Protein Purification by Ion Exchange Chromatography

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Abstract

Separation of similar biomolecules and proteins with little or no differences in molecular weight or without tags can be difficult with chromatographic techniques such as affinity or size exclusion. To circumvent this problem, distinct physicochemical properties of protein molecules have been harnessed for their separation. Since proteins carry overall electrical charges due to their chemical composition; ion exchange chromatography (IEX) uses this property to separate positively or negatively charged molecules via interaction with charged ion exchange resins as stationary media. Charged proteins bind to the resins in normal buffering conditions and can be gradually eluted with increasing salt concentration or by changing the pH of the mobile phase. Depending on the protein's isoelectric point (pI) value, cation or anion exchange chromatography media can be used. If the pH environment of a protein is lower than its pI, it will carry a positive surface charge and strongly bind the cation exchange resins, while proteins with the negative surface charge will bind to the anion exchange counterpart. The purpose of this chapter on ion exchange chromatography is to describe its basic principle, protocols, applications in protein purification as well as provide troubleshooting tips.

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7.1 Introduction

Besides affinity chromatography, one of the most useful liquid chromatographic techniques of protein purification is ion exchange chromatography (IEX). Affinity chromatography has limitations by its requirement of fusion tags during protein purification as discussed in the earlier chapter. Additionally, separation and purification of proteins having similar molecular mass (size) are challenging while working with size exclusion chromatography. In such conditions, IEX is an excellent choice as it separates molecules based on the interaction between the charged solute molecules and the complementarily charged matrix of the column. Moreover, due to high resolving power and capacity, this technique is often used for the isolation and purification of proteins. The mechanism of separation using ion exchange relies on two main factors such as competitive binding (attraction) between opposite charged ions and repulsion between similarly charged ions fixed on the matrix of the column. In addition to the charge variations, a couple of other considerations such as charge density and charge distribution on their surfaces play important roles in determining the degree of separation. The inclusion of these factors enables IEX to efficiently separate proteins even with a single charged residue difference. Thus, IEX is considered one of the most powerful protein purification techniques [1–3].

The concept of ion exchange emerged with the introduction of polystyrene matrix by D'Alelio in 1944, which accelerated the usage of ion exchange as an analytical tool [4]. However, later in 1956, IEX of protein was first successfully demonstrated by Peterson and Sober using cellulose matrix [5]. Thereafter, a series of research endeavors for almost two decades led to the development of a robust ion exchange chromatography system by Bauman and coworkers in 1975 [6]. Within the next 5 years, the latest anion and cation exchange chromatographic tools were also developed leading to a breakthrough in biochemical and industrial research [7–9]. Since then, ion exchange has been considered as one of the most important preparative and analytical tools for the separation, purification, and characterization of nucleic acids, proteins, peptides, and other charged biomolecules [10–12]. This chapter will discuss the basic principles and applications of IEX in recombinant protein purification.

7.2 Basic Principles of Ion Exchange Chromatography

Ion exchange is a process in which ions attached to a matrix are exchanged for other ions in solution [13, 14]. The basic process of ion exchange for a negatively charged resin (i.e., cation exchanger) is provided by Eq. (7.1):

$$(\mathrm{Res}^-)\mathrm{B}^+ + \mathrm{C}^+ \ (\mathrm{soln}) \Leftrightarrow (\mathrm{Res}^-)\mathrm{C}^+ + \mathrm{B}^+(\mathrm{soln}) \tag{7.1}$$

where Res^- represents the negatively charged ion exchange resin, i.e., cation exchanger, B^+ is the counterion of the opposite charge associated with the exchanger matrix, C^+ is the charged molecule (bearing the same charge as the counterion) in the sample to be separated. The positively charged molecule (C^+) can interchange with the counterion (B^+) to find appropriate binding sites on the negatively charged resin. All the other neutral or negatively charged ions present in the buffer do not interact with the exchanger. Bound ions, C^+, can now be eluted from the resin in two ways. The first way of elution includes passing of solvent through the resin with increasing concentrations of B^+. This method increases the possibility that B^+ will replace C^+ by substitution in the above-stated equilibrium due to the presence of B^+ in high concentration. The quantity of charge possessed by C^+ determines the concentration of B^+ required for elution. The greater the charge carried by C^+, the higher the concentration of B^+ required for eluting it. Another way of eluting the bound C^+ ions from the resin is by altering the pH of the solvent in a way such that C^+ is converted to an uncharged moiety. In this case, p*K*a of C^+ is the determining factor to the requirement of the pH of the solvent. The higher the p*K*a value of C^+, the higher pH is required for the elution [3].

Similarly, the method of ion exchange for a positively charged resin (i.e., anion exchanger) is presented by Eq. (7.2):

$$(\mathrm{Res}^+)\mathrm{X}^- + \mathrm{Y}^- \ (\mathrm{soln}) \Leftrightarrow (\mathrm{Res}^+)\mathrm{Y}^- + \mathrm{X}^-(\mathrm{soln}) \tag{7.2}$$

where Res^+ represents the positively charged ion exchange resin, i.e., anion exchanger, X^- is the counterion associated with the exchanger matrix, Y^- is the charged molecule (bearing the same charge as the counterion) in the sample to be separated. To find the appropriate binding sites on the positively charged resin, the negatively charged residues (Y^-) from the solution can interchange with the counterion (X^-). The remaining positively charged and neutral residues will pass through the resin without interacting with the exchanger. Elution of the bound Y^- ions from the resin can be done by using higher concentrations of X^- or by increasing the pH of the solvent. Therefore, the interchange of similarly charged ions between the solvent and the resin through substitution forms the basic principle of ion exchange chromatography (Fig. 7.1) [1–3].

Based on the ion exchange mechanism, IEX separates molecules depending on their net charges [3, 14]. It is a unique type of adsorption chromatography in which ionic solutes (mobile phase) interact with a charged resin (stationary phase) in a reversible electrostatic interaction. The ion exchange resin is composed of an inert,

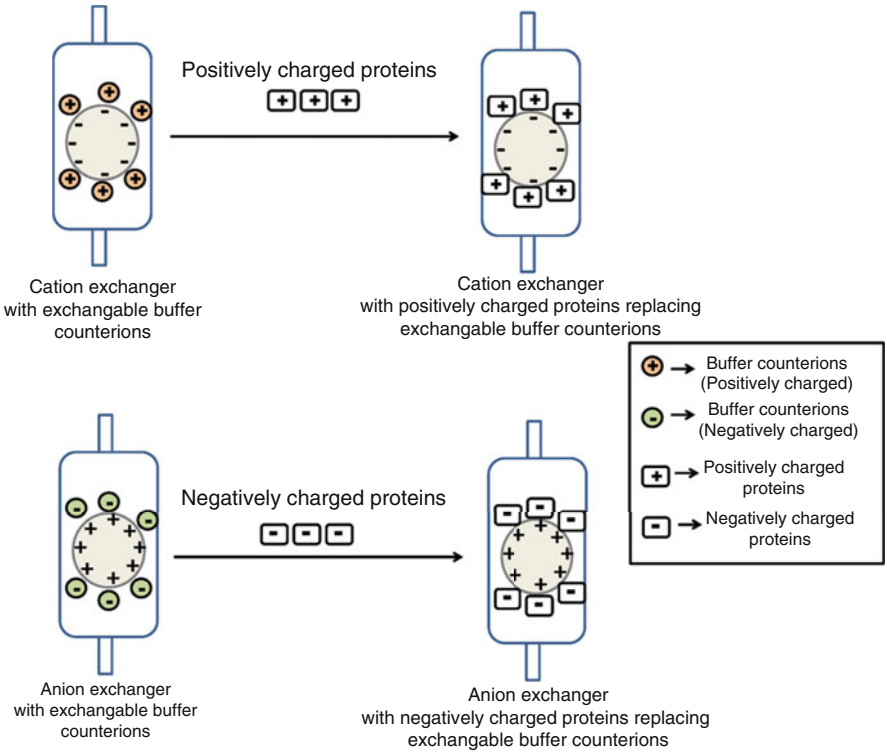

Fig. 7.1 Working principle of cation and anion exchangers. The cation exchangers carry negatively charged functional groups and attract positive charge counterions from the surrounding buffer solution. Due to the presence of positively charged protein in the buffer, these buffer counterions are replaced by positively charged proteins. On the other hand, the anion exchangers carry positively charged functional groups and attract negative charge counterions from the buffer solution. Upon the addition of protein (negatively charged), these buffer counterions are replaced by negatively charged proteins

porous matrix covalently bound to a significant number of specific ionic functional groups [15]. These specific ions are stabilized by equivalent and oppositely charged ions from the buffer specified as counterions. For the purification of proteins, both **cation** and **anion exchangers** are used [15]. As mentioned earlier, **cation exchangers** possess negatively charged groups and therefore attract positively charged cations from the proteins that have an overall positive charge at the pH in which the experiment is conducted. Since their negative charges arise from the ionization of acidic groups, these exchangers are also known as acidic ion exchangers. Conversely, **anion exchangers** that have positively charged groups attract negatively charged anions. Since positive charges on the resin result from the binding of protons with basic groups, these exchangers are often named basic ion exchangers [3, 10, 15]. The working principle of cation and anion exchangers is shown in Fig. 7.1.

Proteins carry many ionizable groups in the charged polar side chains of amino acid residues. The free amino group (α-NH_2) at one end and the free carboxylic group (α-COOH) at the other end of the peptide chain or protein may also ionize. Depending on the pH, the α-NH_2 group and the side-chain groups such as NH_2, imidazole, and guanidino groups may accept protons (H^+) to form cations, while the α-COOH and side-chain COOH groups may donate H^+ to form anions. Thus, a protein shows its amphoteric nature (property to act equally as an acid and a base) by consequently acting both as a donor and an acceptor of H^+. At a specific pH known as the isoelectric pH (pI), the amino acid exists as a dipolar ion or zwitterions carrying equal numbers of positive and negative charges on its ionizable groups so that the net charge is minimum or zero. In a buffered solution, if the pH of the solution is lower than the pI of a protein, the protein exists as a cation by accepting H^+ from the acidic solution and binds to the negatively charged functional groups of a cation exchange resin [2, 16]. On the other hand, if the pH of the buffer solution exceeds the pI of the same protein, it forms an anion by donating H^+ to the alkaline solution and binds to the positively charged functional groups of an anion exchange resin [2, 16]. In the subsequent sections, we will illustrate various components, specific conditions, and protein purification methods using IEX.

7.3 Components and Factors of Ion Exchange Chromatography

7.3.1 Ion Exchange Resins

Ion exchange resins act as the stationary phase of IEX having two major components—a matrix and functional ionic groups. The matrix is an inert, three-dimensional, porous material to which charged groups are bound covalently. It is composed of cross-linked polymers that are typically polystyrene-, cellulose- or agarose-based. The extent of cross-linking of the resin should be sufficient to provide required mechanical stiffness, insolubility, and porosity [17]. However, smaller pore sizes are not conducive to working with macromolecules. Therefore, polystyrene, which is hydrophobic and with less porosity, is suitable for inorganic ions and small molecules [18, 19], while cellulose and agarose are hydrophilic and useful for larger, biologically essential molecules such as proteins and nucleic acids [20–23].

The other constituents of the ion exchange resin are the functional ionic groups. These functional ionic groups are covalently attached to the matrix specifying the nature and strength of the ion exchanger. Depending on their affinity for either positive or negative ions, functional ionic groups are classified as cationic exchangers and anionic exchangers, respectively. Thus, the charge carried by the exchangeable ion defines whether the material is anionic or cationic.

Depending on the ionization state, ion exchange resins are categorized as strong and weak exchangers. The strong ion exchange resins contain strongly ionized groups like sulfopropyl and diethyl-(2-hydroxyl-propyl)-aminoethyl. These get completely ionized and exist in the charged form except at extreme pH values.

The weak ion exchange materials, on the contrary, contain groups such as carboxylate and diethylaminoethyl, whose ionizations are pH-dependent [2, 24].

The ion exchange process, which involves replacing the resin exchangeable ions (A_r) by the counterions (B_s) from a solution, can be expressed as Eq. (7.3):

$$A_r + B_s \Leftrightarrow B_r + A_s \tag{7.3}$$

In this reversible process, the selectivity coefficient (K) for the charged ions is defined as Eq. (7.4):

$$K = [B_r][A_s]/[A_r][B_s] \tag{7.4}$$

where the terms in parentheses reflect the concentrations of ions A and B in the resin or mobile phase, respectively. Relative affinities of ions for a particular resin can be obtained experimentally by determining the standard values of the selectivity coefficient (K). Thus, if the obtained value of K is greater than 1, the resin indicates more affinity for ion B, whereas if the experimental value of K is lesser than 1, the resin exhibits more affinity towards ion A.

7.3.2 Capacity

Another significant feature of ion exchange resin is its capacity. Capacity is characterized as the potential of an ion exchanger to adsorb its counterions [2]. The total capacity of an ion exchanger is the amount of charged groups per unit weight of a dry exchanger. On the other hand, the amount of counterions that can bind to the ion exchanger under specific experimental conditions is the available capacity of the exchanger. The exchange capacity of a cation exchange resin may be calculated experimentally by determining the amount of sodium (Na^+) ion which are absorbed by 1 g of the dry resin in H^+ form. Similarly, the exchange capacity of a strongly basic anion exchange resin is estimated by quantifying the amount of chloride (Cl^-) ion taken up by 1 g of dry resin in OH^- form. The exchange capacity is usually expressed as millimoles per gram of exchanger. In some cases, the porosity of the resin matrix decreases due to the presence of excessive covalent cross-linking. As a result, large molecules cannot enter the matrix and can bind only with surface-charged groups. Thus, in these cases, the available capacity will be significantly reduced in comparison to the total capacity [2].

7.3.3 Selection of the Ion Exchange Resins

During protein purification, the selection of appropriate ion exchange resin primarily confines the folding and stability of target proteins and their relative molecular mass or size. The following criteria are used for the selection of ion exchange resin.

7.3.3.1 Choice of Anionic and Cationic Exchangers

Usually, a cation exchanger should be used if the target protein shows maximum stability below its pI value. Preparing a protein solution under an appropriate buffer condition at a pH lower than its pI results in a net positive charge on the protein, which is then purified using a cation exchanger [2]. On the other hand, when the target protein is most stable above its pI value (giving it a net negative charge), an anion exchanger is used [2]. Some proteins are stable over a broad pH range both above and below their pI value. Either type of exchanger can be used for the purification of these proteins [2].

7.3.3.2 Choice of Strong and Weak Exchangers

The preference between a strong and a weak exchanger is dependent on the target protein stability over various pH ranges. The expressions weak and strong do not refer to the binding strength of a protein to an ion exchanger, but rather to the degree of ionization of the exchanger as a function of pH. Due to their p*K*a values, strong ion exchangers are fully ionized over [3, 10, 15] a broader pH range compared to weak ion exchangers. A strong exchanger can be used if the pH required for binding of the target protein is very acidic (down to pH 2.0) or basic (up to pH 12.0) provided the target protein retains its stability at the working pH [2]. In this case, the functional groups remain charged over a larger pH range. In contrast, weak exchangers might be more suitable for proteins that do not require an extreme pH for binding. Weak exchangers are advantageous in protein purification because the binding tendency of weakly charged impurities is very low, resulting in improved elution characteristics [2]. The routinely used ion exchangers for protein purification are listed in Table 7.1 [25].

Table 7.1 Commonly used ion exchange resins

Sl. no.	Resin name	Type	Functional group	Matrix	Class	Refs.
1	SP-Sephadex	Cation Exchangers	Sulfopropyl	Dextran	Strong	[26]
2	P-Cellulose		Phosphate	Cellulose	Intermediate	[27]
3	Bio-Rex 70		Carboxylate	Acrylic	Weak	[28]
4	CM-Sephacel		Carboxymethyl	Sephacel		[29]
5	CM-Sephadex		Carboxymethyl	Dextran		[30]
6	QAE-Sephadex	Anion Exchangers	Diethyl-(2-hydroxyl-propyl)-aminoethyl	Dextran	Strong	[31]
7	AG3		Tertiary amine	Polystyrene	Weak	[32]
8	DEAE-Sephacel		Diethylaminoethyl	Sephacel		[33]
9	DEAE-Sephadex		Diethylaminoethyl	Dextran		[34]

7.3.3.3 Choice of Particle Size and Porosity of the Resin Matrix

Flow characteristics and chromatographic resolution of an ion exchanger are primarily determined by the particle size and porosity of the matrix particles. The pore size or degree of cross-linking of the resin matrix does not influence the selectivity but impacts the resolution of separation. Smaller pore size offers better resolution but usually imposes lower flow rates. This type of resin is frequently used while working with high-resolution media/buffer as well as in the last phase of preparative chromatography (for purification and separation) using smaller proteins. Viscous samples such as cell lysates or samples containing glycerol and larger proteins often cannot be isolated using small pore size resins due to the elevated backpressure of resins, which might exceed the column's working pressure level. A larger pore size matrix allows for increased flow rates but results in lower resolution. Large pore size resins are mostly employed for the purification of bigger proteins from the cell lysates when IEX is used in the initial steps of the protein purification process. Altogether, the viscosity and size of the protein define the selectivity of the resin porosity for its efficient purification [1–3].

7.3.4 Buffer

Buffers regulate small changes in pH by maintaining solutions within a constant pH range. A buffer system consists either of a weak acid and its conjugate base or a weak base and its conjugate acid. The pH range of a buffer is based on its p$K_{a,}$ which is defined as the pH at which 50% of the molecules are in their acidic, and 50% in their basic forms [35]. The effective range of a buffer to maintain a constant pH of a solution is when the pH of the solution remains at pKa $\pm$ 1. Commonly used buffers

Table 7.2 Recommended buffer substances for cation exchange chromatography

Sl. no.	Effective pH range	Buffer compounds	Conc. (mM)	Counter-ion	pKa (25 °C)	Refs.
1	2.6–3.6	Citric acid	20	Na^+	3.13	[36]
2	3.3–4.3	Lactic acid	50	Na^+	3.86	[37]
3	3.7–4.7	Succinic acid	50	Na^+	4.21	[38]
4	4.3–5.3	Acetic acid	50	Na^+ or Li^+	4.75	[39]
5	5.1–6.1	Succinic acid	50	Na^+	5.64	[40]
6	5.6–6.6	2-(N-morpholino)ethanesulfonic acid (MES)	50	Na^+ or Li^+	6.27	[41]
7	6.7–7.7	Monophosphate	50	Na^+	7.2	[42]
8	7.0–8.0	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	50	Na^+ or li^+	7.56	[43]
9	7.8–8.8	N,N-Bis(2-hydroxyethyl)glycine (BICINE)	50	Na^+	8.33	[44]

Table 7.3 Recommended buffer substances for anion exchange chromatography

Sl. no.	Effective pH range	Buffer compounds	Conc. (mM)	Counter-ion	p*K*a (25 °C)	Refs.
1	4.3–5.3	N-Methylpiperazine	20	Cl^-	4.75	[45]
2	4.8–5.8	Piperazine	20	Cl^- or $HCOO^-$	5.33	[46]
3	5.5–6.5	L-Histidine	20	Cl^-	6.04	[47]
4	6.0–7.0	Bis–Tris	20	Cl^-	6.48	[48]
5	6.2–7.2	Bis–Tris propane	20	Cl^-	6.65	[49]
6	7.3–8.3	Triethanolamine	20	Cl^- or CH_3COO^-	7.76	[50]
7	7.6–8.6	Tris	20	Cl^-	8.07	[51]
8	8.0–9.0	N-Methyldiethanolamine	20 or 50	SO_4^{2-} or cl^- or CH_3COO^-	8.52	[52]
9	8.4–9.4	Diethanolamine	20 or 50	Cl^-	8.88	[53]
10	8.6–9.6	bis-Tris propane	20	Cl^-	9.1	[54]
11	9.0–10.0	Ethanolamine	20	Cl^-	9.5	[55]
12	9.2–10.2	Piperazine	20	Cl^-	9.73	[56]
13	10.6–11.6	Piperidine	20	Cl^-	11.12	[57]

for cation exchange and anion exchange chromatography are enlisted in Tables 7.2 and 7.3, respectively [2].

7.3.5 Selection of Buffer

Buffer selection is an important part of the IEX as the electrostatic interactions between the oppositely charged surface and protein depend on buffer conditions. The choice of buffers broadly relies on the following factors:

7.3.5.1 Buffer Substance

Selecting a proper buffer substance is a critical factor for IEX. Buffering ions should have the same charge as the functional groups on the ion exchanger. Therefore, cationic buffers should be used while working with anionic exchangers and vice versa. If the buffering ions bear an opposite charge to that of the functional groups of exchangers, it participates in the ion exchange process by competing with proteins for binding sites. Thus, this process greatly reduces the capacity of the column. Additionally, it can also cause significant pH fluctuations while elution. Therefore it is always advisable to use identical charged buffering ions as the functional groups on the ion exchanger.

7.3.5.2 pH of the Buffer

Another critical factor for choosing a buffer is its working pH range. The foremost thing is the protein to be separated should be stable in the pH range of the buffer. Ensuring strong electrostatic interactions between the surface and protein, the pH of

the buffer should be kept in between the p*K*a of the surface functional groups and the pI of the protein molecules. Based on this criterion, the p*K*a of the chosen buffer should at least be within 0.7 units of the working pH; however, the ideal value is 0.3 units [58].

7.3.5.3 Ionic Strength of the Buffer

The ionic strength of the buffer has a major impact on protein adsorption, i.e., its attachment to the matrix. With increased ionic strength, the buffer counterions compete with the adsorbed protein molecules to substitute on the surface of the matrix. The salt ions also exhibit a shielding effect by hindering the interactions between the charged groups of proteins and surface binding sites. At a constant pH and ionic strength, the type of ionic species in the buffer might also interfere with protein binding. Sodium chloride (NaCl) is the most common salt to increase the ionic strength of the eluent while using IEC for proteins. The use of NaCl is advantageous as it does not alter the protein structure and is thus considered a gentle eluent. Nevertheless, NaCl is not always the greatest elution choice. It has been demonstrated that the type of the cations and anions influence the separation and chromatographic resolution [2]. Salts containing monovalent cations (such as Na^+, Li^+, and K^+) and monovalent anions (Cl^-, $HCOO^-$, CH_3COO^-) are preferred in the elution buffer [2, 59, 60]. The presence of multivalent ions (Ba^{2+}, Ca^{2+}, Mg^{2+}, Al^{3+}, Th^{4+}, SO_4^{2-}) in the buffer results in a reduced ion exchange rate as well as a considerable decrease in the surface charge of cation and anion exchangers. Thus, the affinity of proteins to the cationic and anionic surface decreases [2, 59–61].

7.3.5.4 Temperature of the Buffer

The p*K*a value of a buffering substance is dependent on temperature [62]. Consequently, the pH of the buffer varies with an increase or decrease in the temperature. For example, Tris has different p*K*a values at different temperatures. At 0 °C it shows a p*K*a value of 8.85, which is distinctly reduced to 8.06 and 7.72 when the temperature increases to 25 °C and 27 °C, respectively. Therefore, at certain temperatures, the buffer exhibits a very low buffering capacity that might result in a working pH different from the desired pH range for IEX. Thus, to evade this trouble, it is recommended to prepare buffer solutions at the same temperature at which they are supposed to be used.

7.4 Protein Purification Using Ion Exchange Chromatography

Purification of proteins by ion exchange chromatography using either cation exchangers or anion exchangers primarily consists of the following steps which are described below.

7.4.1 Equilibration

The first step of an IEX is the equilibration of the resin matrix, which provides a condition to ensure that the target proteins interact effectively with the matrix. In this step, an equilibration or starter buffer is used so that the charged groups in the matrix are surrounded by buffer exchangeable counterions. As the pH and ionic strength of the buffer play crucial roles, they are selectively chosen to ensure the proper binding of the protein of interest.

7.4.2 Loading of Sample

The given protein sample solution comprising the target proteins of interest and bacterial protein impurities is then loaded onto the resin in the same buffer that is used for equilibration. Under experimental conditions, proteins entering the resin may possess a negative charge, positive charge, or neutral charge. Proteins with the opposite charge as the resin bind tightly yet reversibly to the resin. The strength of binding depends on the charge and charge density (amount of charge per unit volume) of the solute. The greater the charge or the charge density, the stronger is the binding.

7.4.3 Washing

Extensive washing is done afterward to remove the nonspecific interaction of the proteins with the resin. Neutral proteins or those with the same charge as the resin do not show any affinity and washes out of the resin.

7.4.4 Elution

The bound proteins can be eluted from the resin using a buffer of increased ionic strength or pH (salt or pH gradient). As ionic strength increases, the proteins with the lowest net charge elute first from the column at the selected pH. Similarly, at a given pH, the proteins with maximum charge content are retained strongly and elute at the end. Choices of elution using increased ionic strength are of two general types such as linear gradient elution and step elution [63]. A linear gradient elution refers to a gradual increase of ionic strength in the elution buffer where weakly bound proteins elute first followed by stronger binding proteins. Linear gradients are ideal for the purification of an unknown sample or if peak resolution is important. In step elution, the ionic strength over which the target protein will elute is already known and it can be easily eluted using that particular ionic strength. Step elutions are often faster to perform and use less total volume to elute the protein in comparison to linear gradient elutions. The linear gradient elution and step elution are shown in Fig. 7.2.

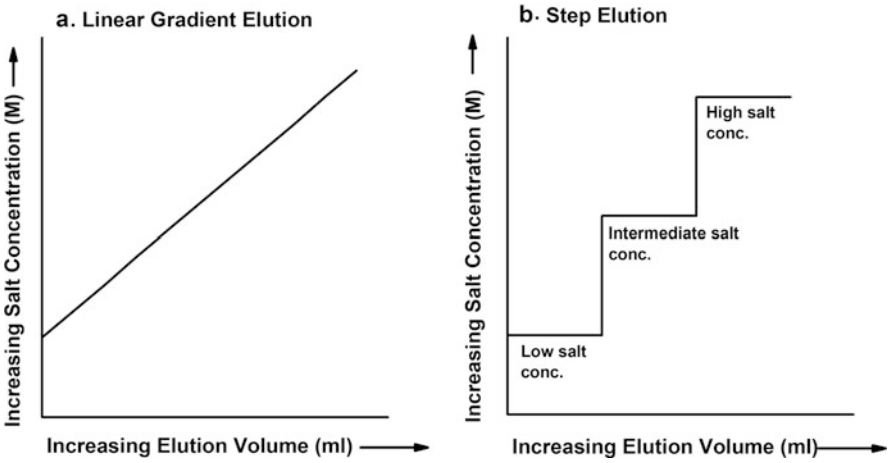

Fig. 7.2 Elution profiles of ion exchange chromatography using increased ionic concentration. Elution of proteins from ion exchange resin can be performed using increased ionic strength or salt concentration. This can be done in two ways such as linear gradient elution (**a**) and step elution (**b**). In linear gradient elution, there is a continuous elution of the proteins from the ion exchanger, whereas the elution of proteins is discontinuous in step elution

7.4.5 Regeneration

In the final step, increased ionic strength or increased pH buffer is used to eliminate any protein still bound to regenerate the resin for further use. Increasing ionic strength of the buffer releases bound proteins by displacement while increasing buffer pH weakens the interaction by lowering the charge on the protein or the resin [64]. All the steps of protein purification using ion exchange chromatography are illustrated in Fig. 7.3.

7.5 Instrumentation for Ion Exchange Chromatography

A typical ion exchange chromatography includes several elements that are schematically presented in Fig. 7.4 and described as follows.

7.5.1 Pump

The pump is used to deliver the mobile phase into the chromatographic system. It ensures a continuous and constant flow of the solvent through the injector, guard column, ion exchange column, and finally to the detector. The recommended flow rate depends on the ion exchange resin and should be included in the supplier's instructions. Usually, the chromatographic steps are carried out at a lower flow rate as compared to the column washing and equilibration steps.

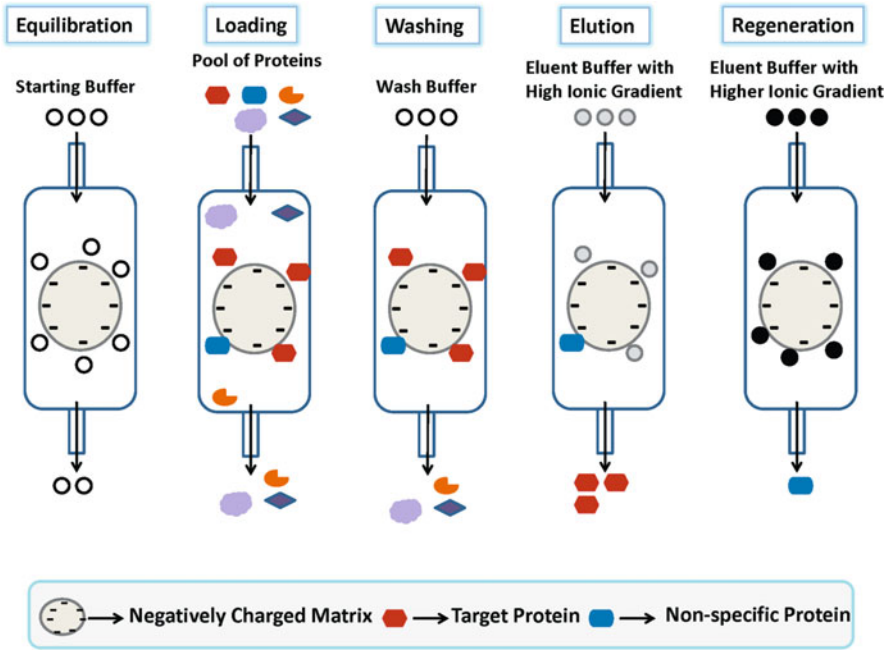

Fig. 7.3 Generic steps of ion exchange chromatography. A schematic model showing different steps involved in ion exchange chromatography. In the first step, equilibrium of the matrix (here, negatively charged) is done followed by loading the pool of protein samples. While the target protein binds to the matrix of the resin, other proteins flow through the column. The washing step ensures the removal of all nonspecific interactions. Elution steps involve deattachment of the target protein from the matrix using different ionic gradients. Finally, the column is regenerated for further use by using a higher ionic strength buffer to release if any proteins are still bound

7.5.2 Injector

An injector introduces the sample into the mobile phase or eluent stream into the column. It is capable of introducing small as well as a large volume of samples depending on the requirement.

7.5.3 Guard Column

A guard column is fitted next to the injector and is placed anterior to the ion exchange column. The role of a guard column is to protect the ion exchange column by filtering impurities and suspended particles that might clog the separation column.

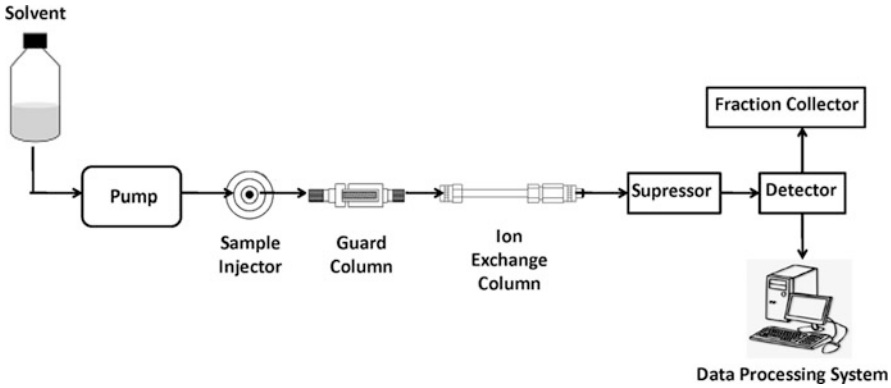

Fig. 7.4 Schematic overview of instrumentation for ion exchange chromatography. Ion exchange chromatography involves an automated system that includes a pump, sample injector, columns, suppressor, fraction collector, and detector coordinated through software. With the help of the pump, the solvent is passed through the guard column and ion exchange column at a certain flow rate. Protein mixture is introduced into the system through a sample injector and is separated by ion exchange columns based on their net charges. A suppressor reduces the conductance of the solvent. The elution of proteins is monitored through data processing by the detector and consequently collected using a fraction collector

7.5.4 Column

The choice of column for IEX depends on the target protein to be separated. If the target protein in the working buffer carries a net positive charge, a cation exchanger should be used, while if the target protein is negatively charged, an anion exchanger is preferred. Similarly, a strong exchanger is selected when the pH required for binding of the target protein is very acidic (down to pH 2.0) or basic (up to pH 12.0). On the contrary, when the pH required for binding of the target protein is moderate (pH 6–8), weak exchangers are chosen. The length-to-diameter ratio of a column varies according to the type of elution. For preparative (purification and separation) purposes using Fast Protein Liquid Chromatography (FPLC) and analytical (characterization) purposes using High Performance Liquid Chromatography (HPLC) most commonly used columns have a length and diameter ratio of 5:1 or lower (Details about FPLC and HPLC have been discussed in Chap. 8) [2]. Frequently used column sizes for preparative purposes of proteins are 10 × 50 mm (vol = 3.9 ml), 16 × 100 mm (vol = 20.1 ml), 26 × 200 mm (vol = 106 ml), or 50 × 300 mm (vol = 589 ml). Smaller columns provide better resolution in step elution whereas longer columns are further appropriate for linear gradient elution [2]. Short columns with a diameter of 5 mm and a length of 50 mm (vol = 1 ml) and 100 mm (vol = 2 ml) are more common for analytical purposes. The column material is usually made up of comparatively inert materials such as stainless steel, titanium, glass, or inert plastics [65].

7.5.5 Suppressor

In IEX, the eluent generally has a high ionic concentration. As a result, the conductivity of eluent increases which decreases the sensitivity of the detector. Conductivity is the measure of the concentration of ions present in the eluent [66]. This is calculated by the ability of the analyte to transmit an electrical current over a defined area. During ion exchange chromatography, a suppressor is installed between the column and the detector to lower the conductivity of the eluent, thus improving the sensitivity of IEX [66].

7.5.6 Detectors

For monitoring the column eluent, UV and conductivity detectors are used. While using UV detectors, two wavelengths are scanned such as 220 nm (for amide peptide bond detection) and 280 nm (for aromatic amino acid residues) [67].

7.5.7 Fraction Collector

An automated fraction collector with tube holders helps in collecting the sample that is eluted from the column.

7.5.8 Data Processing System

Provides a graphical representation of peaks from different fractions, stores data, and provides a graphical representation of the data.

7.6 Protocol for Recombinant Protein Purification

For the purification of a recombinant protein using ion exchange chromatography, the first step is to estimate the isoelectric point (pI) of the target protein to be purified using bioinformatics resources such as the ProtParam feature of ExPASy (Expert Protein Analysis Software) (details mentioned in Chap. 5). The estimated pI of the target protein helps to decide whether the cation or anion exchange chromatography to be used along with the pH of buffers. The detailed manufacturer instructions about the maximum and optimal flow rates of the columns should be carefully followed. A detailed protocol for the purification of recombinant protein using **anion exchange chromatography** is discussed as follows.

7.6.1 Instruments and Materials

- FPLC connected with a pump and a UV detector.
- QAE Sephadex A-25 resin or any other corresponding anion exchange resin.
- Recombinantly expressed protein sample.
- Binding buffer: 20 mM Tris–Cl, pH 7.5.
- Wash buffer: 20 mM Tris–Cl (pH 7.5)/100 mM NaCl.
- Elution buffer: 20 mM Tris–Cl (pH 7.5)/350 mM NaCl.
- Regeneration buffer: 20 mM Tris–Cl (pH 7.5)/2 M NaCl.
- Collection tubes.

7.6.2 Procedure

7.6.2.1 Step 1 (Equilibration of the Column)

- Follow the instructions given by the manufacturer to remove the column storage buffer and place the column in water.
- Equilibrate the resin with a minimum of 2 column volumes (CV) of the binding buffer. Before proceeding, verify that the readings on UV absorption (280 nm and 220 nm) are stable.
- Set the measurement to zero for UV absorption at both wavelengths.

7.6.2.2 Step 2 (Binding of the Protein Sample)

- Before loading, adjust the pH and salt concentration of the protein sample to initial optimal values. Be sure that the buffer composition of the protein sample is the same as the binding buffer used for the equilibration of the column. Else the protein should be dialyzed in the binding buffer.
- A fraction of the sample should be collected before loading into the column for running the SDS-PAGE assay later.
- Load the protein sample into the system by injecting the protein sample through the injector.
- If the pressure of the column goes beyond its maximum pressure, decrease the flow rate.
- A fraction of the flow-through should be collected.

7.6.2.3 Step 3 (Removal of Unbound Proteins)

- Wash the column with a minimum of 2 column volumes of the binding buffer.
- The UV absorbance both at 280 nm and 220 nm should be checked. The washing step should carry on till the absorbance (A_{280} and A_{220}) is zero or close to zero.
- A fraction of the wash flow-through should be collected.

7.6.2.4 Step 4 (Elution of the Bound Protein)

- *Elution with increasing salt concentration:* There are two methods for elution with increasing salt concentration. First, for gradient elution, apply a gradient from 0% (20 mM Tris–Cl, pH 7.5) to 100% (20 mM Tris–Cl, pH 7.5/350 mM

NaCl) of elution buffer over 20 column volumes. Collect each of the fractions. For step elution, apply the elution buffer containing 20 mM Tris–Cl, pH 7.5/ 350 mM NaCl over 10 column volumes.

- *pH-based elution:* For pH-based elution, the pH of the elution buffer is altered. For an anion exchanger, elution occurs when the pH of the elution buffer is decreased and vice versa. So apply 10 column volumes of low pH eluent buffer for elution of the desired protein.
- For both the elutions, the elution buffer should be run till there is no significant absorbance at 280 nm or 220 nm.
- Analyze the fractions (loaded sample, flow-through, washes, eluted protein) using gel electrophoresis (SDS-PAGE).
- Collect the pooled fractions of eluted proteins containing the purified desired protein.

7.6.2.5 Step 5 (Storage of the Protein)

- Dialyze to remove the excess salt from collected pooled fractions of the desired protein.
- Store it at −80 °C or liquid nitrogen as per the requirement.

7.6.2.6 Step 6 (Regeneration of the Column)

- Wash the column with 2–5 column volumes of the regeneration buffer (20 mM Tris–Cl, pH 7.5/2 M NaCl) to remove any very tightly bound species.
- Run 2 column volumes of binding buffer through the column to re-equilibrate the column with the binding buffer.

The protocol for *cation exchange chromatography* is exactly similar to that of anion exchange chromatography being the only differences are in the buffer composition and pH and the selection of appropriate cation exchanger resin.

7.7 Choice of Different Combination of Chromatographic Techniques

With the aim of achieving the highest level of purity and yield, the protein purification from a crude sample is often performed in a multi-step downstream process using the purification strategy of capture, intermediate purification, and polishing [68]. In the capture step, the desired protein is isolated, concentrated, and stabilized. During the intermediate purification step, the objective is to eliminate most bulk contaminants, like other proteins and nucleic acids. In the polishing stage when most of the impurities have already been removed, the goal is to attain high purity levels (>95%) by eliminating residual impurities or closely related moieties.

Ion exchange chromatography (IEX) is frequently used in most multi-step purification systems. If a specific affinity medium is not available or if the desired protein is little understood, IEX should be considered as the initial step towards any purification. This technique is highly flexible to be applied at any stage of

purification, viz., capture, intermediate, or polishing, based on the exact purpose. As IEX offers several selectivities (anion or cation exchangers), and the purification pH may be varied to alter the charge of target proteins, this technique might be used more than once under the same purification schemes. Additionally, IEX can be employed with gradual elution to provide the maximum resolution for a quick capture or with gradient elution in a polishing step.

- *Ion exchange as a capture step*: The goal of using IEX as a capture step is to rapidly adsorb the protein of interest from the crude sample and separate it from critical impurities such as proteases and glycosidases. As a result, the target protein is concentrated and transferred to an environment that will preserve its function. It is usually used in cases where the researcher prefers to purify the protein without a tag and hence affinity chromatography remains no longer an option [69]. This can be followed by hydrophobic interaction chromatography (HIC), reverse phase chromatography (RPC), and/or gel filtration chromatography (GFC) for further purification that has been elaborated in later sections of this book.
- *Ion exchange for intermediate purification*: The aim of using IEX for intermediate purification is to eliminate the majority of the significant impurities such as proteins, nucleic acids, endotoxins, and viruses. The main focus in a typical intermediate purification step is on capacity and resolution to maintain productivity (the amount of target protein processed per column in unit time) and achieve high purity. For example, when a protein after purification through affinity chromatography exhibits impurities comprising proteins of similar molecular weights, then IEX can be used as an intermediate step [70]. Afterward, HIC or RPC can be followed as the final polishing steps if GFC does not work very well.
- *Ion exchange as a polishing step:* Most contaminants are eliminated when IEX is used for polishing, except for trace quantities of closely related moieties such as structural variations of the target protein, nucleic acids, viruses, or endotoxins. The goal of the separation is to minimize these variants and trace impurities to levels that are acceptable for the application. In contrary to capture steps where quick, high-capacity step-elution is usually utilized, a polishing step focuses on producing the maximum possible resolution [71].

Like other chromatographic separation techniques, IEX is rarely sufficient as the sole purification stage for the separation of crude protein mixture samples. Ion exchange is frequently combined with other techniques such as gel filtration chromatography, hydrophobic interaction chromatography, reversed phase chromatography, as well as affinity chromatography for obtaining proteins of very high purity to be used in structural and biophysical studies.

7.8 Advantages and Disadvantages of Ion Exchange Chromatography

Alike other chromatographic techniques, IEX also has some advantages as well as drawbacks. Some of them are discussed below:

7.8.1 Advantages

- It is one of the most effective techniques for separating charged particles and can be employed for nearly any category of charged molecules including large proteins, small nucleotides, and amino acids.
- The purification technique does not require any additional tag and hence is capable of retaining the native architecture of the recombinant protein.
- IEX can be run with high flow rates. The high flow rate results in quick separation and purification of the target protein. Thus, it is crucial for the recovery of active protein.
- A high yield of the desired protein can be achieved through IEX.

7.8.2 Disadvantages

- One main disadvantage of IEX is that it can only separate the charged molecules. Molecules that do not carry any charges cannot be separated through IEX.
- The pH of buffers plays a crucial role in separation through IEX and a little alteration in pH can greatly alter the binding profile of proteins to the ion exchange resin.
- Excessive buffers with different ionic strengths or pH are essentially required while performing IEX which increases the overall cost of protein purification and separation using this technique.
- Since the method requires high salt concentration or high or low pH conditions for elution of desired proteins, the salt concentration and pH must be adjusted before they can be used for biochemical, biophysical, or structural studies.

7.9 Applications of Ion Exchange Chromatography

7.9.1 Purification of Recombinant Proteins

IEX is one of the widely used and efficient techniques for the purification of recombinant proteins. This technique is often combined with other techniques, which separate proteins depending on other parameters such as size (gel filtration), hydrophobicity (hydrophobic interaction chromatography or RPC), or biological activity (affinity chromatography). The protocols of protein purification using cation exchange and anion exchange chromatography have been discussed above. It is a

simple technique that includes loading the sample with the desired protein onto the chromatographic system and collection of the eluent with the aid of a fraction collector.

7.9.2 Purification of Enzymes

In the purification of biologically active enzymes, the retrieval of enzymatic activity is very crucial. Purification of enzymes with high activity recovery can be achieved through IEX. Additionally, the purification of isoenzymes can also be done with IEX. The enzyme isoforms usually have around the same molecular weight. This renders separation by gel filtration chromatography difficult. However, with the slight variations in charge owing to the variation of the amino acid composition, isoenzymes are separated by IEX. As an example, for the diagnosis of hematological malignancy, N-Acetyl β-D-glucosaminidases are intensively explored. An isoenzyme called "Intermediate 1 Form" was described in the case of common acute lymphoblastic leukemia [72]. The separation of these isoenzymes is improved by using chromatography with high-resolution ion exchange.

7.9.3 Miscellaneous Applications

Apart from these, IEX is routinely used for industrial purposes for analytical applications such as quality assessment. It is a useful tool in the downstream process to scrutinize the fermentation process of various enzymes [73, 74]. In clinical studies, this method is used for the separation and purification of blood components such as albumin and recombinant growth factors [75, 76].

7.10 Troubleshooting

Sl. no.	Problems	Possible reasons	Possible solutions
1	Decreased or no flow through the column	Outlet closed or leakage or pumps not working	Open the outlet, check for leakage and pumps
2	Poor resolution	Flow rate is either too fast or too slow	Adjust flow rate
		Suboptimal elution condition	Adjust elution conditions by altering the pH and ionic strength of the buffer
		Column overloaded or sample is too viscous	Decrease sample load or dilute as required
3	Protein does not bind or elute	Incomplete column equilibration	Run more equilibration buffer through the column until the conductivity and pH are stabilized

(continued)

Sl. no.	Problems	Possible reasons	Possible solutions
		Protein may be unstabilized in the elution buffer	Adjust the pH and ionic strength of the buffer
		Protein aggregated in the column	Clean the column and adjust buffer conditions for more protein stability
4	Lower protein yield	Protein degradation by proteases	Add protease inhibitor
		Protein aggregated in the column	Adjust buffer conditions for more protein stability
		Nonspecific adsorption or precipitation of the protein	Reduce the ionic strength of the elution buffer to minimize hydrophobic interaction. Add suitable detergent or organic solvent
5	Protein loses activity during the procedure	Protein is destabilized or aggregated in the column	Adjust buffer conditions for more protein stability
		During purification, a necessary cofactor has been removed	Add cofactor

7.11 Conclusion

In this chapter, we have discussed the separation and purification of proteins by IEX. Ion exchange chromatography is a highly adaptable method for protein purification, which is vital for some studies. The technique can be used at any stage of the purification level. Alongside, the availability of a large variety of resins offers a wide range of selectivity which can be tailored according to the experimental conditions or protein of interest. Although IEX is an incredibly versatile method for protein purification, the ability to separate only the charged molecules limits its use. Besides, the selection of appropriate resin and buffers requires extensive analytical skills of the users for the successful execution of this method. We will discuss more other chromatographic techniques for the purification of proteins in the subsequent chapters.

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Problems

Multiple Choice Questions

1. The capacity of the resin for ion exchange relies on:
   (a) The cumulative molecular mass of the resin
   (b) Length of the ion exchange resin
   (c) The total number of ion active groups
   (d) Solubility of the ion exchange resins
2. The concept of ion exchange chromatography is based on:
   (a) Electrostatic attraction
   (b) Electrical mobility of ionic species
   (c) Adsorption
   (d) Partition
3. In anion exchange chromatography:
   (a) The column contains negatively charged beads where positively charged proteins bind
   (b) The column contains positively charged beads where negatively charged proteins bind
   (c) The column contains both positive and negatively charged beads where proteins bind
   depending on their net charge
   (d) All of these

Subjective Questions

1. A protein has an isoelectric point (pI) of 5.2. What is the net charge on this protein in BICINE [(N, N-bis(2-hydroxyethyl)glycine] buffer (pH 8.5)? Explain.
2. A crude lysate sample comprising four proteins (1, 2, 3, and -galactosidase) is obtained by a protein biochemist. He wants to purify β-galactosidase using ion exchange chromatography. The respective isoelectric points of these proteins are enlisted below:

Protein	Isoelectric point (pI)
1	3.7
2	6.8
3	9.5
β-Galactosidase	5.3

(continued)

He equilibrated an anion exchange column using a buffer of pH 5.0. (A) At this condition, which protein(s) from the lysate sample will bind to the column? (B) How the bound protein(s) can be eluted from the anion exchange column? He then recognized the fraction containing β-galactosidase from the anion exchange column and opted to purify it using a cation exchange column. (C) Explain how a cation exchange column may be used to separate β-galactosidase from any residual contaminated protein (s).

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