20 Protein Purification

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20.1 HISTORY OF PROTEIN PURIFICATION

Early purification methods for proteins very much focused on reproducing and understanding observable physical phenomena.[1] For example, the fact that egg white hardens upon heating led to identifying the heat-coagulable component "albumin" (a name dating back to Pliny the Elder) by 1800. By this point, specific precipitation methods had been identified, including the use of tanning agents, mercury, or lead salts. This represents an early foray into the purification of a protein, even before its molecular features were recognized. Features of egg albumin were attributed to the basis of its behavior when subjected to various chemicals or reagents, including the fact that it contains sulfur. This phenomenology was further generalized to proteins in other contexts, such as those involved in the clotting of blood (i.e., the red water-insoluble fibrin) or curdling of milk (i.e., casein).

The discovery and characterization of the first enzymes in the early 1800's are other early forays into protein purification.[1,2] This included precipitation of the enzyme amylase, or "diastase", as reported in 1833 by Payen and Persoz, which could convert starch into sugar and be heat-inactivated. Pepsin was precipitated soon after that (1836) by Schwann and shown to digest food.

Moving forward, the chromatography techniques that are so ubiquitous in modern protein purification were developed initially as a means to separate pigments in plants (i.e., colored species).[1,3] This was first reported by Tswett in 1903, using a column of calcium carbonate. Color is no longer explicitly required (although certainly, many analyte detection schemes still rely upon specific absorbance behavior), but the name persists.

Electrophoresis also has a history dating back to the early 1800s, with application to proteins beginning in the early 20th century.[4] Its applicability to proteins became much wider in the ~1960's with the development of zonal electrophoresis in gels. The introduction of sodium dodecyl sulfate (SDS) as a means to denature protein complexes in 1969 by Weber and Osborne facilitated the subsequent separation of protein components (subunits) according to their approximate molecular masses.[5] So, gel electrophoresis is a relative newcomer to the suite of applicable techniques for protein purification.

20.2 PRINCIPLE OF PROTEIN PURIFICATION

Protein purification is a multistep process involving cell/tissue lysis and subsequent purification (see general workflow in Figure 20.1 and detailed discussion below). While different purification protocols may be applied for a given target protein, the choice of the exact protocol depends heavily on the type of starting material available and the intended use of the purified protein. Some tasks such as centrifugation, which is crucial for the separation of soluble and precipitated components of a target protein-containing mixture, yielding pelleted (i.e., insoluble) and supernatant (i.e., soluble) components,[4] may have only passing mentions below, they nonetheless demand full attention and perfect execution like any other tasks in the protocols. After release from cells or tissues, proteins are liable to denaturation. Therefore, all steps during cell/tissue lysis and subsequent protein purification should be ideally conducted at low temperature (4°C) to reduce possible protein denaturation. Additionally, the inclusion of one or more protease inhibitors in lysis buffer prior to cell/tissue lysis is also helpful in minimizing protein denaturation.

20.2.1 CELL/TISSUE LYSIS

As proteins are localized inside cells or tissues, the first step for protein purification involves cell/tissue lysis/disruption so that proteins are released for subsequent purification. While a diversity of cell or tissue lysis/disruption protocols are available, in principle, they are either mechanical or non-mechanical.

Mechanical disruption relies on sonication, high-pressure (French press), or mechanical force (grinding, shaking, or stirring in the presence of glass beads) to break up microbe, plant, or animal cells and tissues.[6–8] Sonication produces high-frequency ultrasonic vibrations through resonance (15–25 kHz) of a tuned probe or horn, which cause the collapse of formed microbubbles and generate shock waves to disintegrate cell walls and destroy nucleic acids (leading to reduced viscosity). High-pressure disruption with homogenizers and extruders is carried out by forcing a pressurized cell solution through a narrow opening, such as a needle valve in a French press apparatus or a more complex two-stage design in a Gaulin homogenizer. Cell rupture occurs through a combination of mechanisms, with resulting pressure differentials, fluid shear, impact forces, and/or cavitation being proposed as the major forces responsible for disruption.[9] The use of glass beads to grind cells in suspension is also effective as a mechanical cell extraction technique through bead milling or bead homogenization. Bead milling can be accomplished using a magnetic stirrer, vortex mixer, blender, high-speed mils, agitator, or mixer. Cell concentration, bead size and composition, ratio of beads to suspension, processing duration, and force applied are important factors determining cell disruption.[10] Cells

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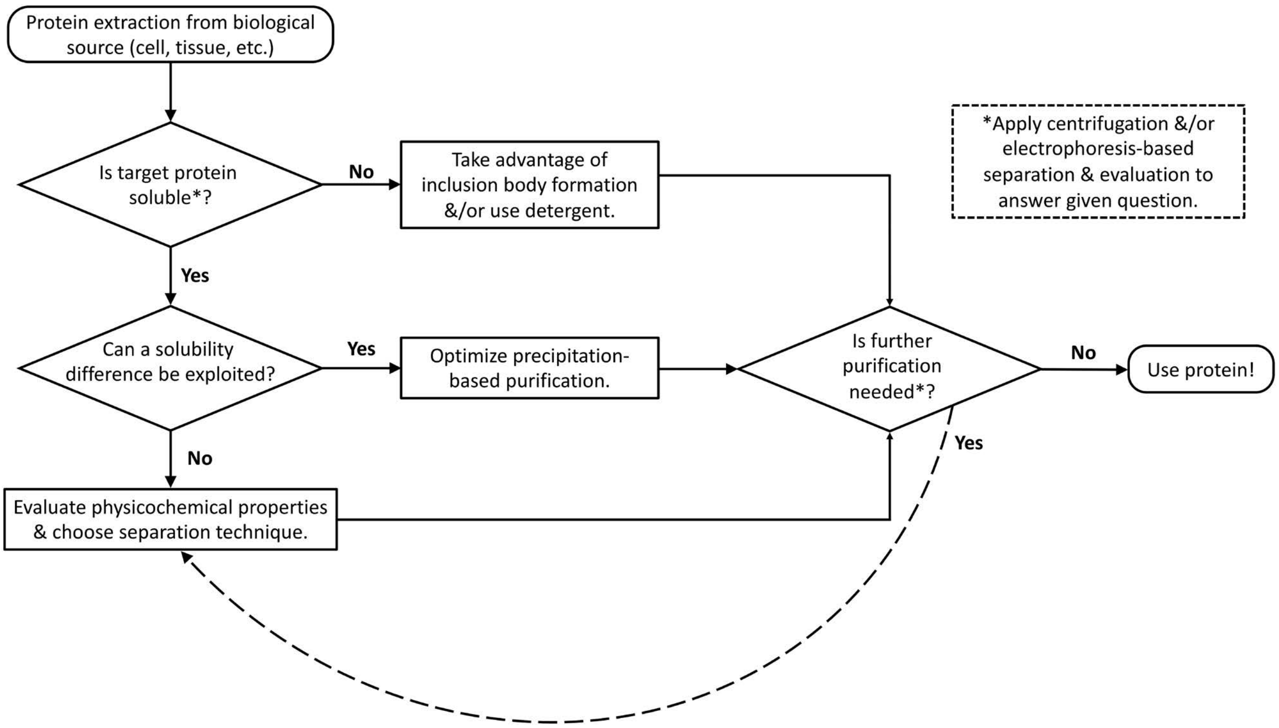

FIGURE 20.1 Flowchart illustrating interplay and relation between protein purification steps and methodologies, starting from the extraction of a target protein from a biological source through to the final production of a pure protein sample for use.

are crushed, ground, and torn apart by abrasive contact and forces between beads, cells, and the reaction chamber itself. Mechanical disruption suffers from the drawbacks of not being easily applied to cell pellets harvested from 5 mL of culture or less and producing excess heat and oxidation. However, small-scale sonicator heads and microplate horns are now available for high throughput and mechanical disruption of samples less than 5 mL.

Non-mechanical lysis employs enzymes (e.g., lysozyme and other lytic enzymes), chemicals (salt, alkali, detergents), or temperature (repeated freezing and thawing) to break up cells and tissues.[11] In the presence of detergents (e.g., SDS, Triton X 100, CHAPS), lysozyme and other lytic enzymes effectively lyse cell wall, and subsequent addition of DNAse helps digest genomic DNA and decrease viscosity.[12] Highly active enzyme mixtures (comprising lysozyme and DNAse) and lysis reagents are available commercially and can be used alone and in combination to streamline cell lysis and free up proteins. In addition, reagents containing active enzymes have been developed to simultaneously lyse *Escherichia coli* cells and release recombinant proteins without cell harvest, thus permitting automation and easy scalability from even very small amounts of starting materials.[13,14] This approach reduces the number of centrifugation steps required to separate cells from culture media and crude extract and minimizes damage associated with mechanical disruption.[15] As the entire cell growth, extraction, and purification process can be done in a single tube, this approach saves time and resources. However, enzymatic lysis has some inherent disadvantages, such as detergent incompatibility in some instances, detergent impurities that can affect the downstream processing, and fractionation methods that depend on hydrophobic interactions. Chemical and heat lysis/disruption include thermal lysis,[16] osmotic shock,[17] and alkaline lysis,[18] which may be considered according to budget, target cell type, protein sensitivity, and duration.[19] Compared to mechanical disruption, non-mechanical lysis (involving enzyme, chemical, or heat) is very easy to use and does not require expensive instruments.

20.2.2 PURIFICATION

After cell or tissue lysis/disruption, the target protein needs to be separated from contaminants in the solution using precipitation, chromatography, inclusion body, or electrophoresis-based purification protocol. Specifically focusing on either (i) target protein or (ii) other contaminating components, precipitation-based purification takes advantage of protein characteristics (e.g., protein fold and amino acid content) that affect protein solubility, leading to partial or complete separation of protein(s) from contaminating components.

20.2.2.1 Precipitation

Precipitation typically involves the use of salts and/or alcohol. Salts can either stabilize soluble proteins by removing charge build-up at the protein surface (low salt concentration; salting in) or destabilize proteins by building up charge at the protein surface (high salt concentration; salting out).[20] The magnitude of the salt-based stabilizing or destabilizing effect varies with the content of hydrophobic residues in a protein. While various salts are useful, ammonium sulfate stands out due to the high stabilizing capabilities of both ammonium and sulfate, high solubility, and cost-effectiveness.[20]

Alcohols are another commonly used reagent type for protein precipitation. High concentrations of ethanol, for example, are thought to interact unfavorably with hydrophilic regions in proteins, promote the formation of α-helical secondary structure, and thus lead to instability in solution.[21] While other solvents can be used for the same purpose, 2-methyl-2,4-pentanediol (MDP) is found to be non-structurally perturbing to RNase-A at up to 60% v/v.[22]

Besides salts and alcohols, proteins can also be precipitated through primary or secondary effect affinity precipitation.[23] Specifically, primary effect affinity precipitation is a quick and simple protocol that results directly from ligand-protein binding, leading to precipitation of the larger complex.[24] Secondary effect affinity precipitation provides a more controlled and fine-tunable protocol. It involves the design of a polymer that complexes the target protein to form a compact hydrophobic affinity macro-ligand, which prevents unwanted interactions and facilitates multiple cycles of protein binding and release once certain conditions are met [23,25].

20.2.2.2 Chromatography

Chromatography takes advantage of a distinct physical property of a target protein for effective separation from impurities and is often done in five different ways (i.e., ion exchange, size exclusion, affinity, hydrophobic interaction, and high-performance liquid; Figure 20.2). The nuances of each protocol are discussed in the sections that follow, with the ultimate preference being guided by careful consideration of the physicochemical properties and behavior of target protein in relation to impurities.

20.2.2.2.1 Ion-Exchange Chromatography (IEC)

Ion-exchange chromatography (IEC) separates biomolecules such as proteins, peptides, or amino acids on the basis of their total charge,[26,27] and depending on the charge of the resin employed, it can involve either anion or cation exchange (Figure 20.2). Specifically, the ionization state of arginine, histidine, and lysine side chains modulates a polypeptide's cationic nature, while the ionization of aspartate, cysteine, glutamate, and tyrosine side chains modulates a polypeptide's anionic nature.[28,29] The net charge of a protein is, thus, pH-dependent, with the pH at which it has zero net

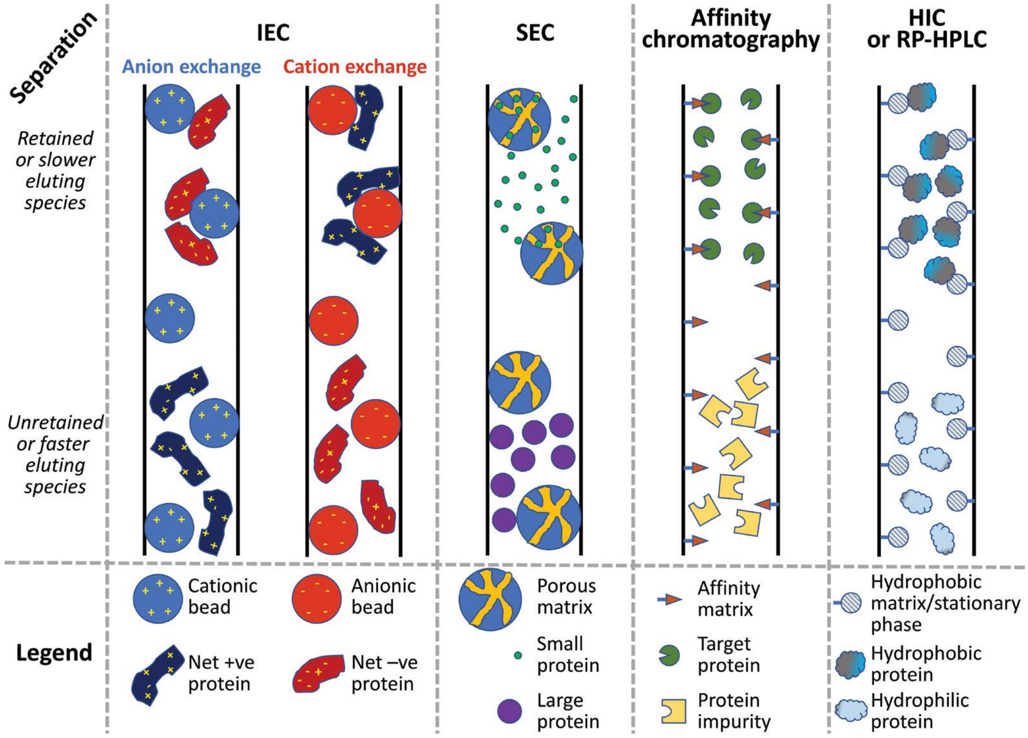

FIGURE 20.2 Illustration of four major classes of chromatographic separation introduced herein: Ion-exchange chromatography (IEC), size exclusion chromatography (SEC), affinity chromatography, and the two hydrophobicity/polarity-based methods: Hydrophilic interaction chromatography (HIC) and reverse phase high-performance liquid chromatography (HPLC). In each instance, two protein species are illustrated: One with physicochemical properties that lead it to be more retained on the stationary phase and more slowly eluted, concentrated toward the top of the column, and a second with properties that lead it to be less retained and more rapidly eluted, concentrated toward the bottom of the column.

charge defined as its isoelectric point (pI). The pI may be estimated based on the amino acid sequence.[30] Above its pI, a protein will have a net negative charge that will allow it to bind to a positively charged anion exchange medium. Conversely, at a pH below its pI, a protein will have a net positive charge and will bind to a negatively charged cation exchange medium. Positively charged diethylaminoethyl (DEAE) and quaternary ammonium (Q) groups are typically employed anion-exchangers, while negatively charged carboxymethyl (CM), sulfomethyl (S), and sulfopropyl (SP) groups are routinely used as cation-exchangers.[27,31] These charged functional groups are covalently bound to a solid matrix such as agarose, cellulose, dextran, polyacrylamide, silica, or others.

When a sample containing a target protein of interest is loaded into an ion exchange column containing a matrix with one of these charged functional groups, the oppositely charged proteins bind to the ionic groups of the resin, whereas uncharged or similarly charged proteins will be washed out through the column. Bound proteins are most frequently eluted by increasing the ionic strength of the buffer or, occasionally, by changing the pH. The salt ions (usually Na^+ and Cl^-) compete with the bound protein to interact with the charged groups immobilized in the matrix.[31,32] A protein with the lowest net charge will be eluted first as ionic strength increases. Proteins with higher net charge require higher ionic strength for elution and will have a greater retention time. The target protein can then be collected as a function of ionic strength in the eluent fractions.

20.2.2.2.2 Size Exclusion Chromatography (SEC)

Size exclusion chromatography (SEC), also referred to as gel filtration chromatography, separates molecules based on their molecular size by filtration through a resin.[33–35] SEC resins may be composed of different materials, including natural polymers, such as cross-linked agarose or dextran, and synthetic polymers, such as polyacrylamide.[36] The resins are physically and chemically stable and form pores of a characteristic size that act effectively as a molecular sieve (maze). Unlike other chromatography techniques, molecules do not bind to SEC resin thus the pore size is the critical factor for separation.[35] Each resin has an "exclusion limit", which indicates the minimum size of a molecule that is excluded from entering into the pores and will therefore elute in the void volume, which is ~30% of the column volume (CV). Conversely, molecules that are small enough to enter the pores of the resin will penetrate deeply and spend more time in the beads, eluting last.[36] These extremes in elution behavior are illustrated in Figure 20.2. Molecules between these sizes are able to partially enter the pores, giving rise to intermediate elution times. The eluent is typically collected in equal-sized fractions from which a target molecule can be recovered according to molecular weight.

If a sample containing a target protein of interest is placed on the column, and a buffer is allowed to flow slowly through the column, different components of the sample flow down the column at different rates depending on their size. Very large proteins cannot enter into the pores and elute faster in the void volume. Conversely, target proteins enter into the pores with varying degrees of effectiveness and separate gradually as they pass through the column. Other small-sized proteins will elute slowly, and salt like NaCl will elute at a volume equal to the CV. As a result, a highly pure protein sample can be collected from the fractions.[34,36]

20.2.2.2.3 Affinity Chromatography

Introduced in 1968 by Cuatrecasas and Wilchek,[37] affinity chromatography is a liquid chromatographic technique based on the highly selective interaction between two molecules[38] (e.g., a protein with the triangular affinity-binding site, contrasting with the semicircular binding site that does not bind, in Figure 20.2). Purification and resolution of "catch-and-release" models like enzyme-substrate, receptor-ligand, or antibody-antigen, etc., are achieved by exploiting specific non-covalent (electrostatic, hydrogen bonding, van der Waals and/or hydrophobic) interactions between the biomacromolecular functional groups in the mobile phase and specific ligand molecule that is covalently attached to a derivatized polymer (agarose, polyacrylamide, etc.) forming the stationary phase.[39] Unlike electrophoresis (Section 20.2.2.4), affinity chromatography is independent of the physical properties of the macromolecule.

Immobilized metal ion affinity chromatography (IMAC) is a very widely used affinity purification technique and is frequently employed to purify recombinant proteins expressed in *E. coli* for biochemical and especially structural studies.[40,41] Chelated metal ion (Cu^{2+}, Ni^{2+}, etc.) complexes, covalently attached to the affinity matrix, mimic bio-specific interaction sites.[42] Short peptide sequences, called fusion tags, engineered onto the N-terminus, C-terminus, both termini and/or within a recombinant protein of interest facilitate the binding of target proteins onto these selective adsorption sites.

Agarose modified with nickel-nitrilotriacetic acid (Ni-NTA) is frequently used as the stationary phase for recombinant proteins carrying a poly-histidine fusion-tag.[42] Metal-protein recognition is facilitated through His residues of the fusion-tag that bind to vacant positions in the coordination sphere of the Ni^{2+} in complex with NTA when a protein preparation (whether cell lysate or downstream from that stage) is passed through the column.[42] While the target protein is retained by the column, all other unbound proteins and contaminants are washed out using a suitable solvent. Bound protein may then be eluted from the column by reducing the binding affinity through alteration in the pH, pI, charge, salt concentration, ionic strength, or through the addition of excess ligand or a competitive binder like imidazole in buffer solution.[43] Hence, this bind-wash-elute procedure serves as a single-step purification technique in recombinant protein production, typically yielding >80% pure protein. Depending on protein location, solubility, accessibility of the His-tag, and desired downstream application, either native or denaturing conditions may be used.

20.2.2.2.4 Hydrophobic Interaction Chromatography (HIC)

Hydrophobic interaction chromatography (HIC) separates proteins on the basis of hydrophobicity, as first introduced by Tiselius, who noted that "proteins may be adsorbed on silica gel and filter paper in salt solutions of concentrations less than those necessary for normal precipitation by salting out".[44] Taking advantage of adsorption behavior of proteins in salt solutions, Amersham Pharmacia Biotech introduced the first generation of commercial HIC adsorbent,[45] which has been widely adopted for both laboratory- and commercial-scale protein purification applications.

Several theories have been proposed to explain protein adsorption onto HIC adsorbents. Srinivasan and Ruckenstein postulated that HIC results from van der Waals attraction between proteins and adsorbents, as the van der Waals force between protein and adsorbent increases when nearby water molecules become increasingly ordered in the presence of kosmotropic salts.[46] An alternative theory is based on the similarity between the effect of kosmotropic salts in salting out and in HIC, with water-solute interactions weakened in favor of the water-salt ion interactions facilitating hydrophobic interaction between protein and adsorbent.[47–49] Alternatively, it is suggested that protein-adsorbent interactions are entropically favorable, as hydrophobic interactions between two or more nonpolar molecules in a polar solution are spontaneous due to displacement of ordered water molecules caging a hydrophobic moiety into bulk water, allowing for an increase in entropy.[50–54]

HIC separation specifically exploits differences in protein hydrophobicity (Figure 20.2). The hydrophobic ligand of the HIC adsorbent establishes a reversible interaction with hydrophobic patches on the surface of a protein; thus, proteins will have different binding profiles highly dependent on their amino acid sequences and tertiary structuring.[45,52,55] The HIC adsorbent-protein interaction is significantly influenced by the presence of kosmotropic salts in the mobile phase, where elevated salt concentrations enhance and decreased salt weakens the interaction. Thus, HIC separation is generally performed by solubilizing proteins in a solution containing a high concentration of kosmotropic salt to promote adsorption. A linear or stepwise decrease in the salt concentration is then applied to cause sequential desorption of proteins dependent on their hydrophobicity. The protein-adsorbent interaction is weakened as the ionic strength of the adsorption medium is reduced, with the least hydrophobic proteins eluting first and the most hydrophobic proteins eluting last (Figure 20.2).

Five key parameters affect HIC separation: ligand, kosmotropic salt, pH, temperature, and solvent additives. Of these, ligands (and their density) and salts (and their concentration) have the most significant influences in determining the final outcome of the HIC purification process.

Typically, HIC adsorbents consist of hydrophobic ligands with alkyl or aryl groups coupled to an inert matrix, which demonstrate high physical and chemical stability capable of maintaining low levels of non-specific protein interactions. Alkyl group ligands exhibit pure hydrophobic interactions, whereas aryl ligands show mixed-mode behavior, with both aromatic and hydrophobic interactions possible.[45,56,57] The choice between alkyl and aryl ligands is empirical and typically evaluated experimentally for the protein of interest. The loading of ligand on the matrix also determines its hydrophobicity,[45,57,58] with protein binding capacity of HIC adsorbents directly correlating with the amount of immobilized hydrophobic ligand and thus higher protein binding capacity in adsorbents with higher ligand loading.

Apart from adsorbent choice, the adsorption of a protein is influenced by kosmotropic salts that enhance water ordering[59] at relatively high concentrations but not at sufficient levels to cause protein precipitation (with the caveat that salting in and salting out may have somewhat distinct considerations relative to kosmotropic and chaotropic features[60]). Both the salt type and its concentration determine the ability of that salt to promote specific interaction between a protein and the adsorbent matrix.[61–63] Kosmotropic salts promote hydrophobic interactions between proteins and HIC ligands by reorganizing the waters of hydration to make hydrophobic regions more accessible. Thus, high ionic strength in the HIC solvent facilitates the hydrophobic interaction between the proteins and the ligands. As with HIC ligands, the choice of salt and its concentration are typically evaluated empirically to ensure proper protein binding while minimizing the binding of impurities.

Introduction of a water-miscible organic solvent weakens protein-ligand interactions in HIC through direct competition with the bound proteins for HIC ligand binding and a decrease in the surface tension of water, thus weakening hydrophobic interactions.[64] In contrast, the effects of pH and temperature in HIC are not as straightforward. In general, an increase in pH weakens the apparent hydrophobic interactions between proteins and hydrophobic ligands as a change in net charge of proteins increases their hydrophilicity, whereas a decrease in pH often strengthens the hydrophobic interactions.[52,65–67] Changes caused by a shift in pH are protein-dependent and rely on the pI of the protein, with pH variation near physiological conditions having little effect on HIC. The effect of temperature in HIC is not trivial, but an increase in temperature often strengthens hydrophobic interactions between proteins and the hydrophobic ligands.[51,68] Changes in pH, temperature, or addition of solvent additives can also affect protein stability, with the risk that proteins may be denatured or inactivated through these factors.

20.2.2.2.5 High Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) makes use of differences in the partitioning of analytes between a solid stationary phase and a liquid mobile phase, with the specific application of pump(s) capable of exerting high-pressure on the mobile phase.[69,70] A stable pumping system, sensitive detector(s), and stationary phase materials that are suitably small in dimensions all contribute to the ability to separate materials efficiently. Both flow rate and column dimensions may be adjusted in order to reduce separation time (i.e., higher flow rate to achieve faster elution) and change scale (i.e., larger column to increase the capacity of material that may be separated; longer column to increase the ability to separate materials), but these changes must be made carefully with consideration of the separation efficiency that will be achieved as reflected in the "theoretical plate number" for the separation. The sensitivity of an HPLC method for protein detection and separation can be further enhanced using the stationary phase material of smaller CV because the sample is more concentrated in the eluent when it reaches the detector. Namely, the smaller the column and the particle size of its stationary phase materials, the more optimal its separation speed is, both in theory and in practice.

The most common separation mode for protein samples is reversed phase HPLC[70,71] (RP-HPLC), which is based on analyte partitioning between a polar mobile phase and a nonpolar stationary phase. This is similar in principle to HIC (Figure 20.2), with distinctions between these techniques detailed below (Section 20.3). In the case of RP-HPLC, the earliest stationary phases were solid particles coated with nonpolar liquids. These were quickly replaced by covalently bonded hydrophobic groups, such as octadecyl (C_{18}) bonded groups on a silica support. Stationary phases with hydrocarbons of lower hydrophobicity (e.g., C_3 or C_4) may be applied, e.g., in instances where the analyte protein is more hydrophobic, such as in the case of membrane proteins.[72]

20.2.2.3 Inclusion Body

The protocols described above generally rely on proteins readily solubilized in non-denaturing buffers or, potentially, an organic solvent system. However, they may encounter failure when working with large proteins prone to aggregation, like spider silk proteins[73], that form insoluble inclusion bodies. Proteins behaving this way can be purified after either (i) solubilization or (ii) solubilization and washing away everything except the protein of interest. As an example for improving solubilization of aggregated proteins, protein-containing solutions can be incubated to increase solubility, as is done with small molecules.[74] However, unlike the solubility of small molecules, protein denaturation leads to a finite increase in solubility before thermal denaturation leads to instability in solution.

Chaotropic compounds (i.e., chemicals that disrupt non-covalent bonds) are widely used for solubilizing proteins. The mechanism has been investigated in depth with urea,[75] which tends to accumulate at the protein surface by displacing water molecules, stabilizing both nonpolar amino acids and the protein backbone. This lowers the energetic drive to bury hydrophobic residues, making protein unfolding energetically favorable and allowing the protein to be solubilized. While urea and guanidinium chloride[75,76] are the most commonly used chaotropic agents in protein purification, other chemicals containing chaotropic anions, such as sodium chloride[77], can be also used to solubilize proteins. Chaotropic ions from salts may promote protein solubility through the process of salting in.[78] At low-to-intermediate concentrations of salt (<1 M) and variable pH-dependent on the isoelectric point of a targeted protein,[78] proteins are stabilized due to the neutralization of surface charge.[20]

Other modifications to solubilization conditions may be necessary for very hydrophobic proteins, which can be solubilized by creating less polar local environments using surfactants. Water-miscible surfactants like Triton,[79] SDS,[80,81] or dodecylphosphocholine (DPC)[81] can form micelles in solution, encapsulating hydrophobic proteins. With a hydrophobic core and hydrophilic surface, these micelles can then remain soluble in aqueous solutions. This approach can also be applied to highly hydrophilic peptides or proteins by precipitating the protein to form an inclusion body and then purifying it in an organic solvent using an ionic surfactant to form the micelle.[82]

An additional step that may be necessary if a targeted protein inclusion body contains cystine residues is to break disulfide bonds. Namely, cysteines can form strong inter- and intramolecular disulfide bonds that would be unperturbed by other solubilization methods, preventing some proteins from unfolding into a soluble state. To break these disulfide bonds, reducing agents such as the thiol-containing 1,4-dithiothreitol (DTT) are generally used to outcompete cystine disulfide bonds at pH ranges typically used in a laboratory setting.[83] Other strong reducing agents, such as tris(2-carboxyethyl)phosphine (TCEP), can reduce disulfide bonds via nucleophilic attack and hydrolysis, leaving only the reduced cystines.[84]

20.2.2.4 Electrophoresis

Electrophoresis utilizes an electric field to separate a mixture on a porous medium in accordance with the physical properties (particularly net surface charge) of its components. The migration rate (ν) of each species, when passed through a porous medium (filter paper, gel matrix, etc.), is dependent on the applied electric field (E), net surface charge on the protein (z) and the frictional coefficient (f) as follows:

$$V = \frac{E_Z}{f} \tag{20.1}$$

where f is determined by the physical characteristics of the protein species (size, isoelectric point, surface charge, and tertiary structure) and on the viscosity (η) and porosity of the electrophoretic medium.[85] Depending on the protein type and experimental goals, different types of electrophoretic techniques may be used. Below, only the most popular electrophoretic technique, i.e., polyacrylamide gel electrophoresis (PAGE), is discussed.

20.2.2.4.1 Native Gel Electrophoresis

Originally developed by Ornstein and Davis in 1964, PAGE enables the separation of proteins with relatively large size differences. A gel matrix, prepared through the co-polymerization of acrylamide and *N',N'*-methylenebisacrylamide, acts as the molecular sieve to facilitate shape- and native charge-based separation. The pore size in the gel depends on the cross-linking agent and concentration of polyacrylamide. For molecules with the same molecular mass and shape, the one with the greater net charge will move faster toward an electrode. While the native conformation and biological activity of the protein are retained in native PAGE, results can be inconclusive when different proteins have a similar mass:charge ratio. Also, this technique can be misleading when tertiary shape and native charge enable the potential for migration to either electrode.

20.2.2.4.2 Denaturing Gel Electrophoresis

To improve the separation of proteins with a similar mass:charge ratio and reduce the potential effect of tertiary shape and native charge on protein migration, Laemlli introduced the denatured PAGE in 1970, where treatment with ionic detergent disrupts the non-covalent interactions within the native protein leading to the breakdown of the multimer and denaturation of the complex tertiary structure. An anionic detergent like SDS, owing to its amphipathic nature, forms an SDS-protein complex that imparts a uniform charge density across the linearized polypeptide chain, which not only improves protein solubility but also ensures that size is the only deciding factor in migration rate. Thiol reagents like β-mercaptoethanol (BME) or dithiothreitol (DTT) are often used to ensure that any disulfide bonds occurring in the protein are reduced, as these will also modulate migration rates.

20.3 UTILITY OF PROTEIN PURIFICATION

Protein purification is a vital biotechnological workflow with a significant bearing on the outcome of any downstream protein-based application. While a diversity of cell/tissue lysis and protein purification protocols are available, the decision to apply individual or combinational protocols to the purification of a given protein should be made on the distinctive physicochemical properties of each protocol and also the intended end use of this protein. Table 20.1 summarizes the principles and approaches of commonly used protein purification protocols, which will aid their selection for a given target protein. Naturally, the properties of the target protein and the impurities need also be taken into account in protocol selection.

Over and above the fundamental principles and approaches (Table 20.1), a variety of other parameters need to be considered in the selection of protein purification protocols (Table 20.2). Of particular note, some protocols require protein denaturation, which may prevent obtaining an active protein following purification.[4] The relative speed, selectivity, and cost of each protocol may also be important considerations. Not included in Table 20.2 is the ability to scale up a protocol worked out at small scale to purify larger amounts of protein. All of the protocols discussed herein are indeed amenable to scale-up with varying degrees of simplicity in this process – of all, electrophoresis is typically the most challenging to scale up.

Cell/tissue lysis. High-pressure homogenization and bead milling are often applied for large-scale protein

TABLE 20.1

Principles and Approaches of Commonly Used Protein Purification Protocols

Protocol	Principle	Approach
Precipitation	Differences in solubility – consider hydrophobicity and stability	Salting in or salting out of target protein; pH adjustment; alcohol precipitation
IEC	Differences in net protein charge and charge distribution – consider pI	Selection of ionic strength and pH on distinct charge of target protein for retention; increasing ionic strength or adjusting pH for elution
SEC	Differences in protein size – consider size (i.e., Stokes radius)	Selection of medium for distinct partitioning, penetration, and elution of target protein
Affinity chromatography	Differences in interaction with immobilized ligand – consider affinity (inherent or by fusion-tag)	Binding of target protein to immobilized affinity ligand; passage of flow-through and wash of impurities; elution of target protein by competition, pH change, etc.
HIC	Differences in binding to hydrophobic adsorbent – consider surface hydrophobicity	Choice of adsorbent to distinctively retain target protein; change of salt, pH, solvent, etc., to elute target protein
RP-HPLC	Differences in partitioning to hydrophobic stationary phase – consider hydrophobicity as a whole and size	Use of appropriate stationary phase and mobile phase combination to separate target protein; use of a solvent gradient to enhance separation
Inclusion body	Aggregation during protein expression leading to inclusion body formation	Differential resolubilization of target protein vs. other impurities in inclusion body
Electrophoresis	Differences in size, shape, and charge – may need to consider some or all of these parameters	Differential migration of proteins in PAGE gel as a function of charge and shape; use of SDS to denature and linearize proteins for size-dependent migration

TABLE 20.2

Key Parameters of Commonly Used Protein Purification Protocols

Protocol	Denaturation Requirement	Speed	Selectivity	Relative Cost[b]
Precipitation	Not if target remains soluble and organic solvent avoided	Fast	Poor	Low
IEC	No[a]	Intermediate	Poor	Intermediate
SEC	No[a]	Slow	Good	Intermediate
Affinity chromatography	No[a]	Intermediate	Outstanding	High
HIC	No[a]	Intermediate	Variable	Intermediate
HPLC	Yes	Intermediate	Good	High
Inclusion body preparation	Yes	Fast	Poor	Low
Electrophoresis	No[a]	Fast	Good	Low

[a] In some instances, denaturing conditions may be applied if beneficial to enhance separation.

[b] Cost of consumables/columns/mobile phase; required instrumentation (FPLC, HPLC, gel apparatus, etc.) is not explicitly considered.

extraction processes because of the ability to quickly and readily handle a variety of process volumes at a low cost. Sonication, chemical reagents including detergents, enzymatic treatments, thermal, and enzymatic plus chemical or physical method combinations are more often used in the lab, especially at the microscale. Comparative studies[86–88] on the effectiveness of these methods for microbial disruption and protein quantitation provide a good basis for selecting and optimizing methods appropriate for specific types of proteins and host cell structuring. These methods have also undergone many changes and improvements to keep up with the demands of modern proteomics studies.

Precipitaion. Bulk precipitation methods such as salting out can be highly beneficial when working with large volumes of sample or when it is desirable to quickly transfer a protein to a different environment due to purification-based (e.g., removal of certain salts) or experimentally-based (e.g., removal of high background buffer components) considerations. While this is a good preliminary purification method impacted by protein-specific factors like size and amino acid content, solubility based on surface charge is generally not variable enough between different proteins to give a high purity separation,[89] thus acting as a supplement for techniques with higher sensitivity like IMAC purification or HPLC.

In contrast, secondary affinity purification can yield selectively targeted proteins under highly controllable conditions. Through the use of polymers[23] or elastin-like polypeptides[90] which undergo phase transition in a temperature-dependent manner, this has been shown to yield highly pure protein without the need for any additional purification.[90] Combining this technique with the principles of salting out, changes in solution temperature can be minimized, allowing controlled precipitation at room temperature.[90,91]

Chromatography. Most of the chromatographic purification methods introduced above (Figure 20.1) may be performed through open column – usually gravity flow – methods, where columns are hand-packed, or through the use of instrumentation such as functional protein liquid chromatography (FPLC) or HPLC systems.[4] Methods that specifically rely upon HPLC or RP-HPLC are exceptions, as the high pressures required specifically rely upon appropriate chromatography instrumentation. Gravity flow-based methods are generally optimal in terms of simplicity and low-cost purification but are manual processes requiring ongoing monitoring of both the mobile phase and eluent. These methods also typically have a lower resolution than well-configured FPLC or HPLC, with reproducibility being more challenging if methods are not strictly controlled and normalized. This being said, methods such as affinity chromatography that inherently have a clear distinction between the unretained and retained analytes, with a specific buffer component added to induce elution, may be frequently optimally performed in a gravity flow manner. In all instances, a method developed using gravity flow can be translated to an FPLC or HPLC purification protocol, given appropriate columns and mobile phase pump capabilities.

Ion-exchange chromatography (IEC) can be applied for the separation and purification of biomolecules with high resolution and is used at scales from analytical to preparative and industrial.[92] Notably, isoforms and variants of proteins that have similar sizes but distinct charge properties can be successfully separated using IEC.[93] Specifically, slight changes in the amino acid composition of a protein may lead to a difference in net charge, with the gain or loss of a single charge group being sufficient to separate these by IEC. This ability to resolve even a single amino acid substitution makes IEC a powerful technique for the discovery and isolation of new proteins with limited structural information but known biological activity.[93]

This chromatography method can purify untagged proteins (for example, without a polyhistidine tag), uses nondenaturing buffers, permits high flow rates, and elutes proteins at high concentrations.[36] The sample loaded on the column must be in a buffer with low ionic strength; otherwise, the protein of interest will not bind to the resin.

Meanwhile, after elution, the protein will be in high salt concentration and should be dialyzed against a suitable buffer before storage.

Size exclusion chromatography (SEC) is used for both analytical and preparative purposes. Analytical SEC is usually performed to test the quality or properties of a sample, as it requires a small sample volume, 0.3% to 0.5% of the CV. Relatively large volume (i.e., 0.5% to 4% of the CV) can be applied in preparative SEC, allowing the isolation of one or more components from the mixture.[36] Notably, this technique can effectively isolate protein monomers and oligomers.[94] SEC is also an important analytical technique to monitor protein aggregation and to isolate soluble, homogeneous proteins from a sample containing a mixture of soluble and aggregated protein components.[95]

Besides purification, SEC can also be used for desalting and buffer exchange of protein samples.[96] The target protein will elute with the buffer used for equilibrating and running the SEC rather than the buffer composition of the sample loaded into the column. This way, a protein can be eluted in a buffer suitable for further application or storage. Moreover, proteins purified under denaturing conditions can be refolded by buffer exchange using SEC.[97,98] When a denatured protein is injected into a column equilibrated with a refolding buffer, the protein will refold while eluting with the refolding buffer or refold in the eluent fractions. Thus, a single run of SEC can refold the protein, remove aggregates, and lead to the elution of the protein in the required buffer for long-term storage or use.

Analytical SEC may also be used to estimate the molecular weight of proteins and protein-protein complexes.[99] To do so, the column is first calibrated with a mixture of proteins of known molecular weight. A calibration curve is determined based on the retention volume and log molecular weight for these known protein markers. By interpolating the elution volume of the target protein(s) with respect to the calibration curve, their molecular weight can be estimated. Adding to the accuracy of this determination and removing reliance on a calibration process, SEC coupled with multiangle laser light scattering is frequently applied to determine the molecular weight of proteins or supramolecular assemblies with high accuracy.[100]

Affinity chromatography. The development of affinity chromatography provides the potential to replace laborious and less efficient multistep procedures through drastic improvements in product recovery and resolution.[101] The design of specific affinity media and the application of appropriate tagging strategies make this a highly efficient and selective technique.[102] A major advantage comes from the high specificity and reusability of the matrix column, enabling sample recovery from very dilute solutions[38] and reducing the procedural cost, respectively.

Apart from the purification of synthetic proteins, affinity chromatography is widely used for nucleic acid purification, fragment screening in pharmaceutical and biomedical applications,[103–105] enzymatic studies, clinical diagnosis,[106,107] and therapeutics. Affinity columns are also now being employed to purify polymer nanoparticles, thus extending the scope of this technique to non-biomolecular systems.[39,108]

Despite the advantages of affinity chromatography for separating the protein of interest from bulk host proteins, this has the downside of requiring the production of fusion proteins that include the affinity tag. This may complicate downstream purification processes or potentially interfere with the biophysical and biochemical properties of the target protein. Thus, it is desirable to produce a tagless target protein by removing the affinity tags during the affinity purification step.

To facilitate the production of a tagless protein during affinity purification, techniques utilizing self-cleavable intein tags have been developed. An intein is an intervening protein sequence that may be embedded within a host protein sequence and undergoes a spontaneous post-translational self-cleavage reaction, concomitantly ligating the flanking host protein sequences to produce the mature protein.[109] Through protein engineering, modified inteins that undergo controllable N- or C-terminal cleavage with cleavage activity that can be induced by pH alteration or through the addition of thiol-containing compounds such as dithiothreitol[110,111] have been developed. More recently, studies have focused on controlling intein activity by reassembling inteins that are either naturally or artificially split into two fragments to minimize and improve the stability of the fusion protein and to allow rapid cleavage after the affinity chromatography purification step.[112–117] Thus, an intein sequence can be introduced as part of a fusion protein to produce a tagless target protein through controllable N- or C-terminal cleavage. Once the fusion protein has been affinity purified, the intein-mediated cleavage is initiated, liberating the target protein from the remainder of the fusion protein and thereby removing the tag along with the intein.[118–123]

Hydrophobic interaction chromatography (HIC) is widely used in analytical and preparative scale purification for protein capture, intermediate purification to remove major impurities, or as a final polishing step to achieve high purity protein products. Bhuvanesh et al. used HIC as a single capture and polishing step for the purification of *Brugia malayi* abundant larval transcript (ALT-2) through a step gradient of 500, 250, 100, 50 and 0 mM ammonium sulfate in 20 mM phosphate buffer at pH 7.2.[124] The final fraction collected at 0 mM ammonium sulfate yielded near 100% pure protein with a calculated overall yield of 60%. Another study by Lavery et al. similarly used HIC as a single capture and polishing step to purify peroxidase from horseradish (*Armoracia rusticana*) roots through a linear gradient of 1 M to 0 M ammonium sulfate in 50 mM phosphate buffer at pH 7.5.[125] The purified peroxidase has a net yield of 71%, gave 291-fold purification from crude material, and specific activity of 772 U per milligram of protein. Another study focused on purifying an antifungal protein from the *Bacillus subtilis* strain EDR4 and employed HIC as an intermediate purification step preceding anion exchange chromatography.[126] A different study looked at a novel protein endo-β-1,4-glucanase obtained from the giant snail (*Achatina fulica frussac*) used HIC as the final polishing step, in which the target enzyme was eluted with a linear gradient of 1.5 M to 0 M ammonium sulfate in 10 mM acetate buffer at pH 5.0.[127]. The enzyme was purified 29.5-fold with a specific activity of 12.3 U per mg protein against CMC, with a recovery of 14.7%.

HIC is emerging as a popular analytical technique for separating the molecular variants of monoclonal antibodies (mAbs) resulting from post-translational modifications. Valliere-Douglass, Wallace, and Balland (2008) found that methionine oxidation products of an antibody could be separated as introducing a foreign oxygen atom reduced the hydrophobicity of the molecule and allowed HIC separation based on the number of oxidation events.[128] They also found that monomeric and dimeric forms could be effectively separated. Moreover, it was found that soluble antibody aggregates could be readily separated, with resolution superior to SEC. Aspartate isomerization and formation of succinimides could also be separated from the native form through hydrophobicity changes in the molecule, affecting the retention time of the particular species.

HIC is also widely used to separate populations of cysteine-linked antibody-drug conjugates (ADCs) that differ in their number of covalently attached drug molecules. Due to the highly abundant cysteine residues in antibodies, drug conjugation often results in a heterogeneous population of ADCs that differ only with respect to the site of conjugation and the number of drugs per antibody.[129] These differences in drug-loading and conjugation sites are key as they can influence the pharmacokinetics, toxicity, clearance, and therapeutic activity of the ADCs.[130–132] From the studies by Wiggins et al. and Le et al., it was shown that HIC could separate these ADCs based on the difference in drug-loading.[129,133] Moreover, Hamblett et al. showed that analysis of an HIC chromatogram allows the characterization of the overall distribution of drug molecules attached to an ADC and allows the determination of the average number of drug molecules attached to the ADC through peak integration.[134]

High-performance liquid chromatography (HPLC) is a versatile analytical and preparative purification technique widely used to analyze pharmaceuticals, biomolecules, polymers, and many organic and ionic compounds.[69–71,135] This technique provides the advantages in application toward diverse analyte types with highly reproducible quantitative analysis. However, it tends to be most successfully applicable to proteins below ~30 kDa in molecular weight.[4] Through optimization of parameters in the analytical scale, appropriate methods for preparative processes can be worked out with low consumption of the material to be separated and mobile phase solvents. Scale-up may then be readily achieved by matching the separation properties employed at the analytical scale to those used at the preparative scale, typically by increasing column diameter with a corresponding increase in mobile phase flow rate. The high separation power of HPLC can also achieve highly sensitive detection through direct coupling with mass spectrometry (HPLC/MS). The limitations of HPLC are the lack of the ideal universal detector, lower separation efficiency than capillary gas chromatography, and difficulty in regulatory or quality control (QC) testing.

As noted above, HIC and RP-HPLC operate on a similar principle in that the lower polarity of the stationary phase relative to the mobile phase leads to partitioning or adsorption of nonpolar species to the stationary phase. Where RP-HPLC typically differs from HIC, however, is that the mobile phase will typically contain an acidic water/organic solvent mixture, with the ratio between solvents either being fixed (i.e., isocratic chromatography conditions) or varied to achieve a gradient of conditions where the mobile phase becomes less polar over the course of the chromatographic separation. HIC, conversely, more typically uses aqueous buffers, meaning that proteins that are not amenable to exposure to organic solvents may still be separated effectively from other species on the basis of hydrophobicity. Notably, in cases where the protein is amenable to RP-HPLC, this technique generally provides a more reproducible and generalizable purification method than HIC.[4]

Inclusion body. The use of chaotropic compounds is a very common approach to solubilizing inclusion body proteins.[75,76] Denaturants such as urea that stabilize hydrophobic interactions could be used at lower concentrations to solubilize less hydrophobic, low molecular weight proteins and separate them from larger proteins containing more hydrophobic character. While urea is a common and highly beneficial purification supplement for protein solubilization, special care can be needed depending on the application. In solution, urea can disassociate into isocyanate and ammonia.[136] Isocyanate then reacts with the N-terminal amine and side chains of lysine and arginine, to form carbamylated products. Beyond potential functional implications, this is problematic for proteomics studies, as it blocks digestion sites and changes the molecular weight of the protein, which can lead to identification issues. Carbamylation has been found to be minimized through the use of high concentrations of ammonium in urea-containing buffers.

When working with particularly hydrophobic components such as membrane proteins and phospholipids, solubilization with the help of detergent micelles is a commonly used technique. Depending on the downstream applications of a protein, many detergent options are possible to increase solubility[137]; however, for purification, Triton compounds are generally used.[73,138] The use of the detergent alone,[138] or in combination with a chaotropic compound like urea,[73] provides a method to tailor inclusion body purifications based on the suspected identities of contaminating proteins, potentially allowing simple, column free purification.

Regardless of the method used to purify an inclusion body, an important consideration is proper refolding of the protein. Each method of solubilizing proteins from inclusion bodies relies on disturbing the structure in the aggregated state. This could lead to multiple tertiary structures that can be adopted due to local energy minima, thereby giving undesirable products. To obtain a protein with the desired functionality, different approaches would be needed depending on the complexity of the protein (i.e., if the protein contains disulfide-bonded cystines in the native fold). To test for deviations from the expected secondary structure of the protein, bulk structural information from techniques like circular dichroism spectroscopy[21] or atomic-level structural information from nuclear magnetic resonance spectroscopy[139] could be compared to spectra of a properly folded version of the protein. If there is a mixture of correctly folded and incorrectly folded proteins, proteins with non-desirable tertiary structure can be separated from the desired protein through techniques like HPLC.

Electrophoresis. Following electrophoretic-based separation, staining, which enables protein visualization, is typically achieved using staining agents including silver or dyes such as Coomassie blue, amido black, etc. Autoradiography may also be employed to detect radioactive labels. To allow for direct inference of molecular weight, particularly in SDS-PAGE, a standard protein mix is typically loaded into one or more of the lanes of the gel and used as a marker to enable the identification of the protein weight.

Electrophoresis is typically used to examine the rate of protein expression, the effectiveness of another purification technique or step, estimate molecular size, estimate purity, determine isoelectric point (pI), and/or monitor the efficiency of enzymatic activity.[140] Taking the technique beyond strictly the analysis of gel features themselves, purified proteins can be extracted and used for downstream applications such as enzymatic assays, immunoblotting, and mass spectrometric analysis in areas like proteomics, forensic analysis, molecular biology, genetics, etc.

Apart from proteins, electrophoresis may also be used to separate and analyze macromolecules like nucleic acids (DNA, RNA), where agarose gels are replaced with polyacrylamide. Recent development of a novel electrophoretic technique, called dielectrophoresis, for the size and shape-dependent separation of materials like nanoparticles and nanotubes is widening the application of this technique.[141,142]

SUMMARY

Protein purification is a multistep process indispensable for any protein-based research and application. Beginning with a brief perspective on the history of protein purification, this chapter examines the key principles underlying cell/tissue lysis (both mechanical and non-mechanical) and subsequent protein purification (precipitation, chromatography, inclusion bodies, and electrophoresis), which are critical steps for obtaining protein(s) of high purity and quality. In the case of chromatography-based purification, the techniques of IEC, SEC, affinity chromatography, hydrophobic interaction chromatography, and HPLC are covered. The chapter ends with a detailed discussion on the utility of various protein purification protocols, emphasizing the physicochemical properties of target protein relative to impurities, which should be considered in helping determine the most appropriate technique for a specific purpose.

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