffer must have a pH greater than 7. To elute the protein, the elution buffer can have a pH of 7 or less. To bind this protein to a cation exchange column, the binding buffer must have a pH less than 7 and to elute it, the buffer can have pH of 7 or more.

Selecting the optimum pH

The pI and pH stability of the protein must be considered when determining the

the elution buffer. Likewise for a cation exchange column, the most weakly bound proteins will be displaced first by the Na^+ in the elution buffer.

Gradient pH elution

For a cation exchange column, elution starts from a low pH to a higher pH. Considering the elution of three proteins with different pI - Protein A – pI 5, Protein B - pI 7 and Protein C – pI 9, from a cation exchange column:

If the elution gradient starts from a low pH the following results would be obtained:

pH	Protein A	Protein B	Protein C
5	pH = pI Protein elutes	pH < pI	pH < pI
6		pH < pI	pH < pI
7		pH = pI Protein elutes	pH < pI
8			pH < pI
9			pH = pI Protein elutes

If the pH < pI, the protein will have a positive charge and the protein will therefore bind to the cation exchange column.

If the gradient proceeds from pH 9 to pH 5, All proteins would elute simultaneously.

pH	Protein A	Protein B	Protein C
9	pH > pI Protein elutes	pH > pI Protein elutes	pH = pI Protein elutes
8			
7			
6			
5			

For an anion exchange column, elution starts from a high pH to a lower pH.

Again, considering the three proteins. For a pH gradient starting from pH 9 and decreases to pH 5, the following elution order would be obtained (C => B => A):

pH	Protein A	Protein B	Protein C
9	pH > pI	pH > pI	pH = pI Protein elutes
8	pH > pI	pH > pI	
7	pH > pI	pH = pI	
6	pH > pI	Protein elutes	
5	pH = pI Protein elutes		

If the pH > pI, the protein will have a negative charge and the protein will therefore bind to the anion exchange column. If the pH gradient proceeds from a low pH to a high pH all three would elute simultaneously:

pH	Protein A	Protein B	Protein C
5	pH = pI Protein elutes	pH < pI Protein elutes	pH < pI Protein elutes

Cation exchange separation

The three steps in ion exchange chromatography are sample application, washing the column to remove weakly bound proteins, and elution of the protein of choice with a properly designed gradient. It is necessary to select a resin and buffer so that the protein of interest will bind to the resin. In a cation exchange separation, the protein of interest needs to be positively charged to bind to the stationary phase in the column.

The column heading indicates the pH for

Table 1. Charge on Proteins for Cation Exchange.

Protein	pI	pH4.8	pH7.2	pH8
Carbonic Anhydrase	7.0	+16.5	-0.4	-2.7
Carboxypeptidase B	6.2	+12.0	-3.3	-6.3
Chymotrypsin	8.0	+9.0	+2.7	0.0
Lysozyme	9.8	+14.1	+7.9	+6.9

the separation and the stationary phase. For example, pH 4.8 cm is a test mixture with a pH 4.8 mobile phase and a carboxymethyl-Sephadex stationary phase.

For the protein mixture in Table 1, all the proteins will bind to the cation exchanger at pH 4.8, since all are positively charged. The more positively charged the protein, the stronger it will bind. Therefore the elution order of the four proteins in this mix when eluted with a 0 to 0.2 M NaCl gradient, will be chymotrypsin, carboxypeptidase B, lysozyme, and carbonic anhydrase. If *pH7.2 cm* is used, then carbonic anhydrase and carboxypeptidase B will elute in the wash (before the gradient is initiated), followed by chymotrypsin, then lysozyme during the salt gradient. In the mixture *pH8 cm*, only the lysozyme will bind to the stationary phase, the other three proteins will elute in the wash.

A similar set of test mixtures may be used for *anion exchange chromatography*. The same four proteins and buffers may be used as are shown in Table 1, but the stationary phase is DEAE-cellulose.

Ion exchange chromatography is commonly used for the separation and purification of proteins, peptides, nucleic acids, polynucleotides and other charged molecules mainly because of its high resolving power and high capacity.

Amino Acid Analysis by Ion Exchange High performance Chromatography

The basic components include pump, injector, column and detector. Amino acid analysis is the determination of what types of amino acids and how many of each composes a protein. Only the amino acid composition is determined, not the order or sequence of amino acid residues. Amino acid analysis involves four basic steps:

1. Hydrolyze a protein to individual constituent amino acids

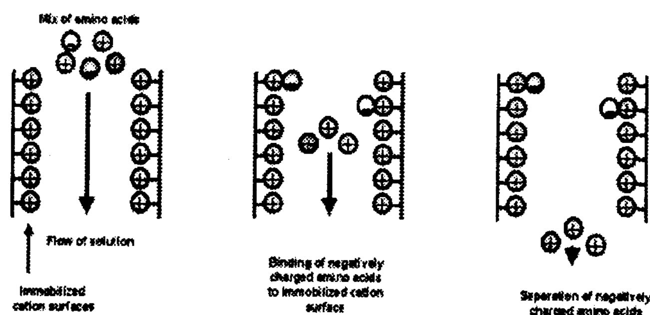


Fig. 3. Ion-exchange chromatography (anion exchange)

2. Label amino acids with a detectable UV-absorbing or fluorescent marker
3. Separate different types of amino acids by chromatography
4. Measure relative amounts of each amino acid type based on intensity of the detectable marker associated with the emergence of each type of amino acid from the chromatographic system
5. The HPLC system is calibrated for quantitative work by the use of standards of each amino acid.

Many refinements have been made through the years to improve amino acid analysis techniques. Most significantly, ion exchange high performance liquid chromatography systems have been optimized by changes in resin characteristics, column sizes, column temperatures, buffer pH and ionic strength to improve resolution (chromatographic separation) and reduce required sample sizes (increase sensitivity).

Sample Preparation and Hydrolysis Methods

Protein and peptide samples must be hydrolyzed to free amino acids. The most widely used agents for hydrolyzing proteins are acids; usually HCl. Hydrolysis with HCl is performed at 110°C for 20-70 hours, with the temperature being accurately controlled. The HCl hydrolysis procedure also minimizes decomposition of reduced S-carboxymethylcysteine and preserves S-carboxymethylated amino acids. After hydrolysis, residual HCl is removed in a rotary evaporator and the residue dissolved in water or buffer, depending on whether the separation method will be ion exchange chromatography or high pressure liquid chromatography (HPLC).

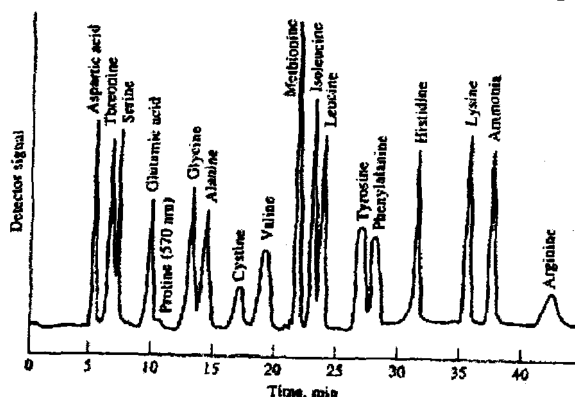


Figure 28-25 Separation of amino acids on an ion-exchange column. Packing: cation exchange with particle size of 8 μm. Pressure: 2700 psi. *(Reprinted with permission from J. R. Benson, Amer. Lab., 1972, 4(10), 60. Copyright 1972 by International Scientific Communications, Inc.)*

Amino Acid Derivatization

Derivatization or chemical labeling of amino acids for detection can be performed either pre- or post-column separation. Ninhydrin is used routinely for post-column derivatization, whereas *o*-phthalaldehyde (OPA) a pre-column derivatization reagent is used often when greater analytical sensitivity is required. OPA imparts fluorescence to the amino acids. Derivatization with phenylisothiocyanate (PITC, also called Edman's Reagent) also provides rapid analysis for amino acids. Proline and hydroxyproline being imino acids are not detected by ninhydrin; they have to be converted into amino acids by reaction with sodium hypochlorite for detection.

Buffer System

Separation of amino acid analysis by ion exchange chromatography is dependent on the buffer pH and ionic strength. The counter ion typically is either sodium or lithium, and buffer changes occur either in a step-wise or gradient manner. Sodium is used for

acid-hydrolyzed samples, and lithium is used for isotonic samples. Citrate is the most commonly used buffer component for systems using a pH <7. Unfortunately, citrate is not suitable for high sensitivity applications because of inherent amino acid contamination. For consistent analyses, high-purity reagents must be used for buffer preparation.

Chromatofocusing

The technique of chromatofocusing, the principle of which is similar to that of isoelectricfocussing is suitable for protein purifications. A linear pH gradient is generated in the column by exploiting the high buffer capacity of an ion exchanger pre-equilibrated to a particular pH. An amphoteric buffer that has even buffering capacity over a range of pH is used. A pH gradient which is 3-4 pH units lower at the top than at the bottom of the column is formed by running the buffer through the column for a pre-determined time. The starting pH of the gradient should be lower than that at which the ion-exchange column has been pre-equilibrated. When a protein is added to this pH gradient, with a buffer whose pH is similar to that at the top of the column, it will migrate down the column as a cation, facing an increasing pH, until it reaches a pH corresponding to its isoelectric point. Beyond this point it will become an anion and will bind to the positive groups of the anion exchanger. As the elution continues with the starting buffer, the existing pH will be lowered, causing the binding of the protein to cease. The protein will continue its movement down the column until once again it encounters a pH slightly above its isoelectric point, when again it will bind. This process is repeated continuously until the protein is eluted at a pH slightly above its isoelectric point. In a mixture of proteins, each protein would elute in the order of its isoelectric point. More protein added at the top of the column during this elution process would automatically catch p with the initial protein, thereby producing a focusing effect and enabling large volumes to be applied to the column, with no deleterious effect. Thus the technique has a high capacity. Chromatofocusing gives a good resolution of quite complex protein mixtures provided that there are discrete differences in their isoelectric point.