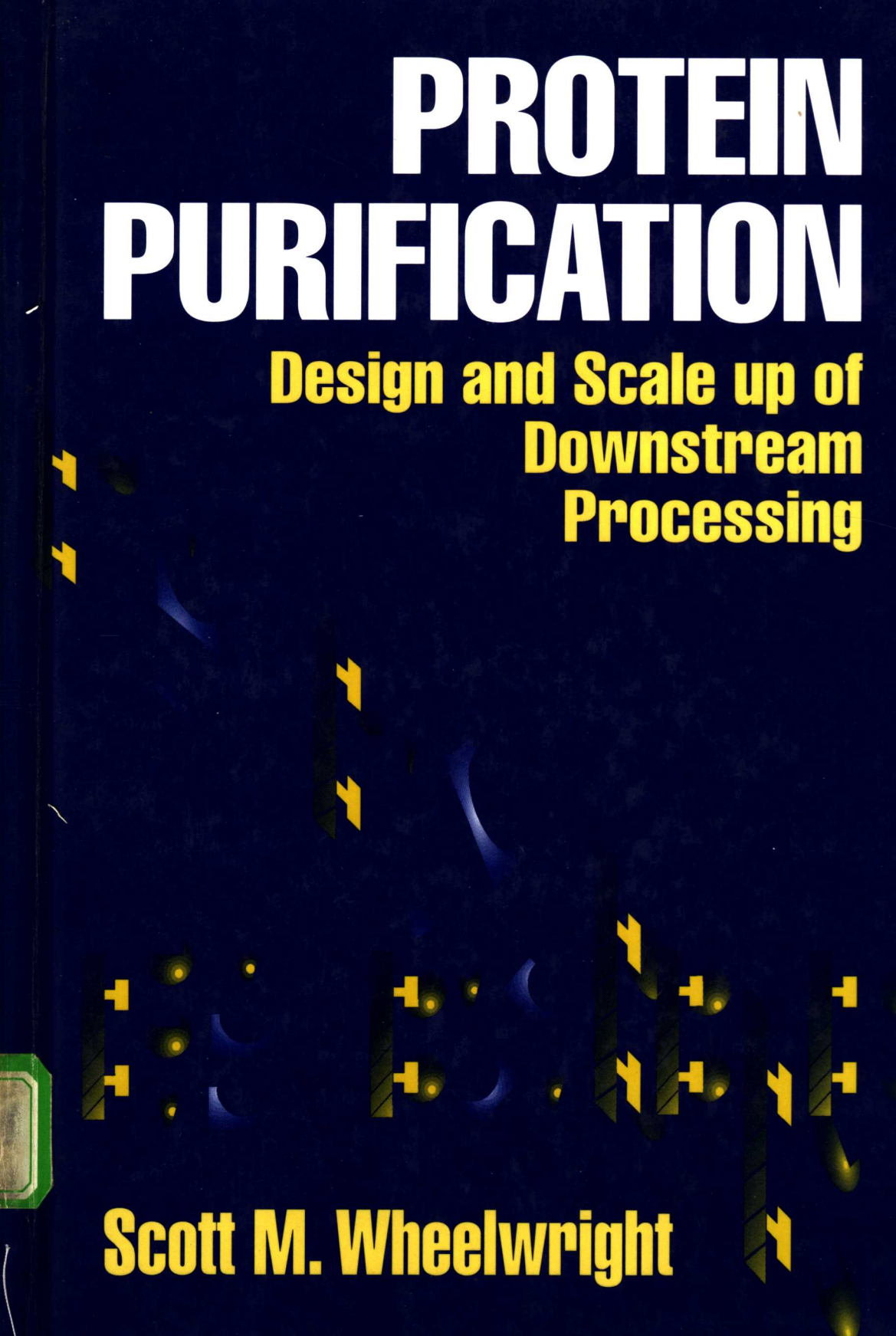
PROTEIN PURIFICATION

Design and Scale up of Downstream Processing



Scott M. Wheelwright

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Design and Scale up of Downstream Processing

SCOTT M. WHEELWRIGHT

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FOREWORD

If one looks at the evolution of great technological innovations, a number of common patterns appear. Two such patterns are the increasing breadth of applications and the increasing economy of using the technology. The evolution of the biotechnology industry has certainly witnessed these trends. Actually, these two trends are probably linked: as the technology becomes more economical it is used more broadly, and as it is used more extensively it becomes more economical.

At the start of the computer age, single computers were the size of large rooms and were used to calculate ballistic trajectories. If one follows the expansion of the computer industry over the last few decades, it is astounding what computers are being used for today, from the ultrasophisticated to the mundane. Airplanes are being built today that cannot be flown by man but must be flown by computers. Medical imaging techniques, such as MRI and CAT scanning, are only possible because of computational power and programming sophistication. Most cars, television sets and microwave ovens have computers or at least microprocessors. Even a $25 wrist watch can have a chip that not only keeps time but is a calculator as well.

While biotechnology is a much younger industry, it already appears to be following the same trends. A decade ago there were no biotech pharmaceuticals and today there are ten. In ten more years, at the turn of the century, there will be many more. More than half of the new drug substances now being submitted for approval to the FDA Center for Biologics Evaluation and Research are produced using either recombinant DNA-derived cells or hybridomas. Even many conventional drug substances are now being discovered and developed using recombinant DNA and monoclonal antibody technology. Receptors and other biological targets such as the HIV polymerase are being purified from cloned sources rather than from naturally-occurring materials.

Cesar Milstein has said that when he and George Koehler discovered monoclonal antibodies in 1976, they did not imagine the myriad uses for these molecules. Fortunately for the rest of us, they published their important work without applying for a patent. Today, monoclonal antibodies are being used as assay reagents in research as well as for clinical diagnostic tests. These tests have become simple and cheap enough to be used in the home, such as the early pregnancy test. Monoclonal antibodies are also being used as "magic bullets"—molecules that can specifically seek out targets in the body to deliver potent drugs for a concentrated local effect. Sometimes, the antibody itself is the drug, as in the case of OKT 3 or individualized monoclonals against B-cell lymphoma. Other times, the antibody is coupled to another effector reagent as in the case of radiolabelled antibodies or antibodies which are coupled to a toxin such as Ricin A

chain. In these cases the antibody's purpose is to direct the site of action of the other entity.

Monoclonals are also being used in the purification of many other products, especially recombinant proteins. Progress in this field has been swift and dramatic. Improvements in the culturing of hybridomas, attachment of monoclonals to support media, and elution and regeneration procedures that extend the lifetime of this media have reduced the cost of using monoclonals by a factor of about one thousand over the past decade. This improved economy coupled with the unique ability to tailor the specificity of monoclonals puts them in the forefront of our recovery and purification tools.

In the same way that monoclonal antibodies can be used to purify recombinant proteins, recombinant proteins can be used to purify monoclonals. Protein A is now made by recombinant DNA technology. Another microbial protein that binds monoclonals, Protein G, has been mutated to delete the albumin-binding domain. This makes it useful as a capture reagent even in the presence of whole serum. This is an example of how the availability of a new technology can act to accelerate its own development in much the same way that each generation of computers is used to design the next generation.

In this historical context, what is the place of the bioprocess industry within the broader biotech industry? A timely example is available in the development of Activase™.

When I started working on the development of the process for TPA in 1982, the dose was estimated to be one milligram. The capacity and cost per gram targets based on this dose estimate did not seem too difficult to achieve. But very shortly the estimates of dose began to rise and there was a simultaneous shift away from an *E. coli* expression system to a mammalian cell culture process. At the time, these changes made the task appear quite a bit more difficult, but still possible. As experience was gained in human clinical trials, the dose continued to rise until it reached 100 mg! If, in 1982, we had known that this was to be the dose, we probably would have given up. Fortunately, progress on the process was made at about the same pace at which the dose rose. Thus, while the dose was rising by 100-fold, the cost per gram was falling by about 100-fold, keeping the cost per dose about the same. If not for all the progress in molecular biology, fermentation and purification science, and manufacturing technology, tissue plasminogen activator would not be available today. Even more important is the fact that this technology is now being used to accelerate the development of a significant number of new molecules as they work their way through development and regulatory approval.

I suspect that the ultimate size of the biotech industry will, to a significant extent, be determined by the success of bioprocess scientists and engineers in making bioproducts at low cost, large scale, and high quality. This will permit the broadest use of these products. There are several projects now under way that represent the current forefront of this effort. One of these, bovine growth hor-

mone, is nearing approval. The market need may be as great as 100,000 kg per year and the production cost has to be 1 to 2 orders of magnitude lower than that for current recombinant human pharmaceutical proteins. Still to be mastered are the challenges presented by recombinant albumin, recombinant hemoglobin and certain monoclonal antibodies given in multigram doses. The production of these proteins will require another order of magnitude improvement in economy and probably at least a similar improvement in purity. While it may be hard to imagine today exactly how this will be done, I am optimistic that great progress can be made.

Scott Wheelwright's book is an unusual accomplishment. It is rare today for an individual scientist to write a book of such breadth and clarity. In addition to covering the fundamentals of the properties of proteins and the unit operations of protein purification, he also addresses the important related issues of process design, integration with upstream operations, cost, and timing to market. I believe that this book can serve as a foundation for someone trying to enter this field or as an enjoyable review for current practitioners. The figures should make it a useful reference source. Hopefully, it will help in the training of more people capable of contributing to the advancement of bioprocess technology. It is my expectation that this in turn will result in the continuing expansion of the whole biotechnology industry.

STUART E. BUILDER

So. San Francisco
January 7, 1991

PREFACE

One of the dominant characteristics of high-technology industries is the overlap of skills needed between disciplines. The boundaries between fields of study are no longer sharp and distinct but rather are blurred to a considerable extent. Biotechnology is not an exception. For products to move from the laboratory to commercial success requires more than just an understanding of the new biology; it also requires an understanding of the fundamentals of engineering and their practice as applied to large-scale protein purification.

The roles of the biochemist and the chemical engineer have been traditionally distinct, but an overlap between these two disciplines has been created by biotechnology. The number of proteins that have been identified as commercial products has generated a need for scientists and technicians with experience in both areas. The purpose of this book is to help bridge the historical gap, to provide the biochemist with an understanding of engineering requirements and the engineer with an understanding of biochemistry requirements for successful scale up of processes for the purification of proteins on a large scale and the commercial success of the new biology.

The hybridization of the biochemist or protein chemist and the chemical engineer or process engineer creates a new discipline, that of protein process designer. This new discipline incorporates both the protein chemist's understanding of protein chemistry—structure, function, characterization, and reaction—and process engineering: mass transfer, hydrodynamics, and thermodynamics. And although the fundamentals underlying this discipline are held in common with other fields, the applications are unique. It is the application of the fundamentals that necessitates this welding of disciplines into a new field.

This book concentrates on these unique applications; for it is the applications that truly set this area apart from other fields. And it is the sheer volume of possible applications or unit operations for protein purification that requires a specialist in this area. Gone are the days when a generalist with an understanding of the fundamentals could pick up and make use of each of these applications without further training. And while it is still possible to intuit each of these applications, starting with first principles, to do so would require a lifetime. Thus this book has been written to provide an introduction to the art and science utilized by the biochemist and process engineer in the design and optimization of large-scale protein-purification processes.

The author wishes to acknowledge the help of many who have brought this work to fruition: my family for their love and support, my colleagues, especially Dr. Ron Johnson, for their encouragement, ideas, suggestions, and helpful pointers on large-scale protein purification, and Scott Bevan for his fine drawings. Parts of this book were previously published: Chapter 2 in the *Journal of Biotechnology* 11 (1989), and Chapter 5 in *Biotechnology Research and Applications*, J. Gavora et al. (eds.), Elsevier Applied Science, London (1988).

Nomenclature

A	=	cross-sectional area
$b_p(G_{sol})$	=	free energy resulting from interaction of salts and bound protein
c	=	solute concentration in fluid phase
c_b	=	concentration in bottom phase
c_f	=	final concentration
c_i	=	initial concentration
c_r	=	reference concentration in fluid phase
c_t	=	concentration in top phase
C_d	=	drag coefficient
C_0	=	total ionic strength of solution
d_p	=	diameter of particle
D	=	diffusivity
$f_p(G_{sol})$	=	free energy resulting from interaction of salts and unbound protein
F_b	=	buoyancy force
F_c	=	centrifugal force
F_d	=	drag force
g	=	gravitational acceleration
h	=	reduced plate height
$HETP$	=	height equivalent to a theoretical plate
J	=	flux
k	=	valence ratio of ions
K	=	equilibrium constant
K_s	=	salting-out constant
L	=	length of column
m	=	mass
M_0	=	zero-order moment
M_1	=	first-order moment
M_2	=	second-order moment
M_i	=	ith-order moment
n	=	exponent in Stokes–Einstein equation
N	=	speed of rotation (number of revolutions per unit time)
NTP	=	number of theoretical plates
P	=	transmembrane pressure
P_f	=	filtrate pressure
P_i	=	inlet pressure
P_o	=	outlet pressure
q	=	solute concentration in solid phase
Q	=	volumetric flow rate
Q_0	=	total number of charge sites in solid phase
r	=	radius
r_1	=	outer radius of centrifuge rotor
r_2	=	inner radius of centrifuge rotor
R	=	constant separation factor

R_g	=	resistance of gel layer
R_m	=	resistance of membrane
RCF	=	relative centrifugal force
S	=	sedimentation coefficient
t	=	time
v	=	volume of sorbent from inlet to point of interest
V	=	volume of fluid fed to column
V_c	=	cumulative volume
V_e	=	elution volume
V_f	=	final volume
V_i	=	volume injected
V_s	=	volume of stationary phase
V_t	=	total volume of column
V_v	=	void volume of column
V_0	=	average sample volume
V'	=	holdup volume
V_0'	=	volume after concentration and dilution
w	=	width of elution curve
x	=	relative concentration in fluid phase
y	=	relative concentration in solid phase
y	=	relative concentration in solid phase
z	=	number of separating spaces in disk-type centrifuge
α	=	selectivity factor
β	=	intercept of salting-in line and normalized solubility line extended to zero concentration
γ	=	surface tension at solvent-air interface
δ	=	Freundlich isotherm exponent
ΔG_{sol}	=	change in free energy
ε	=	void fraction in packed column
κ	=	mass transfer coefficient
Λ	=	slope of salting-in line
μ	=	viscosity
ν	=	velocity
ρ_m	=	density of fluid medium
ρ_p	=	density of particle
ρ_s	=	density of solid phase
σ	=	standard deviation
σ_i	=	molal-surface-tension increment
	=	equivalent clarification area
φ	=	angle of separating space in disk-type centrifuge
ψ	=	liquid-solid contact angle
ω	=	angular velocity
Ω	=	intrinsic salting-out coefficient

CONTENTS

CHAPTER 1

PROCESS DESIGN AND COMPETITIVE ADVANTAGE

The design of a process for the large–scale purification of a protein is one of several complex activities that must be successfully completed to bring a product to market. The complexity is due in large part to the many alternatives from which the designer must select the appropriate recovery steps; but the design is also complicated by its impact on the competitive advantage of the corporation. This book is intended to help the process designer optimize the design so as to provide competitive advantage for the strategy of the corporation: The process is to be designed to give the company the advantage it seeks in the marketplace.

There are three main sources of competitive advantage in the manufacture and sale of high–value protein products: first to market, high quality (in terms of purity, activity, dependability, or flexibility), and low cost. These alternatives are illustrated in Figure 1.1. Each of these offers advantages that can be realized only at the expense of the others. To decide which process steps are appropriate, the company must define the advantage sought. The optimal process is that which gives the best performance with respect to the desired advantage.

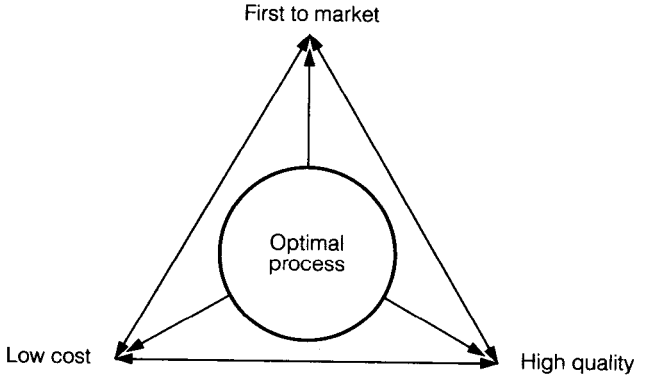

Figure 1.1. The optimal process must be defined within the context of competing goals or sources of competitive advantage.

1.1 SOURCES OF COMPETITIVE ADVANTAGE

Competitive advantage may arise from many sources. Anything that provides an edge over competitors or serves to differentiate the product from similar products is a source of advantage. As described by Porter (1985), in the world at large the opportunities for creating competitive advantage are almost limitless. Within the field of high–value proteins the three categories already mentioned cover most of the common sources of advantage.

Perusal of business journals that cover the industry shows that the company that is the first to market a new protein enjoys a substantial advantage over the competition, particularly if being first to market includes being first with patent protection. Large sums are spent in drug research, for example, to be first with blockbuster money makers. The company that finds itself in second place may net only one–tenth the sales of the company that got there first. With this difference, it obviously makes little sense to spend years developing the lowest cost process if long–term development means losing the race. On the other hand, if the product is not unique or if it can exist in many forms, then a greater advantage may be obtained by differentiation of quality (for example, activity or dependability) than by being first to market.

Many products must share the market with competitors for various reasons, such as insufficient patent protection to keep all competition off the market. In such cases being first to market may not be enough to ensure competitive advantage, and product differentiation may be obtained by varying the characteristics of the product. A protein that is more pure, is more active, has greater lot–to–lot consistency, or is improved by some other measure of performance may outshine the competition. Similarly, a company that can configure its product in more alternatives to suit individual customers shows greater flexibility and may gain an advantage.

As a product acquires competitors, price becomes a greater issue with customers, and lower–cost products gain an advantage. Of course, the lower the cost is from the beginning, the higher the margin and the greater the profit. Thus the company whose process designer has initially developed a lower cost process prevails over the company that must redesign to lower its cost. Regulatory restrictions impose such a high cost on process change that good original design provides a competitive edge.

Once the source of competitive advantage has been identified, it must be maintained as the criterion for evaluation of process design because it defines the goal. The optimal process is the process that minimizes time to market, maximizes a differentiating characteristic, or minimizes cost within the constraints of product specifications and available resources.

1.2 PRODUCT SPECIFICATIONS

With this definition of the goal in mind, we can review the product specifications. The purity required of the product depends on its intended use, as in Figure 1.2, which shows the increase in purity expected in products as a function of dosage. As the dosage increases, so does the purity requirement. Agricultural products and specialty chemicals require only 90 to 95 per cent purity, as do in vitro diagnostics. In vivo diagnostics and other parenterals, such as vaccines, that require only a few injections, do not need so high a purity as do drugs that are administered in large quantities.

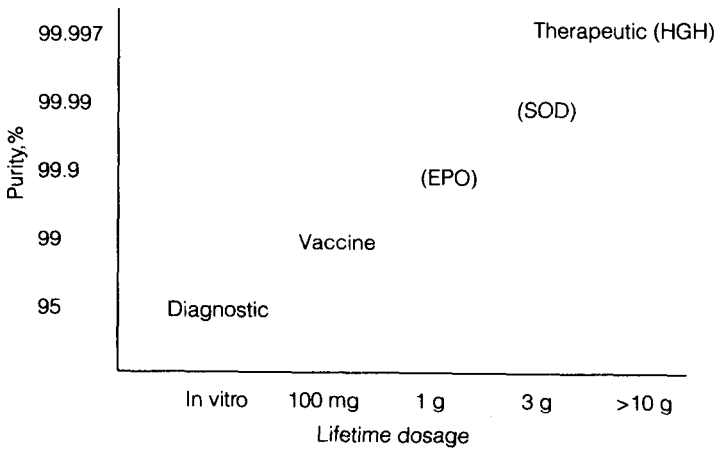

Figure 1.2. Required purity as a function of dosage for selected examples of biotechnology products.

The level of purity required also varies with the nature of the contaminants: Endotoxins, nucleic acid, and anaphylactic agents must be reduced to very low levels, whereas precursors to, or fragments of, the product may be allowable at higher levels.

1.3 FIRST TO MARKET

Being first to market requires short process–development times. In a regulated industry (such as pharmaceuticals) it also means that approval must be granted first. A sloppy process that does not provide consistent product may result in delayed product licensing. This delay can be longer than it would be in the case of a better process. Of course, selecting early license

application or more complete process design is a judgment that must be made on the basis of experience and is part of what makes process design complex.

In a regulated industry changes in manufacturing methods tend to be expensive, as they often require repeated studies of safety and efficacy as well as delays resulting from seeking approval for the changes. Builder et al. (1988) estimate, for a licensed product, a minimum of two years from the time of the conception of a process change until the point when a license amendment may be granted and a cost for the entire effort of millions of dollars. Thus designs may be difficult to alter, and rushing in with an incomplete or ill–defined process may cause considerable difficulties once the product is on the market.

1.4 QUALITY

Quality has almost as many different definitions as there are people who define it. A practical definition in the production of biologicals is that the higher the quality, the less the loss incurred by the customer in using the product. As stated by Taguchi (1986), the magnitude of quality is inversely proportional to the loss caused by the use of that product. In biologicals this frequently means that consistency between lots is more valuable than increased purity; a customer incurs less loss when each batch of the product is equivalent (say, 95 per cent pure) than when the purity (and impurities, side effects, and the like) varies (say, from 92 to 98 per cent). As Taguchi writes, "an article with good quality performs its intended functions without variability, and causes little loss through harmful side effects, including the cost of using it. If cost control is concerned with reducing the various losses that may occur before the product is shipped, quality control is concerned with reducing the losses that it may cause to society after it is shipped." Anything that can be done to establish the quality of a product when it is still in the design stage is many times less expensive than trying to fix problems in the manufacturing stage.

The quality of the product is dependent upon the process by which it is made. Quality cannot be inspected into a product but must be designed into it via the process. Two important factors enter into design for quality: design for manufacturing and robust design.

The unit operations available for protein purification vary in many ways. Some are limited to small–scale operation, others to large–scale operation; some have few operating variables, others many; some are simple to control, others difficult. Design for manufacturing means that the limitations of large–scale manufacturing are considered before the process is implemented