T. Gu,H. Chen, Z. Su and G. Ma, "Selection of Biochemical Separation Processes," in Perry's Handbook of Chemical Engineering, 8th Ed., McGraw-Hill, New York, pages 20-71 to 20-84 (2008).

Section 20

Alternative Separation Processes*

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CRYSTALLIZATION FROM THE MELT

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SUPERCRITICAL FLUID SEPARATION PROCESSES

ALTERNATIVE SOLID/LIQUID SEPARATIONS

MEMBRANE SEPARATION PROCESSES

SELECTION OF BIOCHEMICAL SEPARATION PROCESSES

of water from salt solution, the reverse of the actions in the diluate compartment (but having equal free energy). *Schaffer & Mintz* develop that change, and after solving the appropriate material balances, they arrive at a practical simplified equation for a monovalent ion salt, where activities may be approximated by concentrations:

$$\Delta G = RT(C_f - C_d)\left(\frac{\ln C_{fc}}{C_{fc}-1} - \frac{\ln C_{fd}}{C_{fd}-1}\right);\ C_{fc} = \frac{C_f}{C_c};\ C_{fd} = \frac{C_f}{C_d} \quad (20\text{-}108)$$

where C_f is the concentration of ions in the feed, C_d is the concentration in the diluate, and C_c is the concentration in the concentrate, all in kmol/m^3. When $C_{fc} \to 1$ and $C_{fd} \to$ infinity, the operation is one approximating the movement of salt from an initial concentration into an unlimited reservoir of concentrate, while the diluate becomes pure. This implies that the concentrate remains at a constant salt concentration. In that case, Eq. (20-108) reduces to $RT(C_f - C_d)$. As a numerical example of Eq. (20-108) consider the desalting of a feed with initial concentration 0.05 M to 0.005 M, roughly approximating the production of drinking water from a saline feed. If 10 ℓ of product are produced for every 3 ℓ of concentrate, the concentrate leaves the process at 0.2 M. The energy calculated from Eq. (20-108) is 0.067 kWh/m^3 at 25°C. If the concentrate flow is infinite, $C_c = 0.05$ M, and the energy decreases to 0.031 kWh/m^3.

This minimum energy is that required to move ions only, and that energy will be proportional to the ionic concentration in the feed. It assumes that all resistances are zero, and that there is no polarization. In a real stack, there are several other important energy dissipaters. One is overcoming the electrical resistances in the many components. Another is the energy needed to pump solution through the stack to reduce polarization and to remove products. Either pumping or desalting energy may be dominant in a working stack.

Energy Not Transporting Ions Not all current flowing in an electrodialysis stack is the result of the transport of the intended ions. Current paths that may be insignificant, minor, or significant include electrical leakage through the brine manifolds and gaskets, and transport of co-ions through a membrane. A related indirect loss of current is water transport through a membrane either by osmosis or with solvated ions, representing a loss of product, thus requiring increased current.

Pump Energy Requirements If there is no forced convection within the cells, the polarization limits the current density to a very uneconomic level. Conversely, if the circulation rate is too high, the energy inputs to the pumps will dominate the energy consumption of the process. Furthermore, supplying mechanical energy to the cells raises the pressure in the cells, and raises the pressure imbalance between portions of the stack, thus the requirements of the confining gear and the gaskets. Also, cell plumbing is a design problem made more difficult by high circulation rates.

A rule of thumb for a modern ED stack is that the pumping energy is roughly 0.5 kWh/m^3, about the same as is required to remove 1700 mg/ℓ dissolved salts.

Equipment and Economics A very large electrodialysis plant would produce 500 ℓ/s of desalted water. A rather typical plant was built in 1993 to process 4700 m^3/day (54.4 ℓ/s). Capital costs for this plant, running on low-salinity brackish feed were $1,210,000 for all the process equipment, including pumps, membranes, instrumentation, and so on. Building and site preparation cost an additional $600,000. The building footprint is 300 m^2. For plants above a threshold level of about 40 m^3/day, process-equipment costs usually scale at around the 0.7 power, not too different from other process equipment. On this basis, process equipment (excluding the building) for a 2000 m^3/day plant would have a 1993 predicted cost of $665,000.

The greatest operating-cost component, and the most highly variable, is the charge to amortize the capital. Many industrial firms use capital charges in excess of 30 percent. Some municipalities assign long amortization periods and low-interest rates, reflecting their cost of capital. Including buildings and site preparation, the range of capital charges assignable to 1000 m^3 of product is $90 to $350.

On the basis of 1000 m^3 of product water, the operating cost elements (as shown in Table 20-32) are anticipated to be

TABLE 20-32 Electrodialysis Operating Costs

$ 66	Membrane-replacement cost (assuming seven-year life)
32	Plant power
16	Filters and pretreatment chemicals
11	Labor
8	Maintenance
$133	Total

These items are highly site specific. Power cost is low because the salinity removed by the selected plant is low. The quality of the feed water, its salinity, turbidity, and concentration of problematic ionic and fouling solutes, is a major variable in pretreatment and in conversion.

SELECTION OF BIOCHEMICAL SEPARATION PROCESSES

General References: Ahuja (ed.), *Handbook of Bioseparations*, Academic Press, London, 2000. Albertsson, *Partition of Cell Particles and Macromolecules*, 3d ed., Wiley, New York, 1986. Belter, Cussler, and Hu, *Bioseparations*, Wiley Interscience, New York, 1988. Cooney and Humphrey (eds.), *Comprehensive Biotechnology*, vol. 2, Pergamon, Oxford, 1985. Flickinger and Drew (eds.), *The Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparations*, Wiley, New York, 1999. Harrison, Todd, Scott, and Petrides, *Bioseparations Science and Engineering*, Oxford University Press, Oxford, 2003. Hatti-Kaul and Mattiasson (eds.), *Isolation and Purification of Proteins*, Marcel Dekker, New York, 2003. Janson and Ryden (eds.), *Protein Purification*, VCH, New York, 1989. Ladisch, *Bioseparations Engineering: Principles, Practice, and Economics*, Wiley, New York, 2001. Scopes, *Protein Purification*, 2d ed., Springer-Verlag, New York, 1987. Stephanopoulos (ed.), *Biotechnology*, 2d ed., vol. 3, VCH, Weinheim, 1993. Subramanian (ed.), *Bioseparation and Bioprocesss—A Handbook*, Wiley, New York, 1998. Zaslavsky, *Aqueous Two-Phase Partitioning—Physical Chemistry and Bioanalytical Applications*, Marcel Dekker, New York, 1995.

GENERAL BACKGROUND

The biochemical industry derives its products from two primary sources. Natural products are yielded by plants, animal tissue, and fluids, and they are obtained via fermentation from bacteria, molds, and fungi and from mammalian cells. Products can also be obtained by recombinant methods through the insertion of foreign DNA directly into the hosting microorganism to allow overproduction of the product in this unnatural environment. The range of bioproducts is enormous, and the media in which they are produced are generally complex and ill-defined, containing many unwanted materials in addition to the desired product. The product is almost invariably at low concentration to start with. The goals of downstream processing operations include removal of these unwanted impurities, bulk-volume reduction with concomitant concentration of the desired product, and, for protein products, transfer of the protein to an environment where it will be stable and active, ready for its intended application. This always requires a multistage process consisting of multiple-unit operations. A general strategy for downstream processing of biological materials and the types of operations that may be used in the different steps is shown in Fig. 20-86 [see also Ho, in M. R. Ladisch et al. (eds.), *Protein Purification from Molecular Mechanisms to Large-Scale Processes, ACS Symp. Ser.*, **427,** ACS, Washington, D.C. (1990), pp. 14–34]. Low-molecular-weight products, generally secondary metabolites such as alcohols, carboxylic and amino acids, antibiotics, and vitamins, can be recovered by using many of the standard operations such as liquid-liquid partitioning, adsorption, and ion exchange, described elsewhere in this handbook. Biofuel molecules also belong

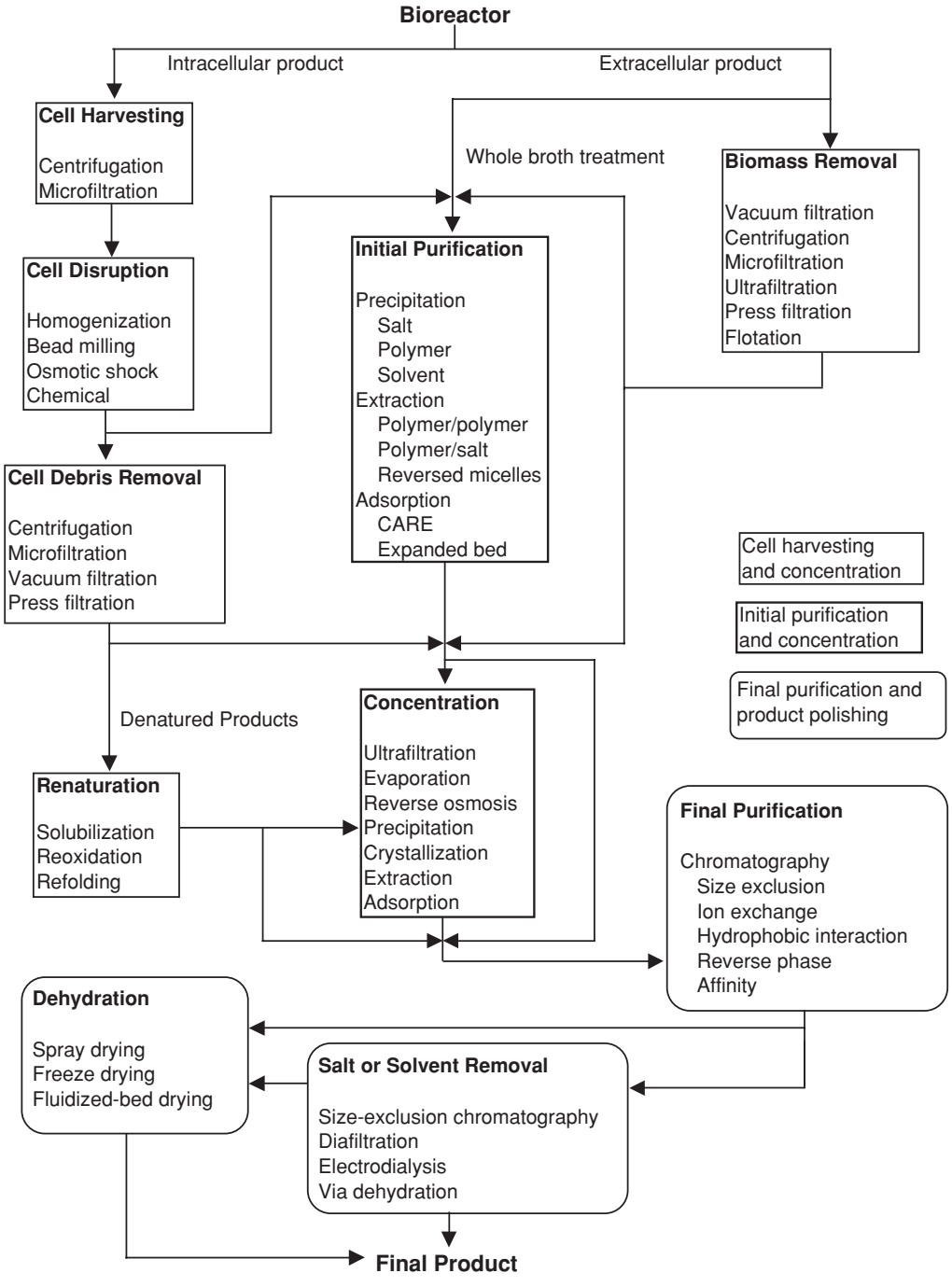


FIG. 20-86 General stages in downstream processing for protein production indicating representative types of unit operations used at each stage.

to this category. They are becoming more and more important. Less expensive and high-throughput unit operations are needed to make a biorefinery process economically feasible. Proteins require special attention, however, as they are sufficiently more complex, their function depending on the integrity of a delicate three-dimensional tertiary structure that can be disrupted if the protein is not handled correctly. For this reason, this section focuses primarily on protein separations.

Techniques used in bioseparations depend on the nature of the product (i.e., the unique properties and characteristics which provide a "handle" for the separation) and on its state (i.e., whether soluble or insoluble, intra- or extracellular, etc.). All early isolation and recovery steps remove whole cells, cellular debris, suspended solids, and colloidal particles; concentrate the product; and in many cases, achieve some degree of purification, all the while maintaining high yield. For intracellular compounds, the initial harvesting of the cells is important for their concentration prior to release of the product. Following this phase, a range of purification steps are employed to remove the remaining impurities and enhance the product purity; this purification phase, in turn, is followed by polishing steps to remove the last traces of contaminating components and process-related additions (e.g., buffer salts, detergents) and to prepare the product for storage and/or distribution. The prevention and/or avoidance of contamination is another important goal of downstream processing. Therapeutic proteins require very high purities that cannot be measured by weight percentages alone. To avoid potential harmful effects in humans, the levels of pyrogens and microbial and viral contaminates in final products must meet stringent safety and regulatory requirements.

Even for good yields of 80 to 95 percent per step, the overall yield can be poor for any process that requires a large number of steps. Thus, careful consideration must be given to optimization of the process in terms of both the unit operations themselves and their sequencing. A low-yield step should be replaced if improvement is unattainable to eliminate its impact on the overall yield. It is usually desirable to reduce the process volume early in the downstream processing, and to remove any components that can be removed fairly easily (particulates, small solutes, large aggregates, nucleic acids, etc.), so as not to overly burden the more refined separation processes downstream. Possible shear and temperature damage, and deactivation by endogenous proteases, must be considered in the selection of separation processes. Protein stability in downstream processing was discussed in depth by Hejnaes et al. [in Subramanian (ed.), vol. 2, op. cit., pp. 31–66]

INITIAL PRODUCT HARVEST AND CONCENTRATION

The initial processing steps are determined to a large extent by the location of the product species, and they generally consist of cell/broth separation and/or cell debris removal. For products retained within the biomass during production, it is first necessary to concentrate the cell suspension before homogenization or chemical treatment to release the product. Clarification to remove the suspended solids is the process goal at this stage.

Regardless of the location of the protein and its state, cell separation needs to be inexpensive, simple, and reliable, as large amounts of fermentation-broth dilute in the desired product may be handled. The objectives are to obtain a well-clarified supernatant and solids of maximum dryness, avoiding contamination by using a contained operation. Centrifugation or crossflow filtration is typically used for cell separation, and both unit operations can be run in a continuous-flow mode [Datar and Rosen, in Stephanopoulos (ed.), op. cit., pp. 369–503]. In recent years, expanded-bed adsorption has become an alternative. It combines broth clarification and adsorption separation in a single step.

Intracellular products can be present either as folded, soluble proteins or as dense masses of unfolded protein (inclusion bodies). For these products, it is first necessary to concentrate the cell suspension before effecting release of the product. Filtration can result in a suspension of cells that can be of any desired concentration up to 15 to 17 percent and that can be diafiltered into the desired buffer system. In contrast, the cell slurry that results from centrifugation will be that of either a dry mass (requiring resuspension but substantially free of residual broth, i.e., from a tubular bowl centrifuge) or a wet slurry (containing measurable residual broth and requiring additional resuspension). During the separation, conditions that result in cell lysis (such as extremes in temperature) must be avoided. In addition, while soluble protein is generally protected from shear and external proteolysis, these proteins are still subject to thermal denaturation.

Cell Disruption Intracellular protein products are present as either soluble, folded proteins or inclusion bodies. Intracellular protein products are very common because *Escherichia coli* is a main workhorse for recombinant proteins. *E. coli* is a gram-negative bacterium that precipitates recombinant proteins in the form of inclusion bodies. Release of folded proteins must be carefully considered. Active proteins are subject to deactivation and denaturation and thus require the use of "gentle" conditions. In addition, due consideration must be given to the suspending medium; lysis buffers are often optimized to promote protein stability and protect the protein from proteolysis and deactivation. Inclusion bodies, in contrast, are protected by virtue of the protein agglomeration. More stressful conditions are typically employed for their release, which includes going to higher temperatures if necessary. For "native" proteins, gentler methods and temperature control are required.

The release of intracellular protein product is achieved through rupture of the cell walls, and release of the protein product to the surrounding medium, through either mechanical or nonmechanical means, or through chemical, physical, or enzymatic lysis [Engler, in Cooney and Humphrey (eds.), op. cit., pp. 305–324; Schutte and Kula, in Stephanopoulos (ed.), op. cit., pp. 505–526]. Mechanical methods use pressure, as in the Manton/APV-Gaulin/French Press, or the Microfluidizer, or mechanical grinding, as in ball mills, the latter being used typically for flocs and usually only for natural products. Nonmechanical means include use of desiccants or solvents, while cell lysis can also be achieved through physical means (osmotic shock, freeze/thaw cycles), chemical (detergents, chaotropes), or enzymatic (lysozyme, phages).

In a high-pressure homogenizer, a pressurized cell suspension is forced through a valve and undergoes a rapid pressure change from up to 50 MPa to the atmospheric pressure. This results in the instant rupture of cells. Product release, which generally follows first-order kinetics, occurs through impingement of the high-velocity cell suspension jet on the stationary surfaces, and possibly also by the high-shear forces generated during the acceleration of the liquid through the gap. While sufficiently high pressures can be attained using commercially available equipment to ensure good release in a single pass, the associated adiabatic temperature increases (~1.8°C/1000 psig) may cause unacceptable activity losses for heat-labile proteins. Further denaturation can occur on exposure to the lysis medium. Thus, multiple passes may be preferred, with rapid chilling of the processed cell suspension between passes. The number of passes and the heat removal ability should be carefully optimized. The efficiency of the process depends on the homogenizing pressure and the choice of the valve unit, for which there are many designs available. Materials of construction are important to minimize erosion of the valve, to provide surface resistance to aggressive cleaning agents and disinfectants, and to permit steam cleaning and sanitization.

The release of inclusion bodies, in contrast, may follow a different strategy. Since inclusion bodies are typically recovered by centrifugation, it is often advantageous to send the lysate through the homogenizer with multiple passes to decrease the particle size of the cell debris. Since the inclusion bodies are much denser than the cell debris, the debris, now much reduced in size, can be easily separated from the inclusion bodies by centrifugation at low speeds. The inclusion bodies may be resuspended and centrifuged multiple times (often in the presence of low concentrations of denaturants) to clean up these aggregates. Since the inclusion bodies are already denatured, temperature control is not as important as in the case of native proteins.

Another popular method for cell disruption is to use a bead mill. In a bead mill, a cell suspension is mixed with glass or metal beads and agitated by using a rotating agitator at high speed. Bead mills have a controllable residence time compared with high-pressure homogenization.

However, they are susceptible to channeling and also fracturing of the beads. Tough cells require multiple passes to achieve a desired yield.

Chemical lysis, or solubilization of the cell wall, is typically carried out by using detergents such as Triton X-100, or the chaotropes urea, and guanidine hydrochloride. This approach does have the disadvantage that it can lead to some denaturation or degradation of the product. While favored for laboratory cell disruption, these methods are not typically used at the larger scales. Enzymatic destruction of the cell walls is also possible, and as more economical routes to the development of appropriate enzymes are developed, this approach could find industrial application. Again, the removal of these additives is an issue.

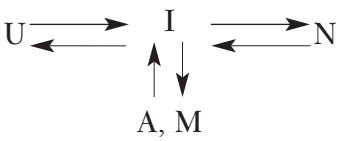
Physical methods such as osmotic shock, in which the cells are exposed to high salt concentrations to generate an osmotic pressure difference across the membrane, can lead to cell wall disruption. Similar disruption can be obtained by subjecting the cells to freeze/thaw cycles, or by pressurizing the cells with an inert gas (e.g., nitrogen) followed by a rapid depressurization. These methods are not typically used for large-scale operations.

On homogenization, the lysate may drastically increase in viscosity due to DNA release. This can be ameliorated to some extent by using multiple passes to reduce the viscosity. Alternatively, precipitants or nucleic acid digesting enzymes can be used to remove these viscosity-enhancing contaminants.

For postlysis processing, careful optimization must be carried out with respect to pH and ionic strength. Often it is necessary to do a buffer exchange. Cell debris can act as an ion exchanger and bind proteins ionically, thus not allowing them to pass through a filtration device or causing them to be spun out in a centrifuge. Once optimal conditions are found, these conditions can be incorporated in the lysis buffer by either direct addition (if starting from cell paste) or diafiltration (if starting from a cell concentrate).

Protein Refolding Although protein refolding is not a bioseparation operation, it is an integral part of a downstream process for the production of an inactive (typically intracellular) protein. So far, it still remains a challenging art casting an uncertainty on the success of a process. Early in the product development stage, animal cell culture may be required to produce many bioactive candidates for initial screening of efficacy. To commercialize the product protein, it may be reexpressed in a far more economic and productive host such as *E. coli.* The commercial products of recombinant DNA technology are frequently not produced in their native, biologically active form, because the foreign hosts such as *E. coli* in which they are produced lack the appropriate apparatus for the folding of the proteins. Thus, the overproduced proteins are generally recovered as refractile or inclusion bodies, or aggregates, typically 1 to 3 μm in size, and all cysteine residues are fully reduced. It is necessary at some stage in the processing to dissolve the aggregates and then refold them to obtain the desired biologically active product [Cleland and Wang, in Stephanopoulos (ed.), op. cit., pp. 527–555].

Advantages of inclusion bodies in the production stage are their ease of separation by centrifugation following cell disruption, because of their size and density, and their provision of excellent initial-purification possibilities, as long as impurities are not copurified to any significant extent with the inclusion bodies. They also provide a high expression level and prevent endogenous proteolysis. There can be, however, significant product loss during protein refolding to the active form. Figure 20-87 shows a typical process for refolding. The inclusion body which is released from the host cell by cell disruption is washed and then solubilized by using a denaturant such as guanidine hydrochloride (4 to 9 *M*), urea (7 to 8 *M*), sodium thiocyanate (4 to 9 *M*), or detergents such as Triton X-100 or sodium dodecyl sulfate. This step disrupts the hydrogen and ionic bonds to obtain fully denatured and stretched peptide chains. For proteins with disulfide bonds, addition of appropriate reducing agents (e.g., beta mercaptoethanol) is required to break all incorrectly formed intramolecular disulfide bonds. To permit proper refolding of the protein, it is necessary to remove the denaturant or detergent molecules from the surroundings of the stretched and solubilized peptides. This will initiate self-refolding of the protein molecules. For proteins with disulfide bonds, an oxidative reaction with oxygen or other oxidants is required to join two free SH groups to form an S—S covalent bond. This in vitro refolding operation is traditionally achieved by dilution with a refolding buffer. However, misfolding or aggregation is usually found in the refolding process. Analysis of in vitro refolding kinetics shows that there is at least an intermediate (I) between the unfolded protein (U) and the fully active refolded native protein (N), as illustrated below [Kuwajima and Arai, in R. H. Pain (ed.), *Mechanism of Protein Folding*, 2d ed., Oxford University Press, Oxford, 2000, pp. 138–171; Tsumoto et al., *Protein Expr. Purif.*, **28,** 1–8 (2003)].



The process to convert U to I may be fast. The process for I to N, however, may be slow and highly reversible. Some intermediate molecules may form aggregates (A) or misfolded proteins (M). Figure 20-87 is a simplified refolding pathway. In reality, the situation can be more complicated where several intermediates (I_1, I_2, I_3, etc.) are present with numerous possibilities of aggregation and misfolding. For most proteins, refolding is a self-assembly process that follows a first-order kinetics. Aggregation, on the other hand, involves interactions between two or more molecules and follows the second- or higher-order kinetics. Therefore, in vitro refolding at higher protein concentrations would lead to the formation of more aggregates. Many observations have shown that a low final protein concentration, usually 10 to 100 μg/mL, is required for dilution refolding [Schlegl et al., *Chem. Engng. Sci.,* **60,** 5770–5780 (2005)]. In an industrial process, this strategy generally features large volumes of buffers, exerting an extra burden for subsequent purification steps because the concentration is low. Optimization of dilution strategy, such as the way of dilution, the speed of dilution, and the solution composition of a refolding buffer, is beneficial for an increased refolding yield.

A number of studies [Kuwajima and Arai, loc. cit.; Sadana, *Biotech. Bioeng.*, **48,** 481–489 (1995)] demonstrated that the presence of some molecules in the refolding buffer may suppress misfolding or aggregation. Molecular chaperones such as GroES and GroEL can promote

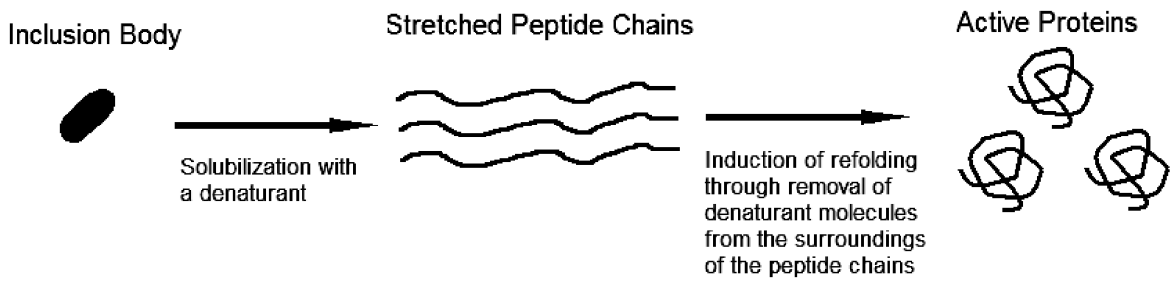


FIG. 20-87 Illustration of a refolding process for a protein from inclusion body.

correct refolding, but they are very expensive. Other molecules have been tried as artificial chaperones. The most commonly used molecules are polyethylene glycol (PEG) of various molecular weights and concentrations, L-arginine (0.4 to 1 *M*), low concentrations of denaturants such as urea (1 to 2 *M*) and guanidine hydrochloride (0.5 to 1.5 *M*), and some detergents such as SDS, CTAB, and Triton X-100. The effects of these additives on protein refolding are still under investigation. A new direction of protein refolding involves the use of chromatographic techniques [Li et al., *Protein Expr. Purif.*, **33,** 1–10 (2004)]. Size exclusion, ion exchange, hydrophobic interaction, and metal chelate affinity chromatographic techniques have been studied with successful results. Chromatographic refolding explores the interaction between the protein molecule to be refolded and the packing medium in the column. It may reduce the interaction between protein molecules and increase the chance of self-assembly with the aid of the functional groups and pores in the matrix of the packing medium. An advantage for chromatographic refolding is the availability of gradient elution that creates a gradual change of the solution environment, leading to a gentle removal of the denaturant and a gradual change of favorable conditions such as pH and the artificial chaperone concentration. Simultaneous refolding and partial purification are possible with this new technique.

For extracellular products, which are invariably water-soluble, the first step is the removal of cells and cell debris using a clarification method and, in the case of typical protein products, the removal of dissolved low-molecular-weight compounds. This must be done under relatively gentle conditions to avoid undesired denaturation of the product. Again, either filtration or centrifugation can be applied. Filtration results in a cell-free supernatant with dilution associated with the diafiltration of the final cell slurry. Centrifugation, regardless of the mode, will result in a small amount of cells in the centrate, but there is no dilution of the supernatant. During the process development careful studies should be conducted to examine the effects of pH and ionic strength on the yield, as cells and cell debris may retain the product through charge interactions. If the broth or cell morphology does not allow for filtration or if dry cell mass is required, tubular-bowl centrifugation is typically utilized. Note that plant and animal cells cannot sustain the same degree of applied shear as can microbial cells, and thus crossflow filtration or classical centrifugation may not be applicable. Alternatives using low-shear equipment under gentle conditions are often employed in these situations.

For whole broths the range of densities and viscosities encountered affects the concentration factor that can be attained in the process, and can also render crossflow filtration uneconomical because of the high pumping costs, and so on. Often, the separation characteristics of the broth can be improved by broth conditioning using physiochemical or biological techniques, usually of a proprietary nature. The important characteristics of the broth are rheology and conditioning.

Clarification Using Centrifugation Centrifugation relies on the enhanced sedimentation of particles of density different from that of the surrounding medium when subjected to a centrifugal force field [Axelsson, in Cooney and Humphrey (eds.), op. cit., pp. 325–346] (see also Sec. 18, "Liquid-Solid Operations and Equipment"). Advantages of centrifugal separations are that they can be carried out continuously and have short retention times, from a fraction of a second to seconds, which limit the exposure time of sensitive biologicals to shear stresses. Yields are high, provided that temperature and other process conditions are adequately controlled. They have small space requirements, and an adjustable separation efficiency makes them a versatile unit operation. They can be completely closed to avoid contamination, and, in contrast to filtration, no chemical external aids are required that can contaminate the final product. The ability now to contain the aerosols typically generated by centrifuges adds to their operability and safety.

Sedimentation rates must be sufficiently high to permit separation, and they can be enhanced by modifying solution conditions to promote the aggregation of proteins or impurities. An increase in precipitation of the contaminating species can often be accomplished by a reduction in pH or an elevation in temperature. Flocculating agents, which include polyelectrolytes, polyvalent cations, and inorganic salts, can cause a 2000-fold increase in sedimentation rates. Some examples are polyethylene imine, EDTA, and calcium salts. Cationic bioprocessing aids (cellulosic or polymeric) reduce pyrogen, nucleic acid, and acidic protein loads which can foul chromatography columns. The removal of these additives during both centrifugation and subsequent processing must be clearly demonstrated.

There are many different types of centrifuges, classified according to the way in which the transport of the sediment is handled [Medronho, in Hatti-Kaul and Mattiasson (eds.), op. cit., pp. 131–190]. The selection of a particular centrifuge type is determined by its capacity for handling sludge; the advantages and disadvantages of various separator types are discussed by Axelsson [in Cooney and Humphrey (eds.), op. cit., pp. 325–346]. Solids-retaining centrifuges are operated in a semibatch mode, as they must be shut down periodically to remove the accumulated solids; they are primarily used when solids concentrations are low, and they have found application during the clarification and simultaneous separation of two liquids. In solids-ejecting centrifuges, the solids are removed intermittently either through radial slots or axially while the machine is running at full speed. These versatile machines can be used to handle a variety of feeds, including yeast, bacteria, mycelia, antibiotics, enzymes, and so on. Solids-discharging nozzle centrifuges have a large capacity and can accommodate up to 30 percent solids loading. Decanter centrifuges consist of a drum, partly cylindrical and partly conical, and an internal screw conveyor for transport of the solids, which are discharged at the conical end; liquids are discharged at the cylindrical end. Levels within the drum are set by means of external nozzles.

Continuous-flow units, the scroll decanter and disk-stack centrifuges, are easiest to use from an operational perspective; shutdown of the centrifuge during the processing of a batch is not expected. While the disk-stack centrifuge enjoys popularity as a process instrument within the pharmaceutical and biotechnology industries, the precise timing of solids ejection and the continuous high-speed nature of the device make for complex equipment and frequent maintenance. It is often used to harvest cells, since the solids generated are substantially wet and could lead to measurable yield losses in extracellular product systems. For intracellular product processing, the wet cell sludge is easily resuspended for use in subsequent processing.

The tubular bowl, in contrast, is a semibatch processing unit owing to the limited solids capacity of the bowl. The use of this unit requires shutdown of the centrifuge during the processing of the batch. The semibatch nature of these centrifuges can thus greatly increase processing cycle times. The introduction of disposable sheets to act as bowl liners has significantly impacted turnaround times during processing. The dry nature of the solids generated makes the tubular-bowl centrifuge well suited for extracellular protein processing, since losses to the cell sludge are minimal. In contrast, the dry, compact nature of the sludge can make the cells difficult to resuspend. This can be problematic for intracellular protein processing where cells are homogenized in easily clogged, mechanical disrupters.

Clarification Using Microfiltration Crossflow filtration (microfiltration includes crossflow filtration as one mode of operation in "Membrane Separation Processes," which appears earlier in this section) relies on the retention of particles by a membrane. The driving force for separation is pressure across a semipermeable membrane, while a tangential flow of the feed stream parallel to the membrane surface inhibits solids settling on and within the membrane matrix (Datar and Rosen, loc. cit.).

Microfiltration is used for the removal of suspended particles, recovery of cells from fermentation broth, and clarification of homogenates containing cell debris. Particles removed by microfiltration typically average greater than 500,000 nominal molecular weight [Tutunjian, in Cooney and Humphrey (eds.), op. cit., pp. 367–381; Gobler, in Cooney and Humphrey (eds.), op. cit., pp. 351–366]. Ultrafiltration focuses on the removal of low-molecular-weight solutes and proteins of various sizes, and it operates in the less than 100,000 nominal-molecular-weight cutoff (NMWCO) range [Le and Howell, in Cooney and Humphrey (eds.), op. cit., pp. 383–409]. Both operations consist of a concentration segment (of the larger particles) followed by diafiltration of the retentate [Tutunjian, in Cooney and Humphrey (eds.), op. cit., pp. 411–437].

Generally, the effectiveness of the separation is determined not by the membrane itself, but rather by the formation of a secondary or dynamic membrane caused by interactions of the solutes and particles

with the membrane. The buildup of a gel layer on the surface of an ultrafiltration membrane owing to rejection of macromolecules can provide the primary separation characteristics of the membrane. Similarly, with colloidal suspensions, pore blocking and bridging of pore entries can modify the membrane performance, while molecules of size similar to the membrane pores can adsorb on the pore walls, thereby restricting passage of water and smaller solutes. Media containing poorly defined ingredients may contain suspended solids, colloidal particles, and gellike materials that prevent effective microfiltration. In contrast to centrifugation, specific interactions can play a significant role in membrane separation processes.

The factors to consider in the selection of crossflow filtration include the flow configuration, tangential linear velocity, transmembrane pressure drop (driving force), separation characteristics of the membrane (permeability and pore size), size of particulates relative to the membrane pore dimensions, low protein-binding ability, and hydrodynamic conditions within the flow module. Again, since particle-particle and particle-membrane interactions are key, broth conditioning (ionic strength, pH, etc.) may be necessary to optimize performance.

Selection of Cell-Separation Unit Operation The unit operation selected for cell separations can depend on the subsequent separation steps in the train. In particular, when the operation following cell separation requires cell-free feed (e.g., chromatography), filtration is used, since centrifugation is not absolute in terms of cell separation. In addition, if cells are to be stored (i.e., they contain the desired product) because later processing is more convenient (e.g., only 2-shift operation, facility competes for equipment with other products, batch is too big for single pass in equipment), it is generally better to store the cells as a frozen concentrate than a paste, since the concentrate thaws more completely, avoiding small granules of unfrozen cell solids that can foul homogenizers, columns, and filters. Here the retentate from filtration is desired, although the wet cell mass from a disc stack-type centrifuge may be used.

Centrifugation is generally necessary for complex media used to make natural products, for while the media components may be sifted prior to use, they can still contain small solids that can easily foul filters. The medium to be used should be tested on a filter first to determine the fouling potential. Some types of organisms, such as filamentous organisms, may sediment too slowly owing to their larger cross sections, and they are better treated by filtration (mycelia have the potential to easily foul tangential-flow units; vacuum-drum filtration using a filter aid, e.g., diatomaceous earth, should also be considered). Often the separation characteristics of the broth can be improved by broth conditioning using physicochemical or biological techniques, usually of a proprietary nature.

Regardless of the machine device, centrifuges are typically maintenance-intensive. Filters can be cheaper in terms of capital and maintenance costs and should be considered first unless centrifugal equipment already exists. Small facilities (< 1000 L) use filtration, since centrifugation scale-down is constrained by equipment availability. Comparative economics of the two classes of operations are discussed by Datar and Rosen (loc. cit.).

INITIAL PURIFICATION

Initial purification is the rough purification (considered by many people as isolation) to prepare a feed for subsequent high-resolution steps. In initial purification steps the goal is to obtain concentration with partial purification of the product, which is recovered as a precipitate (precipitation), a solution in a second phase (liquid-liquid partitioning), or adsorbed to solids (adsorption, chromatography).

Precipitation Precipitation of products, impurities, or contaminants can be induced by the addition of solvents, salts, or polymers to the solution; by increasing temperature; or by adjusting the solution pH (Scopes, op. cit., pp. 41–71; Ersson et al., in Janson and Ryden, op. cit., pp. 3–32). This operation is used most often in the early stages of the separation sequence, particularly following centrifugation, filtration, and/or homogenization steps. Precipitation is often carried out in two stages, the first to remove bulk impurities and the second to precipitate and concentrate the target protein. Generally, amorphous precipitates are formed, owing to occlusion of salts or solvents, or to the presence of impurities.

Salts can be used to precipitate proteins by "salting out" effects. The effectiveness of various salts is determined by the Hofmeister series, with anions being effective in the order citrate > PO_4^{2-} > SO_4^{2-} > CH_3COO^- > Cl^- > NO_3^-, and cations according to NH_4^+ > K^+ > Na^+ (Ersson et al., op. cit., p. 10; Belter et al., op. cit., pp. 221–236).

Salts should be inexpensive owing to the large quantities used in precipitation operations. Ammonium sulfate appears to be the most popular precipitant because it has an effective cation and an effective anion, high solubility, easy disposal, and low cost. Drawbacks to this approach include low selectivity, high sensitivity to operating conditions, and downstream complications associated with salt removal and disposal of the high-nitrogen-content stream. Generally, aggregates formed on precipitation with ammonium sulfate are fragile, and are easily disrupted by shear. Thus, these precipitation operations are, following addition of salt, often aged without stirring before being fed to a centrifuge by gravity feed or using low-shear pumps (e.g., diaphragm pumps).

The organic solvents most commonly used for protein precipitation are acetone and ethanol (Ersson et al., op. cit.). These solvents can cause some denaturation of the protein product. Temperatures below 0°C can be used, since the organic solvents depress the freezing point of the water. The precipitate formed is often an extremely fine powder that is difficult to centrifuge and handle. With organic solvents, in-line mixers are preferred, as they minimize solvent-concentration gradients and regions of high-solvent concentrations, which can lead to significant denaturation and local precipitation of undesired components typically left in the mother liquors. In general, precipitation with organic solvents at lower temperature increases yield and reduces denaturation. It is best carried out at ionic strengths of 0.05 to 0.2 *M*.

Water-soluble polymers and polyelectrolytes (e.g., polyethylene glycol, polyethylene imine polyacrylic acid) have been used successfully in protein precipitations, and there has been some success in affinity precipitations wherein appropriate ligands attached to polymers can couple with the target proteins to enhance their aggregation. Protein precipitation can also be achieved by using pH adjustment, since proteins generally exhibit their lowest solubility at their isoelectric point. Temperature variations at constant salt concentration allow for fractional precipitation of proteins.

Precipitation is typically carried out in standard cylindrical tanks with low-shear impellers. If in-line mixing of the precipitating agent is to be used, this mixing is employed just prior to the material's entering the aging tank. Owing to their typically poor filterability, precipitates are normally collected by using a centrifugal device.

Liquid-Liquid Partitioning Liquid-liquid partitioning (see also Sec. 15, "Liquid-Liquid Extraction") involving an organic solvent is commonly known as solvent extraction or extraction. Solvent extraction is routinely used to separate small biomolecules such as antibiotics and amino acids. However, it is typically not suitable for protein fractionation with only a few exceptions because organic solvents may cause protein denaturation or degradation. A recent review of solvent extraction for bioseparations including a discussion on various parameters that can be controlled for solvent extraction was given by Gu [in Ahuja (ed.), op. cit., pp. 365–378]. As a replacement of solvent extraction, aqueous two-phase partitioning is typically used for protein purification. It uses two water-soluble polymers (and sometimes with some salts when polyelectrolytes are involved) to form two aqueous phases [Albertsson, op. cit.; Kula, in Cooney and Humphrey (eds.), op. cit., pp. 451–471]. Both phases contain water but differ in polymer (and salt) concentration(s). The high water content, typically greater than 75 percent, results in a biocompatible environment not attainable with traditional solvent extraction systems. Biomolecules such as proteins have different solubilities in the two phases, and this provides a basis for separation. Zaslavsky (op. cit., pp. 503–667) listed 163 aqueous two-phase systems including PEG-dextran-water, PEG-polyvinylmethylether-water, PEG-salt-water, polyvinylpyrrolidone-dextran-water, polyvinylalcohol-dextran-water, and Ficoll-dextran-water systems. Partitioning between the two aqueous phases is controlled by the polymer molecular weight and concentration, protein net charge and size, and hydrophobic and electrostatic interactions. Aqueous

two-phase polymer systems are suitable for unclarified broths since particles tend to collect at the interface between the two phases, making their removal very efficient. They can also be used early on in the processing train for initial bulk-volume reduction and partial purification. One of the drawbacks of these systems is the subsequent need for the removal of phase-forming reagents.

Affinity partitioning is carried out by adding affinity ligands to an aqueous two-phase partitioning system. The biospecific binding of a biomolecule with the ligand moves the biomolecule to a preferred phase that enhances the partitioning of the biomolecule [Johansson and Tjerneld, in Street (ed.), *Highly Selective Separations in Biotechnology*, Blackie Academic & Professional, London, 1994, pp. 55–85]. Diamond and Hsu [in Fiechter (ed.), *Advances in Biochemical Engineering/Biotechnology*, vol. 47, Springer-Verlag, Berlin-New York, 2002, pp. 89–135] listed several dozens of biomolecules, many of which are proteins that have been separated by using affinity partitioning. Fatty acids and triazine are the two common types of affinity ligand while metallated iminodiacetic acid (IDA) derivatives of PEG such as Cu(II)IDA-PEG can be used for binding with proteins rich in surface histidines. The drawbacks of affinity partitioning include the costs of ligands and the need to couple the ligands to the polymers used in the aqueous two-phase partitioning.

Product recovery from these systems can be accomplished by changes in either temperature or system composition. Composition changes can be affected by dilution, backextraction, and micro- and ultrafiltration. As the value of the product decreases, recovery of the polymer may take on added significance. A flow diagram showing one possible configuration for the extraction and product and polymer recovery operations is shown in Fig. 20-88 [Greve and Kula, *J. Chem. Tech. Biotechnol.*, **50,** 27–42 (1991)]. The phase-forming polymer and salt are added directly to the fermentation broth. The cells or cell debris and contaminating proteins report to the salt-rich phase and are discarded. Following pH adjustment of the polymer-rich phase, more salt is added to induce formation of a new two-phase system in which the product is recovered in the salt phase, and the polymer can be recycled. In this example, disk-stack centrifuges are used to enhance the phase separation rates. Other polymer recycling options include extraction with a solvent or supercritical fluid, precipitation, or diafiltration. Electrodialysis can be used for salt recovery and recycling.

Reversed micellar solutions can also be used for the selective extraction of proteins [Kelley and Hatton, in Stephanopoulos (ed.), op. cit., pp. 593–616]. In these systems, detergents soluble in an oil phase aggregate to stabilize small water droplets having dimensions similar to those of the proteins to be separated. These droplets can host hydrophilic species such as proteins in an otherwise inhospitable organic solvent, thus enabling these organic phases to be used as protein extractants. Factors affecting the solubilization effectiveness of the solvents include charge effects, such as the net charge determined by the pH relative to the protein isoelectric point; charge distribution and asymmetry on the protein surface; and the type (anionic or cationic) of the surfactant used in the reversed micellar phase. Ionic strength and salt type affect the electrostatic interactions between the proteins and the surfactants, and affect the sizes of the reversed micelles. Attachment of affinity ligands to the surfactants has been demonstrated to lead to enhancements in extraction efficiency and selectivity [Kelley et al., *Biotech. Bioeng.*, **42,** 1199–1208 (1993)].

Product recovery from reversed micellar solutions can often be attained by simple backextraction, by contacting with an aqueous solution having salt concentration and pH that disfavors protein solubilization, but this is not always a reliable method. Addition of cosolvents such as ethyl acetate or alcohols can lead to a disruption of the micelles and expulsion of the protein species, but this may also lead to protein denaturation. These additives must be removed by distillation, e.g., to enable reconstitution of the micellar phase. Temperature increases can similarly lead to product release as a concentrated aqueous solution. Removal of the water from the reversed micelles by molecular sieves or silica gel has also been found to cause a precipitation of the protein from the organic phase.

Extraction using liquid emulsion membranes involves the use of a surface-active agent such as a surfactant to form dispersed droplets that encapsulate biomolecules. Its economic viability for large-scale applications is still weak [Patnaik, in Subramanian (ed.), op. cit., vol. 1, pp. 267–303]. Another less-known method involving the use of a surfactant is the foam fractionation method that has seen limited applications. Ionic fluids have found commercial applications in chemical reactions by replacing volatile solvents. They are emerging as an environmentally friendly solvent replacement in liquid-liquid phase partitioning. Room-temperature ionic liquids are low-melting-point salts that stay as liquids at room temperature. Partition behavior in a system involving a room-temperature ionic fluid and an aqueous phase is influenced by the type of ionic liquid used as well as pH change (Visser et al., *Green Chem.*, Feb. 1–4, 2000). An ionic fluid was also reportedly used in the mobile phase for liquid chromatography

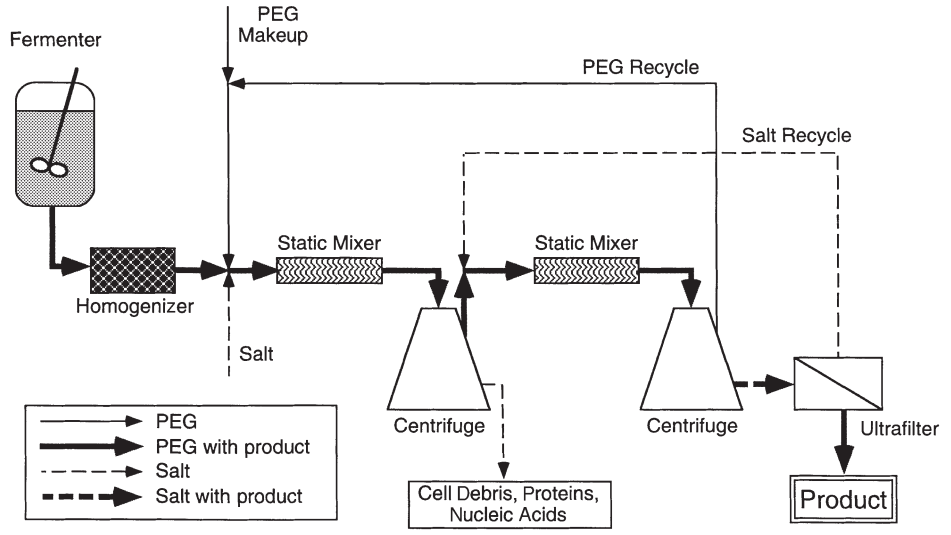


FIG. 20-88 Process scheme for protein extraction in aqueous two-phase systems for the downstream processing of intracellular proteins, incorporating PEG and salt recycling. [*Reprinted from Kelly and Hatton in Stephanopoulos (ed.), op. cit.; adapted from Greve and Kula, op. cit.*]

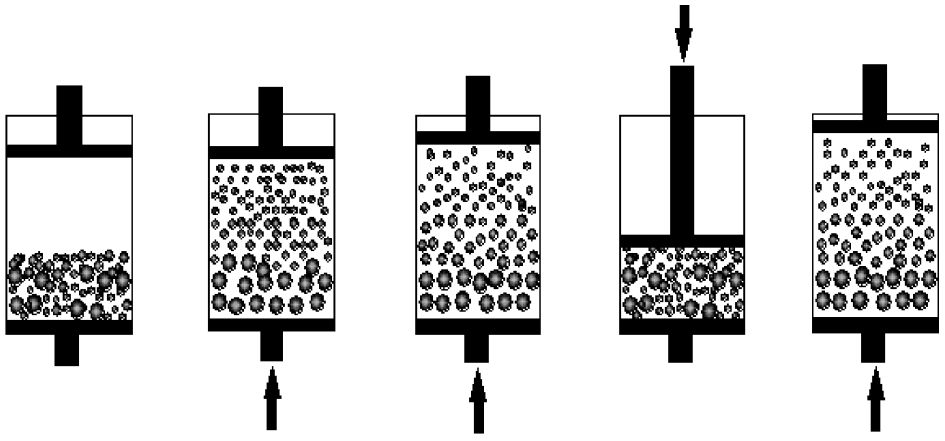


FIG. 20-89 An operation cycle in expanded-bed adsorption.

[He et al., *J. Chromatogr. A*, **1007,** 39–45 (2003)]. More research needs to be done in this area to develop this new green technology [Freemantle, *C&EN*, **83,** 33–38 (2005).]

Aqueous-detergent solutions of appropriate concentration and temperature can phase-separate to form two phases, one rich in detergents, possibly in the form of micelles, and the other depleted of the detergent [Pryde and Phillips, *Biochem. J.*, **233,** 525–533 (1986)]. Proteins distribute between the two phases, hydrophobic (e.g., membrane) proteins reporting to the detergent-rich phase and hydrophilic proteins to the detergent-free phase. Indications are that the size-exclusion properties of these systems can also be exploited for viral separations. These systems would be handled in the same way as the aqueous two-phase systems.

On occasion, for extracellular products, cell separation can be combined with an initial volume reduction and purification step by using liquid-liquid extraction. This is particularly true for low-molecular-weight products, and it has been used effectively for antibiotic and vitamin recovery. Often scroll decanters can be used for this separation. The solids are generally kept in suspension (which requires that the solids be denser than heavy phase), while the organic phase, which must be lighter than water (cells typically sink in water), is removed. Experience shows that scrolls are good for handling the variability seen in fermentation feedstock. Podbielniak rotating-drum extraction units have been used often, but only when solids are not sticky, gummy, or flocculated, as they can get stuck in perforations of the concentric drums, but will actually give stages to the extraction in short-residence time (temperature-sensitive product). The Karr reciprocating-plate column can handle large volumes of whole-broth materials efficiently, and it is amenable to ready scale-up from small laboratory-scale systems to large plant-scale equipment.

Adsorption Adsorption (see also Sec. 16, "Adsorption and Ion Exchange") can be used for the removal of pigments and nucleic acids, e.g., or can be used for direct adsorption of the desired proteins. Stirred-batch or expanded-bed operations allow for presence of particulate matter, but fixed beds are not recommended for unclarified broths owing to fouling problems. These separations can be effected through charge, hydrophobic, or affinity interactions between the species and the adsorbent particles, as in the chromatographic steps outlined below. The adsorption processes described here are different from those traditionally ascribed to chromatography in that they do not rely on packed-bed operations.

In continuous affinity recycle extraction (CARE) operations, the adsorbent beads are added directly to the cell homogenate, and the mixture is fed to a microfiltration unit. The beads loaded with the desired solute are retained by the membrane, and the product is recovered in a second stage by changing the buffer conditions to disfavor binding.

Expanded-bed adsorption (EBA) has gained popularity in bioprocessing since its commercial introduction in the 1990s because of its ability to handle a crude feedstock that contains cells or other particulates. EBA eliminates the need for a dedicated clarification step by combining solid-liquid separation and adsorption into a single-unit operation [Hjorth et al., in Subramanian (ed.), op. cit., vol. 1, pp. 199–226; Mattiasson et al., in Ahuja (ed.), op. cit., pp. 417–451]. A typical EBA operation cycle is illustrated in Fig. 20-89. A bed packed with an adsorption medium (or a resin), usually spherical particles of different sizes, is expanded by an upward-flow liquid stream from the bottom. An unclarified feed is introduced after a stable expansion of the bed is achieved. Particulates pass through the void spaces between the resin particles, while the soluble product molecules are adsorbed by the resin and retained in the column. After a washing step, the resin particles are left to settle in the column to form a fixed bed. The product molecules are then eluted out with a mobile phase entering from the top of the column in a way similar to that in conventional fixed-bed chromatography, to achieve a high-resolution separation. The elution can also be performed in expanded mode if needed. The regeneration step in the expanded mode flushes away residual particulates and refreshes the media for the next cycle. The difference between EBA and conventional fluidized-bed adsorption lies in the adsorption resin. In conventional fluidized-bed adsorption, the resin particles are randomly distributed in the column. In EBA, however, the resin particles are distributed vertically with large ones near the column bottom and the small ones near the top. There is no backmixing along the axial direction of the vertically standing column, thus achieving adsorption similar to that in a fixed-bed column. The resin particles have to be prepared to possess a suitable size distribution, or alternatively a distribution based on density differences if the particles sizes are uniform. An EBA column should have a length typically 3 to 4 times of the settled bed height to allow for bed expansion. An adjustable adapter at the top is needed to push the resin downward for elution in the fixed-bed mode. A proper design of the bottom frit is critical. Its holes must be smaller enough to retain the smallest resin particles but large enough to allow the particulates to enter and exit the column freely. In practical applications, plugging of the frit and non-uniform upward flow tend to be problematic, especially in columns with large diameters. To mitigate this problem, some EBA columns use an optimized inlet design and a mechanical stirrer at the bottom.

The advantages of EBA are its ability to adsorb the soluble product molecules directly from a cell suspension, a cell homogenate, or a

crude biological fluid containing various particulates, thus making the "whole-broth processing" concept a reality. EBA eliminates the solid-liquid separation step (such as microfiltration and centrifugation) and enables a fast, more compact process requiring fewer steps and less time. It performs solid removal, concentration, and purification, all in a single-unit operation. By doing so, it can also minimize the risk of proteolytic breakdown of the product.

Membrane Ultrafiltration Membrane ultrafiltration is often one of the favored unit operations used for the isolation and concentration of biomolecules because they can be easily scaled up to process large feed volumes at low costs. Toward the end of an ultrafiltration operation, additional water or buffer is added to facilitate the passage of smaller molecules. This is known as diafiltration. Diafiltration is especially helpful in the removal of small contaminating species such as unspent nutrients including salts and metabolites. Salt removal is usually necessary if the next step is ion-exchange or reverse-phase chromatography. For a protein that is not very large, two ultrafiltration steps can be used in sequence. In the first one, the protein ends up in the permeate, allowing the removal of large contaminating molecules including pyrogens and also viral particles. In the second ultrafiltration, the protein stays in the retentate. This removes small contaminating molecules and concentrates the feed up to the protein's solubility limit. Membrane materials, configurations, and design considerations were discussed earlier in this section. Proper membrane materials must be selected to avoid undesirable binding with proteins. External fouling, pore blockage, and internal fouling were discussed by Ghosh (*Protein Bioseparation Using Ultrafiltration*, Imperial College Press, London, 2003).

FINAL PURIFICATION

The final purification steps are responsible for the removal of the last traces of impurities. The volume reduction in the earlier stages of the separation train is necessary to ensure that these high-resolution operations are not overloaded. Generally, chromatography is used in these final stages. Electrophoresis can also be used, but since it is rarely found in process-scale operations, it is not addressed here. The final product preparation may require removal of solvent and drying, or lyophilization of the product.

Chromatography Liquid chromatography steps are ubiquitous in the downstream processing. It is the most widely used downstream processing operation because of its versatility, high selectivity, and efficiency, in addition to its adequate scale-up potential based on wide experience in the biochemical processing industries. As familiarity is gained with other techniques such as liquid-liquid extraction, they will begin to find greater favor in the early stages of the separation train, but are unlikely to replace chromatography in the final stages, where high purities are needed.

Chromatography is typically a fixed-bed adsorption operation, in which a column filled with chromatographic packing materials is fed with the mixture of components to be separated. Apart from size-exclusion (also known as gel-permeation or gel-filtration) chromatography, in the most commonly practiced industrial processes the solutes are adsorbed strongly to the packing materials until the bed capacity has been reached. The column may then be washed to remove impurities in the interstitial regions of the bed prior to elution of the solutes. This latter step is accomplished by using buffers or solvents which weaken the binding interaction of the proteins with the packings, permitting their recovery in the mobile phase. To minimize product loss of a high-value product, a small load far below the saturation capacity is applied to the column. A complete baseline separation can then be achieved after elution. Gradient elution uses varied modifier strength in the mobile phase to achieve better separations of more chemical species.

Types of Chromatography Practiced Separation of proteins by using chromatography can exploit a range of different physical and chemical properties of the proteins and the chromatography adsorption media [Janson and Ryden, op. cit.; Scopes, op. cit.; Egerer, in Finn and Prave (eds.), *Biotechnology Focus 1*, Hanser Publishers, Munich, 1988, pp. 95–151]. Parameters that must be considered in the selection of a chromatographic method include composition of the feed, the chemical structure and stability of the components, the electric charge at a defined pH value and the isoelectric point of the proteins, the hydrophilicity and hydrophobicity of the components, and molecular size. The different types of interactions are illustrated schematically in Fig. 20-90.

Ion-exchange chromatography relies on the coulombic attraction between the ionized functional groups of proteins and oppositely charged functional groups on the chromatographic support. It is used to separate the product from contaminating species having different charge characteristics under well-defined eluting conditions, and for concentration of the product, owing to the high-adsorptive capacity of most ion-exchange resins and the resolution attainable. Elution is carried out by using a mobile phase with competing ions or varied pH. Ion-exchange chromatography is used effectively at the front end of a downstream processing train for early volume reduction and purification.

The differences in sizes and locations of hydrophobic pockets or patches on proteins can be exploited in *hydrophobic interaction chromatography* (*HIC*) and *reverse-phase chromatography* (*RPC*); discrimination is based on interactions between the exposed hydrophobic residues and hydrophobic ligands which are distributed evenly throughout a hydrophilic porous matrix. As such, the binding characteristics complement those of other chromatographic methods, such as ion-exchange chromatography. In HIC, the hydrophobic interactions are relatively weak, often driven by salts in high concentration, and depend primarily on the exposed residues on or near the protein surface; preservation of the native, biologically active state of the protein is a desirable feature of HIC. HIC's popularity is on the rise in recent years because of this feature. Elution can be achieved differentially by decreasing salt concentration or increasing the concentration of polarity perturbants (e.g., ethylene glycol) in the eluent.

Reverse-phase chromatography relies on significantly stronger hydrophobic interactions than in HIC, which can result in unfolding and exposure of the interior hydrophobic residues, i.e., leads to protein denaturation and irreversible inactivation; as such, RPC depends on total hydrophobic residue content. Elution is effected by organic solvents applied under gradient conditions. RPC is the most commonly used analytical chromatographic method due to its ability to separate a vast array of chemicals with high resolutions. Denaturation of proteins does not influence the analytical outcome unless protein precipitation in the mobile phase becomes a problem.

HIC typically uses polymer-based resins with phenyl, butyl, or octyl ligands while RPC uses silica beads with straight-chain alkanes with 4, 8, or 18 carbons. Larger ligands provide stronger interactions. Polymeric beads are used in RPC when basic pH is involved because silica beads are unstable at such pH. In HIC, the mobile phase remains an aqueous salt solution, while RPC uses solvent in its mobile phase to regulate binding. Raising the temperature increases the hydrophobic interactions at the temperatures commonly encountered in biological processing.

HIC is most effective during the early stages of a purification strategy and has the advantage that sample pretreatment such as dialysis or desalting after salt precipitation is not usually required. It is also finding increased use as the last high-resolution step to replace gel filtration. It is a group separation method, and generally 50 percent or more of extraneous impurities are removed. This method is characterized by high adsorption capacity, good selectivity, and satisfactory yield of active material.

Despite the intrinsically nonspecific nature of ion-exchange and reversed-phase/hydrophobic interactions, it is often found that chromatographic techniques based on these interactions can exhibit remarkable resolution. This is attributed to the dynamics of multisite interactions being different for proteins having differing surface distributions of hydrophobic and/or ionizable groups.

Size-exclusion chromatography's (SEC's) separation mechanism is based on the sizes and shapes of proteins and impurities. The effective size of a protein is determined by its steric geometry and solvation characteristics. Smaller proteins are able to penetrate the small pores in the beads while large proteins are excluded, making the latter elute out of column more quickly. To suppress the ion-exchange side effects, a salt is typically added to the mobile phase. Ammonium carbonate or bicarbonate is used is if the salt is to be removed by sublimation alone during lyophilization. In rare cases, a solvent at a low

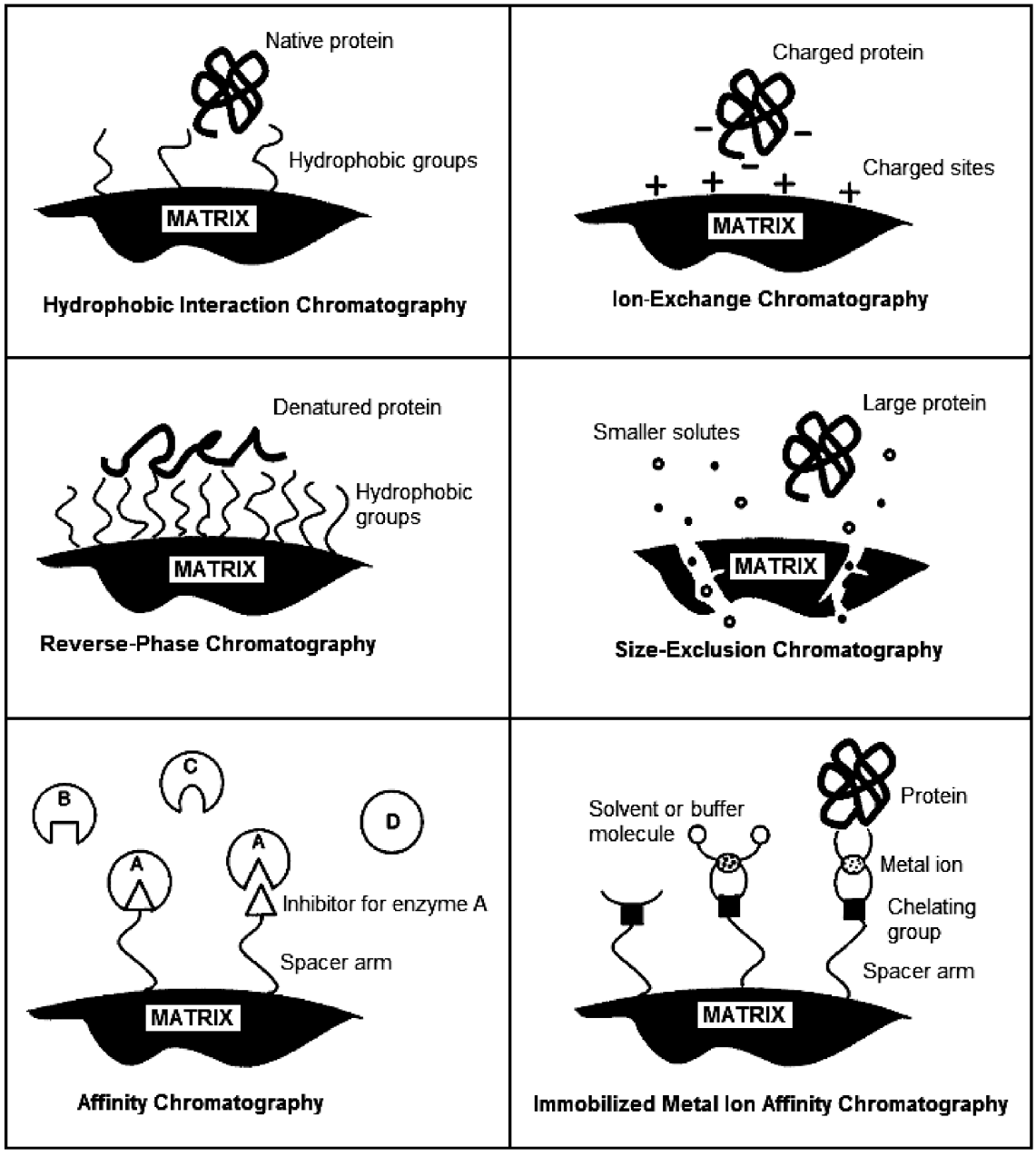


FIG. 20-90 Schematic illustration of the chromatographic methods most commonly used in downstream processing of protein products.

concentration is added to the mobile phase to suppress hydrophobic interactions between the protein molecules and the stationary phase. In industrial production processes, SEC columns are used to separate small molecules from proteins. It is also a choice for desalting and buffer exchange in the product polishing stage. SEC columns are typically very large because the feed loads to SEC columns are limited to 3 to 5 percent of the bed volumes. This loading capacity is far less than those with packings that have binding interactions.

Protein affinity chromatography can be used for the separation of an individual compound, or a group of structurally similar compounds from crude-reaction mixtures, fermentation broths, or cell lysates by exploiting very specific and well-defined molecular interactions between the protein and affinity groups immobilized on the packing-support material. Examples of affinity interactions include antibody-antigen, hormone-receptor, enzyme-substrate/analog/inhibitor, metal ion–ligand, and dye-ligand pairs. Monoclonal antibodies are particularly effective as biospecific ligands for the purification of pharmaceutical proteins. Affinity chromatography may be used for the isolation of a pure product directly from crude fermentation mixtures in a single chromatographic step. Immunosorbents should not be subjected

to crude extracts, however, as they are particularly susceptible to fouling and inactivation. Despite its high resolution and the ability to treat a very dilute feed, affinity chromatography is still costly on the process scale if protein ligands such as protein A or protein G is to be used, or a custom affinity matrix is required. Considerable research efforts are devoted to its development in part due to the increased number of protein pharmaceuticals produced at low concentrations.

After more than two decades of development, membrane chromatography has emerged as an attractive alternative to packed column chromatography. Using a porous membrane as the stationary phase in liquid chromatography has several potential advantages that include a very high flow rate through a very short and wide bed with only a modest transmembrane pressure drop. Elimination or minimization of diffusional mass-transfer resistance shifts the rate-controlling step to faster-binding kinetics, resulting in adsorptive separation of proteins in a fraction of the time required by conventional packed columns. To achieve sufficient adsorptive separation, it is necessary to use a medium that binds strongly with target molecules when a very short flow path is involved. Thus, membrane chromatography typically uses an affinity membrane, and the combination of membrane chromatography with affinity interaction provides high selectivity and fast processing for the purification of proteins from dilute feeds. To a much less extent, ion-exchange, hydrophobic interaction, and reverse-phase membrane chromatography have also been reported [Charcosset, *J. Chem. Technol. Biotechnol.* **71,** 95–110 (1998)].

Figure 20-91 shows the interaction between proteins in the mobile phase and the affinity membrane matrix. The mechanical strength, hydrophobicity, and ligand density of the membranes can be engineered through chemical modifications to make them suitable for affinity membrane chromatography. A preferred membrane medium to be prepared for affinity membrane chromatography should provide (1) desirable physical characteristics such as pore structure and mechanical strength, allowing fast liquid flow at a small pressure loss; (2) reactive groups (such as —OH, —NH_2, —SH, —COOH) for coupling ligands or spacer arms; (3) physical and chemical stabilities to endure heat or chemical sterilization; and (4) a nondenaturing matrix to retain protein bioactivity [Zou et. al., *J. Biochem. Biophys. Methods,* **49,** 199–240 (2001)]. Base membranes can be chosen from commercial membrane materials including organic, inorganic, polymeric, and composite materials. The selected membrane is first activated to create functional groups for chemical attachment of affinity ligands. If there is steric hindrance to binding between the immobilized ligand and the target molecule, a suitable spacer arm is used to bridge the activated membrane surface and the ligand. The ligand should retain its reversible binding capacity after immobilization onto the support membrane. Affinity ligands typically fall into two categories: (1) those derived from enzyme/substrate, antibody/antigen pairs that are capable of very strong and highly biospecific binding and (2) protein A and protein G, coenzyme, lectin, dyes, and metal chelates, etc., each capable of binding with a whole class of molecules. A highly specific ligand provides an unsurpassed resolution and an ability to handle a large volume of a dilute feed. However, they are typically fragile and expensive and may be unavailable off the shelf due to their narrow applications involving just one or a few molecules that can bind. Elution can also prove to be a difficult task because some biospecific bindings can be extremely tenacious. In contrary, a somewhat less specific ligand has a much wider market and thus is considerably less expensive. Various membrane cartridges have been used for affinity membrane chromatography including those with multiple layers of flat sheet membranes, hollow fibers, and spiral-wound and Chromarod membranes (Zou et al., loc. cit.).

Immobilized metal-ion affinity chromatography (*IMAC*) relies on the interaction of certain amino acid residues, particularly histidine, cysteine, and tryptophan, on the surface of the protein with metal ions fixed to the support by chelation with appropriate chelating compounds, invariably derivatives of iminodiacetic acid. Commonly used metal ions are Cu^{2+}, Zn^{2+}, Ni^{2+}, and Co^{2+}. Despite its relative complexity in terms of the number of factors that influence the process, IMAC is beginning to find industrial applications. The choice of chelating group, metal ion, pH, and buffer constituents will determine the adsorption and desorption characteristics. Elution can be effected by several methods, including pH gradient, competitive ligands, organic solvents, and chelating agents.

Following removal of unbound materials in the column by washing, the bound substances are recovered by changing conditions to favor desorption. A gradient or stepwise reduction in pH is often suitable. Otherwise, one can use competitive elution with a gradient of increasing concentration. IMAC eluting agents include ammonium chloride, glycine, histamine, histidine, or imidazol. Inclusion of a chelating agent such as EDTA in the eluent will allow all proteins to be eluted indiscriminately along with the metal ion.

Chromatographic Development The basic concepts of chromatographic separations are described elsewhere in this handbook. Proteins differ from small solutes in that the large number of charged

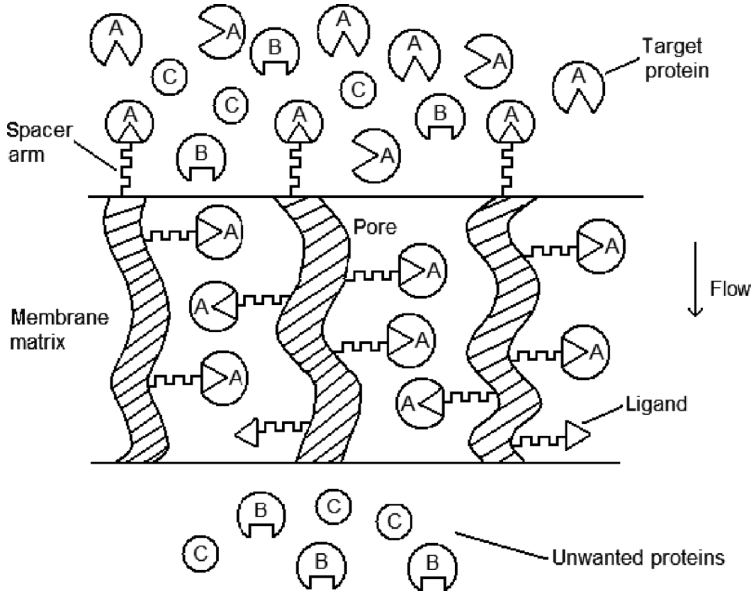


FIG. 20-91 Illustration of the interactions between proteins and membrane matrix in affinity membrane chromatography.

and/or hydrophobic residues on the protein surface provide multiple binding sites, which ensure stronger binding of the proteins to the adsorbents, as well as some discrimination based on the surface distribution of amino acid residues. The proteins are recovered by elution with a buffer that reduces the strength of this binding and permits the proteins to be swept out of the column with the buffer solution. In isocratic elution, the buffer concentration is kept constant during the elution period. Since the different proteins may have significantly different adsorption isotherms, the recovery may not be complete, or it may take excessive processing time and cause excessive band spreading to recover all proteins from the column. In gradient elution operations, the composition of the mobile phase is changed during the process to decrease the binding strength of the proteins successively, the more loosely bound proteins being removed first before the eluent is strengthened to enable recovery of the more strongly adsorbed species. The change in eluent composition can be gradual and continuous, or it can be stepwise. Industrially, in large-scale columns it is difficult to maintain a continuous gradient owing to difficulties in fluid distribution, and thus stepwise changes are still used. In some adsorption modes, the protein can be recovered by the successive addition of competing compounds to displace the adsorbed proteins. In all cases, the product is eluted as a chromatographic peak, with some possible overlap between adjacent product peaks.

Displacement chromatography relies on a different mode of elution. Here a displacer that is more strongly adsorbed than any of the proteins is introduced with the mobile phase. As the displacer concentration front develops, it pushes the proteins ahead of itself. The more strongly adsorbed proteins then act as displacers for the less strongly bound proteins, and so on. This leads to the development of a displacer train in which the different molecules are eluted from the column in abutting roughly rectangular peaks in the reverse order of their binding strength with the column's stationary phase. Many displacers have been developed in the past two decades including both high- and low-molecular-weight molecules. Displacement chromatography may be practical as an earlier chromatography step in a downstream process when baseline separations are not necessary. It has been considered for some industrial processes. However, displacer reuse and possible contamination of the protein product in addition to its inherent inability to achieve a clear-cut baseline separation make the use of displacement chromatography still a challenge. So far, no FDA-approved process exists [Shukla and Cramer, in Ahuja (ed.), op. cit., pp. 379–415].

For efficient adsorption it is advisable to equilibrate both the column and the sample with the optimum buffer for binding. Prior to this, the column must be cleaned to remove tightly bound impurities by increasing the salt concentration beyond that used in the product elution stages. At the finish of cleaning operation, the column should be washed with several volumes of the starting buffer to remove remaining adsorbed material. In desorption, it is necessary to drive the favored binding equilibrium for the adsorbed substance from the stationary to the mobile phase. Ligand-protein interactions are generally a combination of electrostatic, hydrophobic, and hydrogen bonds, and the relative importance of each of these and the degree of stability of the bound protein must be considered in selecting appropriate elution conditions; frequently compromises must be made. Gradient elution often gives good results.

Changes in pH or ionic strength are generally nonspecific in elution performance; ionic strength increases are effective when the protein binding is predominantly electrostatic, as in IEC. Polarity changes are effective when hydrophobic interactions play the primary role in protein binding. By reducing the polarity of the eluting mobile phase, this phase becomes a more thermodynamically favorable environment for the protein than adsorption to the packing support. A chaotropic salt (KSCN, KCNO, KI in range of 1 to 3 *M*) or denaturing agent (urea, guanidine HCl; 3 to 4 *M*) in the buffer can also lead to enhanced desorption. For the most hydrophobic proteins (e.g., membrane proteins) one can use detergents just below their critical micelle concentrations to solubilize the proteins and strip them from the packing surface. Specific elution requires more selective eluents. Proteins can be desorbed from ligands by competitive binding of the eluting agent (low concentration of 5 to 100 m*M*) either to the ligand or to the protein.

Specific eluents are most frequently used with group-specific adsorbents since selectivity is greatly increased in the elution step. The effectiveness of the elution step can be tailored by using a single eluent, pulses of different eluents, or eluent gradients. These systems are generally characterized by mild desorption conditions. If the eluting agent is bound to the protein, it can be dissociated by desalting on a gel filtration column or by diafiltration.

Column Packings The quality of the separation obtained in chromatographic separations will depend on the capacity, selectivity, and hydraulic properties of the stationary phase, which usually consists of porous beads of hydrophilic polymers filled with the solvent. The xerogels (e.g., cross-linked dextran) shrink and swell depending on solvent conditions, while aerogels have sizes independent of solution conditions. A range of materials are used for the manufacture of gel beads, classified according to whether they are inorganic, synthetic, or polysaccharides. The most widely used materials are based on neutral polysaccharides and polyacrylamide. Cellulose gels, such as cross-linked dextran, are generally used as gel filtration media, but can also be used as a matrix for ion exchangers. The primary use of these gels is for desalting and buffer exchange of protein solutions, as nowadays fractionation by gel filtration is performed largely with composite gel matrices. Agarose, a low-charge fraction of the seaweed polysaccharide agar, is a widely used packing material.

Microporous gels made by point cross-linking dextran or polyacrylamides are used for molecular-sieve separations such as size-exclusion chromatography and gel filtration, but are generally too soft at the porosities required for efficient protein chromatography. Macroporous gels are most often obtained from aggregated and physically cross-linked polymers. Examples include agarose, macroreticular polyacrylamide, silica, and synthetic polymers. These gels are good for ion-exchange and affinity chromatography as well as for other adsorption chromatography techniques. Composite gels, in which the microporous gel is introduced into the pores of macroreticular gels, combine the advantages of both types.

High matrix rigidity is offered by porous inorganic silica, which can be derivatized to enhance its compatibility with proteins, but it is unstable at alkaline pH. Hydroxyapatite particles have high selectivity for a wide range of proteins and nucleic acids.

Traditional porous media have pore sizes typically in the range of 100 to 300 Å. To reduce intraparticle diffusion for fast chromatography, column packing materials can be made either nonporous or extremely porous. Gustavsson and Larsson [in Hatti-Kaul and Mattiasson (eds), op. cit., pp. 423–454] discussed various chromatography media for fast chromatography of proteins. Nonporous particles such as the popular 5-μm modified silica beads for reverse-phase chromatography are excellent for analytical applications. However, due to their limited binding sites that exist only on the outer surface of the particles, nonporous particles are not suitable for preparative- or large-scale applications. Gigaporous media are gaining momentum in recent years. They are particles with pore sizes above 1000 Å. Some have large enough interconnecting pores in the range of 4000 to 8000 Å that allow even convective flow inside the particles. POROS® perfusion chromatography media are the first commercial products in this category introduced in the 1980s. A few more products are being commercialized. POROS® media are synthesized in two steps. Nanosize subparticles are first synthesized and then polymerized to form 10- to 50-μm particles in a second step. In recent years, advances in suspension polymerization produced a new type of integral gigaporous media with improved physical strength. The spherical particles are formed in a single polymerization step. An oil phase (dispersed phase) consisting of a monomer (such as styrene), a cross-linking agent (such as divinylbenzene), an initiator, a diluent, and a special porogen is used. By dispersing the oil phase in a water phase containing a stabilizer, a suspension can be obtained. The suspension polymerization is carried out at an elevated temperature above the decomposition temperature of the initiator to obtain the polymer particles. The diluent and porogen in the particles form smaller diffusion pores and much larger through-pores, respectively. Figure 20-92 shows such a particle with both conventional diffusion pores and gigaporous

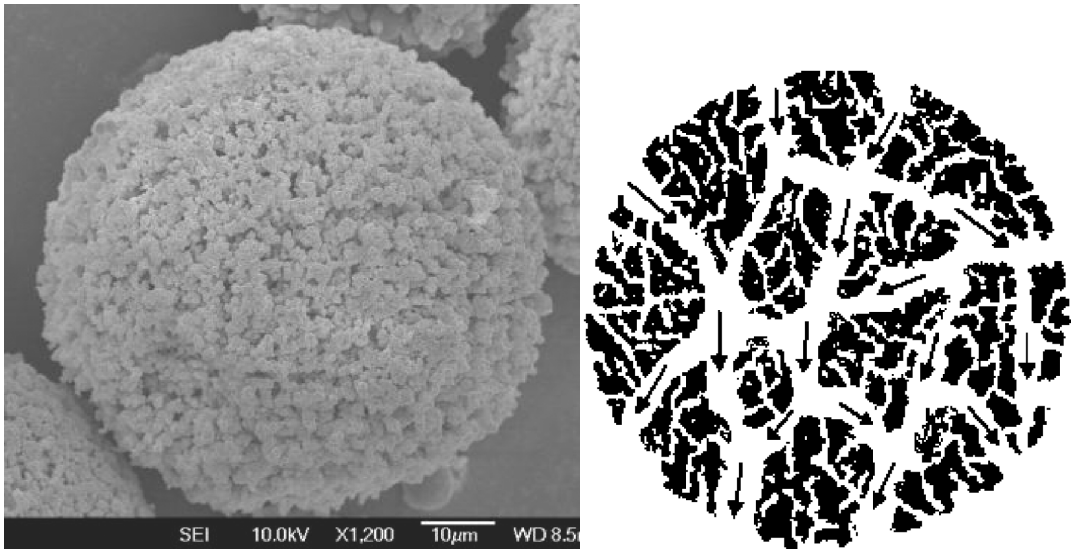


FIG. 20-92 SEM image of a poly(styrene-*co*-divinylbenzene) gigaporous particle synthesized from suspension polymerization and schematic of a gigaporous particle showing through-pores and diffusion pores. (*From Gu et al., Particuology, 3, 349-353 (2005) with permission.*)

through-pores. The through-pores allow convective flow to improve mass transfer, and the diffusion pores provide an overall large surface area required for a large binding capacity.

In the scale-up of a chromatographic column with conventional packing media, the column length scale-up is limited because of the subsequently increased column pressure. To increase the feed load, the column is typically scaled up by enlarging the column diameter after the column length reaches a certain limit. The resultant pancake-shaped column leads to a deteriorating resolution due to poor flow distribution in the column cross-section. With rigid gigaporous particles, axial direction scale-up becomes possible because the flow rate can be up to 40 times higher than that for the conventional media. This kind of scale-up is far superior because of the enhanced resolution together with increased feed capacity when the column axial length is increased. With the advances made in rigid gigaporous media, chromatography columns can potentially process large volumes of very dilute feeds when combined with strong binding kinetics such as those involved in affinity chromatography. This may have a significant impact on the overall downstream process design.

Alternative Chromatographic Columns Commercial radial-flow chromatography (RFC) columns first appeared in the mid-1980s. RFC is an alternative to the conventional axial-flow chromatography for preparative- and large-scale applications. In a RFC column, the mobile phase flows in the radial direction rather than the axial direction. Computer simulation proves that RFC is somewhat equivalent to a pancakelike axial-flow column [Gu, in Flickinger and Drew (eds.), op. cit., pp. 627–639]. Both configurations offer a short flow path and thus a low pressure drop.

Continuous chromatography can be achieved by using a simulated moving-bed (SMB) process shown in Sec. 16, in which several columns are linked with a switching device to simulate a continuous countercurrent flow process. This design maximizes productivity while minimizing eluent consumption [Nicoud, in Ahuja (ed.), op. cit., pp. 475–509]. It is suitable for a simple feed that results in only a limited fraction [Imamoglu, in Freitag (ed.), op. cit., pp. 212–231]. By switching the feed, eluant input positions and raffinate, extract output positions periodically in a series of columns to simulated countercurrent movement of the liquid phase and the solid (resin) phase, continuous chromatography is obtained through the use of multiple columns in series. A four-zone SMB consisting of four columns is capable of producing two fractions as a pair of raffinate and exact. To have two pairs (with a total of four fractions) of raffinate and extract outputs an eight-zone SMB system is needed. Another alternative design described in Sec. 16 is the so-called annual-flow column that rotates continuously in the angular direction. Despite its obvious advantage of being straightforwardly continuous, it suffers from angular dispersion and reduced bed volume. This design so far has seen very limited application since its commercial introduction in 1999 [Wolfgang and Prior, in Freitag (ed.), op. cit., pp. 233–255].

In the past decade, monolithic columns have gained popularity for analytical applications. Instead of using discrete packing particles, a whole polymer block is used as a column. Their continuous homogeneous structure provides fast mass-transfer rates and very high flow rates inside the column [Strancar et al., in Freitag (ed.), op. cit., pp. 50–85]. Thin monolithic disks with affinity binding are capable of fast chromatographic separations. They act much as affinity membrane chromatography cartridges do. To utilize long monolithic columns for process-scale separations, a breakthrough in column fabrication is needed to produce large columns suitable for commercial applications.

Sequencing of Chromatography Steps The sequence of chromatographic steps used in a protein purification train should be designed such that the more robust techniques are used first, to obtain some volume reduction (concentration effect) and to remove major impurities that might foul subsequent units; these robust units should have high chemical and physical resistance to enable efficient regeneration and cleaning, and they should be of low material cost. These steps should be followed by the more sensitive and selective operations, sequenced such that buffer changes and concentration steps between applications to chromatographic columns are avoided. Frequently, ion-exchange chromatography is used as the first step. The elution peaks from such columns can be applied directly to hydrophobic interaction chromatographic columns or to a gel filtration unit, without the need for desalting of the solution between applications. These columns can also be used as desalting operations, and the buffers used to elute the columns can be selected to permit direct application of the eluted peaks to the next chromatographic step.

Factors to be considered in making the selection of chromatography processing steps are cost, sample volume, protein concentration and sample viscosity, degree of purity of protein product, presence of nucleic acids, pyrogens, and proteolytic enzymes. Ease with which

different types of adsorbents can be washed free from adsorbed contaminants and denatured proteins must also be considered.

Scale-up of Liquid Chromatography The chromatography columns in downstream processing typically are operated in the nonlinear region due to a concentrated or overloaded feed. Their scale-up remains a challenging task. There are two general approaches: (1) the rule-based method using equations for column resizing (Ladisch, op. cit., pp. 299–448) and (2) the computer simulation method using rate models (Gu, *Mathematical Modeling and Scale-Up of Liquid Chromatography*, Springer-Verlag, Berlin-New York, 1995) with simulation software such as Chromulator® to predict column performance for a particular column setting and operating conditions.

PRODUCT POLISHING AND FORMULATION

The product from the final purification unit operation is typically in a liquid fraction containing water, a solvent, or a buffer. Based on the requirement for the final product, they may need to be removed. A solid protein is usually far more stable with a much longer shelf life. Product formulation may also require an excipient to be added. Thus, additional unit operations are needed after the final purification step.

Lyophilization and Drying After the last high-performance purification steps it is usually necessary to prepare the finished product for special applications. For instance, final enzyme products are often required in the form of a dry powder to provide for stability and ease of handling, while pharmaceutical preparations also require high purity, stability during formulation, absence of microbial load, and extended shelf life. This product formulation step may involve drying of the final products by freeze drying, spray drying, fluidized-bed drying, or crystallization (Golker, in Stephanopoulos, op. cit., pp. 695–714). Crystallization can also serve as an economical purification step [Lee and Kim, in Hatti-Kaul and Mattiasson (ed.), op. cit., pp. 277–320] in addition to its role as a unit operation for product polishing.

Freeze drying, or lyophilization, is normally reserved for temperature-sensitive materials such as vaccines, enzymes, microorganisms, and therapeutic proteins, as it can account for a significant portion of total production cost. This process is characterized by three distinct steps, beginning with freezing of the product solution, followed by water removal by sublimation in a primary drying step, and ending with secondary drying by heating to remove residual moisture.

Freezing is carried out on cooled plates in trays or with the product distributed as small particles on a drum cooler; by dropping the product solution in liquid nitrogen or some other cooling liquid; by cospraying with liquid CO_2 or liquid nitrogen; or by freezing with circulating cold air. The properties of the freeze-dried product, such as texture and ease of rehydration, depend on the size and shape of the ice crystals formed, which in turn depend on the degree of undercooling. It is customary to cool below the lowest equilibrium eutectic temperature of the mixture, although many multicomponent mixtures do not exhibit eutectic points. Freezing should be rapid to avoid effects from local concentration gradients. Removal of water from solution by the formation of ice crystals leads to changes in salt concentration and pH, as well as enhanced concentration of the product, in the remaining solution; this in turn can enhance reaction rates, and even reaction order can change, resulting in cold denaturation of the product. If the feed contains a solvent and an acid, the solvent tends to sublimate faster than the acid, causing acidic damage to the protein. With a high initial protein concentration the freeze concentration factor and the amount of ice formed will be reduced, resulting in greater product stability. For aseptic processing, direct freezing in the freeze-drying plant ensures easier loading of the solution after filtration than if it is transferred separately from remote freezers.

In the primary drying step, heat of sublimation is supplied by contact, conduction, or radiation to the sublimation front. It is important to avoid partial melting of the ice layer. Many pharmaceutical preparations dried in ampoules are placed on heated shelves. The drying time depends on the quality of ice crystals, indicating the importance of controlling the freezing process; smaller crystals offer higher interfacial areas for heat and mass transfer, but larger crystals provide pores for diffusion of vapor away from the sublimation front.

A high percentage of water remains after the sublimation process, present as adsorbed water, water of hydration, or dissolved in the dry amorphous solid; this is difficult to remove. Usually, shelf temperature is increased to 25 to 40°C and chamber pressure is lowered as far as possible. This still does not result in complete drying, however, which can be achieved only by using even higher temperatures, at which point thermally induced product degradation can occur.

Excipients can be used to improve stability and prevent deterioration and inactivation of biomolecules through structural changes such as dissociation from multimeric states into subunits, decrease in α-helical content accompanied by an increase in β-sheet structure, or complete unfolding of helical structure. These are added prior to the freeze-drying process. Examples of these protective agents include sugars, sugar derivatives, and various amino acids, as well as polymers such as dextran, polyvinyl pyrrolidone, hydroxyethyl starch, and polyethylene glycol. Some excipients, the lyoprotectants, provide protection during freezing, drying, and storage, while others, the cryoprotectants, offer protection only during the freezing process. Spray drying can use up to 50 percent less energy than freeze-drying operations and finds application in the production of enzymes used as industrial catalysts, as additives for washing detergents, and as the last step in the production of single-cell protein. The product is usually fed to the dryer as a solution, a suspension, or a free-flowing wet substance.

Spray drying is an adiabatic process, the energy being provided by hot gas (usually hot air) at temperatures between 120 and 400°C. Product stability is ensured by a very short drying time in the spray-drying equipment, typically in the subsecond to second range, which limits exposure to the elevated temperatures in the dryer. Protection can be offered by addition of additives (e.g., galactomannan, polyvinyl pyrrolidone, methyl cellulose, cellulose). The spray-drying process requires dispersion of the feed as small droplets to provide a large heat- and mass-transfer area. The dispersion of liquid is attained by using rotating disks, different types of nozzles, or ultrasound, and is affected by interfacial tension, density, and dynamic viscosity of the feed solution, as well as the temperature and relative velocities of the liquid and air in the mixing zone. Rotating-disk atomizers operate at 4000 to 50,000 rpm to generate the centrifugal forces needed for dispersion of the liquid phase; typical droplet sizes of 25 to 950 μm are obtained. These atomizers are especially suitable for dispersing suspensions that would tend to clog nozzles. For processing under aseptic conditions, the spray drier must be connected to a filling line that allows aseptic handling of the product.

Thickeners and binders such as acacia, agar, starch, sodium alignate, gelatin, methyl cellulose, bentonite, and silica are used to improve product stability and enhance the convenience of the administration of a liquid formulation. Surface-active agents, colors, flavors; and preservatives may also be used in the final formulation (Garcia et al., *Bioseparation Process Science*, Blackwell Science, Malden, Mass., 1999, p. 374).

INTEGRATION OF UNIT OPERATIONS IN DOWNSTREAM PROCESSING

Generally speaking, a typical downstream process consisting of four stages: removal of isolubles, isolation, purification, and polishing (Belter et al., op. cit., p. 5). Cell disruption is required for intracellular products. One or two, and sometimes more chromatography steps will serve as the center-stage unit operations. The steps before them serve the purposes of feed volume reduction and removal of the majority of impurities. The steps after them are polishing and formulation operations. Based on these general outlines, a few rules of thumb may be used (Harrison et al., op. cit., p. 322; Garcia et al., op. cit., p. 358): (1) Reduce the feed volume early in the process, (2) remove the most abundant impurities or the easy-to-remove impurities first, (3) reduce the amount of impurities as much as possible before the delicate high-resolution chromatography steps, and (4) sequence the unit operations that exploit different separation mechanisms.

The purification of proteins to be used for therapeutic purposes presents more than just the technical problems associated with the separation process. Owing to the complex nature and intricate three-dimensional structure, the routine determination of protein structure

as a quality control tool, particularly in its final medium for use, is not well established. In addition, the complex nature of the human immune system allows for even minor quantities of impurities and contaminants to be biologically active. Thus, regulation of biologics production has resulted in the concept of the process defining the product since even small and inadvertent changes in the process may affect the safety and efficacy of the product. Indeed, it is generally acknowledged that even trace amounts of contaminants introduced from other processes, or contaminants resulting from improper equipment cleaning, can compromise the product. From a regulatory perspective, then, operations should be chosen for more than just efficiency. The consistency of the unit operation, particularly in the face of potentially variable feed from the culture/fermentation process, is the cornerstone of the process definition. Operations that lack robustness or are subject to significant variation should not be considered. Another aspect of process definition is the ability to quantify the operation's performance. Finally, the ease with which the equipment can be cleaned in a verifiable manner should play a role in unit operation selection. Obviously, certain unit operations are favored over others because they are easier to validate. Process validation was covered in a book edited by Subramanian (op. cit., vol. 2, pp. 379–460).

Keep in mind that some unit operations are not as scalable as others. The evolution of a bench-scale process to production scale will see changes in the types and number of unit operations selected. To configure an effective and efficient bioseparations process, a thorough understanding of the various unit operations in downstream processing described above is a prerequisite. Different process-scale and purity requirements can necessitate changes and may result in quite different configurations for the same product. Existing process examples and past experiences help greatly. Due to regulatory restrictions, once a process is approved for a biopharmaceutical, any change in unit operations requires time-consuming and costly new regulatory approval. This means that an optimized process design is much desired before seeking approval. The involvement of biochemical engineers early in the design process is highly recommended.

INTEGRATION OF UPSTREAM AND DOWNSTREAM OPERATIONS

Upstream fermentation (or cell culture) has a direct impact on the design and optimization of its downstream process. Different media or different operating conditions in fermentation result in a different feed for the downstream process. In the development of new products, optimization of the fermentation medium for titer only often ignores the consequences of the medium properties on subsequent downstream processing steps such as filtration and chromatography. It is imperative, therefore, that there be effective communication and understanding between workers on the upstream and downstream phases of the product development if rational tradeoffs are to be made to ensure overall optimality of the process. One example is to make the conscious decision, in collaboration with those responsible for the downstream operations, of whether to produce a protein in an unfolded form or in its native folded form. The purification of the aggregated unfolded proteins is simpler than that of the native protein, but the refolding process itself to obtain the product in its final form may lack scalability or certainty in its method development.

In some instances, careful consideration of the conditions used in the fermentation process, or manipulation of the genetic makeup of the host, can simplify and even eliminate some unit operations in the downstream processing sequence [Kelley and Hatton, *Bioseparation,* **1,** 303–349 (1991)]. Some of the advances made in this area are the engineering of strains of *E. coli* to allow the inducible expression of lytic enzymes capable of disrupting the wall from within for the release of intracellular protein products, the use of secretion vectors for the expression of proteins in bacterial production systems. Fusion proteins can be genetically engineered to attach an extra peptide or protein that can bind with an affinity chromatography medium [Whitmarsh and Hornby, in Street (ed.), op. cit., pp. 163–177]. This can enhance the purification of an otherwise difficult to purify protein greatly. The cell culture medium can be selected to avoid components that can hinder subsequent purification procedures. Integration of the fermentation and initial separation/purification steps in a single operation can also lead to enhanced productivity, particularly when the product can be removed as it is formed to prevent its proteolytic destruction by the proteases which are frequently the by-product of fermentation processes. The introduction of a solvent directly to the fermentation medium (e.g., phase-forming polymers), the continuous removal of products by using ultrafiltration membranes, and the use of continuous fluidized-bed operations are examples of this integration.

Process economics for biological products was discussed by Harrison et al. (op. cit., pp. 334–369) and Datar and Rosen [in Asenjo (ed.), *Separation Processes in Biotechnology*, Dekker, New York, 1990, pp. 741–793] at length, and also by Ladisch (op. cit., pp. 401–430). They provided some illustrative examples with cost analyses. Bioprocess design software can also prove helpful in the overall design process (Harrison et al., op. cit., pp. 343–369).