PROTEIN PURIFICATION PROCESS ENGINEERING

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Series Introduction

Bioprocess technology encompasses all the basic and applied sciences as well as the engineering required to fully exploit living systems and bring their products to the marketplace. The technology that develops is eventually expressed in various methodologies and types of equipment and instruments built up along a bioprocess stream. Typically in commercial production, the stream begins at the bioreactor, which can be a classical fermentor, a cell culture perfusion system, or an enzyme bioreactor. Then comes separation of the product from the living systems and/or their components followed by an appropriate number of purification steps. The stream ends with bioproduct finishing, formulation, and packaging. A given bioprocess stream may have some tributaries or outlets and may be overlaid with a variety of monitoring devices and control systems. As with any stream, it will both shape and be shaped with time. Documenting the evolutionary shaping of bioprocess technology is the purpose of this series.

Now that several products from recombinant DNA and cell fusion techniques are on the market, the new era of bioprocess technology is well established and validated. Books of this series represent developments in various segments of bioprocessing that have paralleled progress in the life sciences. For obvious proprietary reasons, some developments in industry, although validated, may be published only later, if at all. Therefore, our continuing series will follow the growth of this field as it is available from both academia and industry.

W. Courtney McGregor

Foreword

In the 20 years since the invention of genetic engineering, manufacture of recombinant proteins has become a mature industry impinging on almost every important aspect of our daily lives. Products range from extremely expensive diagnostic and therapeutic proteins to low-cost substances such as chymosin used in the manufacture of cheese. These latter substances compete successfully with traditional products in the commercial marketplace, on the basis of both quality and price. The number of commercial recombinant proteins continues to grow and both naturally occurring and genetically engineered molecules are now produced on a routine basis.

In order to maintain this remarkable momentum it is essential to solidify the technological base upon which this new industry is built. Almost obscured by the glamor surrounding these products is the simultaneous development of separation techniques needed for their production. Some of these techniques, such as large-scale cell disruption, exist only in biotechnology, and others have evolved into highly effective and specialized forms unique to protein purification. Rapid development of these processing techniques has given us little chance to consolidate and organize our understanding of them.

Although much information concerning protein purification is available, it has not been easy for the engineer or chemist to get the kind of detailed and explicit descriptions needed to develop effective expertise in either manufacturing or process development. It is particularly unfortunate that these newly important unit operations are being largely ignored in our educa-

tional institutions at all levels. A primary reason for this is the lack of reliable, readable, and well-organized texts.

Downstream processing, or recovery and purification, is particularly important because it typically accounts for nearly three-fourths of manufacturing costs in this new industry and because reliable and effective purification can be of the utmost importance to the user. Moreover, the developers of commercial purification processes must operate quickly and under heavy regulatory constraints. It is increasingly recognized that there must be effective communication among those engaged in research, process development, and manufacturing, from early stages of basic research through commercial production. At present, this is seldom the case.

Protein Purification Process Engineering is directed toward meeting these unfulfilled needs. The book begins with a basic overview of the facilities needed to work in this new area and the ways in which process development should be organized and implemented. The remainder of the text is devoted to individual separation and analytical techniques that are important to protein processing but are covered inadequately in other existing texts.

Analytical techniques are given strong emphasis. These techniques are essential in these systems, where the complexity of the product itself and the process streams can make it extremely difficult to close material balances and to characterize purity. Yields in the multistage purification chains characteristic of protein manufacture are usually low, and it is important to know where and why the losses occur, as well as to characterize the nature of the impurities. Moreover, analysis and quality control are expensive, often about one-third of total manufacturing costs in both upstream and downstream processing.

The most commonly used processing steps, described in the remainder of the text, all present unresolved engineering problems, and their effective use requires a great deal of judgment and experience. This is true for the humble but important mechanical rupture of cell walls as well as for more sophisticated techniques such as bioaffinity chromatography. Membrane filtration is an excellent example, as the potential economy and simplicity of these processes is clouded by poor understanding of boundary-layer behavior, fouling, and irreversible degradation. Liquid extraction is a potentially attractive process that suffers from both lack of suitable processing equipment and inadequate experience with solvent systems. Experience in the more conventional process industry cannot be simply translated into the complex chemistry and small flow rates of modern biotechnology.

The authoritative discussion of precipitation is also welcome. Selective precipitation is among the most powerful and potentially cheapest of all separation techniques, and it is widely used in selected protein purification processes. Extension to other applications is desirable, and the chief barrier is the complexity of this process for large unstable molecules such as proteins. The warning in the introductory chapter to pay close attention to

biochemistry is highly appropriate, and success will require close cooperation between protein chemists and engineers.

The most difficult separation problems are almost always solved by some form of chromatography, and two chapters discuss these highly selective but complex and expensive processes. Chapter 7 is devoted primarily to the details of column design and operation, which tend to be neglected in similar monographs but are of great importance in day-to-day operations. This discussion provides both a useful qualitative introduction as well as an appreciation for orders of magnitude, and it should prove especially helpful for those new to protein processing technology. Chapter 8 deals with the highly selective and somewhat mysterious processes of biospecific adsorption. This chapter reinforces the introductory chapter's advice that system biochemistry must be understood as thoroughly as possible.

This ambitious and comprehensive text concludes with an eminently practical discussion of freeze drying, an important process but often neglected in discussions of downstream processing. Freeze drying is normally the last step in the manufacturing process, and the care with which it is carried out can have a great impact on product stability and quality.

The authors are all recognized leaders in their areas of expertise; all have had a great deal of experience. They have produced a coherent product with its own "personality," one which should fill an important niche. They have provided the book with detailed information and given it a practical emphasis. Moreover, the bibliographies are extensive, permitting readers to delve deeper into topics of their own interest. The text will supplement more theoretically oriented monographs and should prove to be highly useful.

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Preface

The biotechnology industry, which originated in the late 1970s, is now well into the commercialization stage. A significant segment of this new industry is dedicated to bringing to market purified proteins. The economics of the processes to produce these proteins tend to be dominated by purification—typically, 80–90% of the manufacturing cost is for downstream recovery and purification. Thus, it is essential that a very efficient protein purification process be developed in order for the overall process to be cost competitive.

Protein Purification Process Engineering focuses on providing guidance for the substantial effort required in developing protein purification processes for large scale, commercial operation. It is written primarily for those engaged in this or related efforts in the industry. Readers doing research on protein purification in both industry and academia will find this book very useful as they work to improve existing processes or develop new ones.

Chapters cover various aspects related to protein purification: process development, scale-up, mathematical descriptions of processes and phenomena, technology, and applications. These topics fall within the general field of process engineering. Most of the technologies currently used at the commercial scale are covered in this book. Some of the chapters, particularly those discussing precipitation and affinity chromatography, have greater emphasis on the basic science involved. This is primarily because these technologies require a deeper science base to understand and utilize them. The chapters are also of varying length because some fields of protein purification are newer and less developed than others. Included is a chapter

on protein analytical methods by industrial practitioners, as these methods are essential to protein purification and should be in place before process development work begins.

The contributors were carefully selected, based on their substantial experience and expertise in their subject areas. The chapters are new, original treatments of the authors' respective subjects, thus constituting a new resource for those readers in the field.

I am grateful for the stimulating environment in purification process engineering at the Upjohn Company and Phillips Petroleum Company that helped lead to the idea for this book. Encouragement to pursue this project from colleagues in the School of Chemical Engineering and Materials Science at the University of Oklahoma is appreciated.

Roger G. Harrison

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1

Organization and Strategy

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I. INTRODUCTION

During the development of a process to produce a protein, the initial emphasis of the work naturally is on the biological process. This focus on the biological process is often prolonged, because the time to develop this process can be lengthy. As a consequence, there can be a delay in shifting attention to the purification of the protein. However, it is important to realize that key organizational steps should be taken before experimental work on the purification process even begins, and that once the protein purification process development starts, the strategy to use in this development is crucial to the success of the project.

Several factors must be considered that relate to the organization of the work: The facilities and equipment must be appropriate for the job to be performed. The impact and applicability of the Current Good Manufacturing Practices (CGMP) regulations of the Food and Drug Administration (FDA) must be evaluated. The desirability of using a project team approach should be considered.

The complexity of most protein purification processes gives added importance to strategy considerations in the development of these processes. Purification processes for proteins nearly always involve more than one step and frequently involve a multitude of steps. Therefore decisions must be made about which individual unit operations to use and the order in which to use them. This effort is called process synthesis. The economics of the process should be evaluated at various times in the synthesis of the process in order to insure that the process is economically viable.

In addition to the strategy for the overall process synthesis, the strategy to apply in developing each individual process step is important. Four of these strategy considerations stand out, based on the author's practical experience.

In this chapter, elaboration of these organization and strategy considerations is given.

II. FACILITIES AND EQUIPMENT CONSIDERATIONS

Even before the actual process development work on purification begins, the issue of whether present facilities are adequate for the task needs to be addressed. Two situations need to be considered: laboratory scale work and pilot plant scale work.

For process development work at the laboratory scale, a good starting point is a typical protein chemistry laboratory. This would include a spectrophotometer with a UV lamp; a refrigerated centrifuge with centrifuging ability, expressed as relative *g* force times capacity in liters, on the order of 10,000–15,000; a wide variety of sizes of chromatography columns (glass or plastic) with adjustable plungers; a fraction collector with a UV monitor; a peristaltic chromatography pump; and a homogenizer for disrupting cells (1). Analytical equipment should include a system for analytical gel electrophoresis. In some cases, it may be highly desirable to have an analytical high-performance liquid chromatograph (HPLC) on hand for analyzing samples soon after they are taken.

Numerous operations for the purification of proteins need to be done at near 0°C to minimize proteolytic degradation and bacterial growth. The two options that arise for the lab scale are doing these operations in a refrigerated room and doing them in a chromatography refrigerator. The author has used only the latter option for lab process development work and found this to perfectly satisfactory. Chromatography refrigerators with glass doors, electrical outlets, and access portholes can be obtained with up to at least 75 cu. ft. of capacity.

The pilot plant is in essence a large-scale laboratory. Because of its larger scale, pilot plant equipment often must be constructed differently from laboratory equipment. Some equipment such as columns can be made of glass as in the lab. Other equipment such as vessels must be constructed of stainless steel or a plastic that has good chemical resistance, such as polypropylene. It is advantageous for each vessel to have its own pH probe for local and/or remote reading of pH. This is commonly done with an Ingold-type pH probe, which is made of glass and is capable of being sterilized. For applications involving food or pharmaceuticals, vessels should be of the "sanitary" design, which means that there are no threads on product contact surfaces and that surfaces must be smooth (150 grit or better finish). The sanitary equipment design standards usually employed are the "3A

Sanitary Standards" that are published by the *Journal of Food Protection*, Ames, Iowa.

Pumps are needed in the pilot plant for a variety of operations. All should have sanitary designs and, in some instances, should be able to be sterilized. For low-pressure and very low flow rate applications such as feeding a chromatography column, peristaltic tubing pumps are commonly used. Three of the most widely used sanitary pumps are centrifugal pumps, positive displacement rotary pumps, and flexible impeller pumps; factors in the selection of these pumps have been discussed in detail by Horwitz (2). A less frequently used pump is the diaphragm pump, which has the advantage of being able to be sterilized. This can be obtained with a double diaphragm so that the pump's hydraulic fluid will not contaminate the product when a diaphragm rupture occurs.

As to the means of keeping process liquids in the pilot plant refrigerated, one common practice is to use jacketed equipment with circulation of a suitable coolant (methanol-water, for example) through the jackets. However, pilot plants have been built with all or part of the equipment in cold rooms. Before using the cold room approach, the processes to be used should be analyzed to determine if the heat transfer will be adequate to maintain temperatures at near 0°C. Pilot plant processes that have appreciable heat generation will generally need to be done in jacketed equipment because the heat transfer coefficient for air in free convection is only a small fraction of the heat transfer coefficient for liquids in forced convection (3). Also, some operations such as precipitation with organic solvents must often be done below 0°C, which means that operation in a jacketed tank will be mandatory.

A number of utilities are needed in the laboratory and pilot plant. The ones required have been listed by Barrer (4). To provide flexibility in using equipment in the pilot plant, it is a good idea for utilities to be distributed to a number of locations, sometimes called utility stations, in the pilot plant. Each utility station contains appropriate outlets for many or all of the pilot plant utilities. If the equipment is modular and on casters, then a great amount of flexibility can be achieved in configuring pilot plant processes. The author both designed and used a pilot plant with utility stations and mobile equipment and found this concept to work out extremely well.

If solvents will be used in the pilot plant, then all the equipment should be designed to explosion-proof specifications. The National Electrical Code classification for electrical equipment and instrumentation is Class I, Group D for the solvents that would potentially be present in a pilot plant to purify proteins (5). If it is possible that solvents may be used at some point during the life of the pilot plant, serious consideration should be given to obtaining explosion-proof equipment initially because of the cost and inconvenience of converting non-explosion-proof equipment later.

III. GOOD MANUFACTURING PRACTICES

The Current Good Manufacturing Practices (CGMP) regulations issued by the FDA can have a large impact on how the purification process work is organized, depending on the end use of the product under development. In general, the CGMP regulations cover the design, validation, and operation of a pharmaceutical manufacturing facility. CGMP regulations have been issued in the United States *Code of Federal Regulations*, Title 21, which is published yearly. The most widely applicable CGMP regulations are those in Part 211 of Title 21 that govern the manufacture of drug products for administration to humans or animals (6). There are supplementary CGMPs for the manufacture of medicated animal feeds (part 225), medicated premixes (part 226), blood and blood components (part 606), and medical devices (part 820).

The FDA has issued helpful guidelines on how to apply and interpret the CGMP regulations. In 1991 a guide for inspection of bulk pharmaceutical chemical manufacturing was issued (7). Bulk pharmaceutical chemicals (BPCs) are defined as being made by chemical synthesis, recombinant DNA technology, fermentation, enzymatic reactions, recovery from natural materials, or combinations of these processes. On the other hand, finished drug products are usually the result of formulating bulk materials whose quality can be measured against fixed specifications. Thus BPCs are components of drug products. This guideline states that "there are many cases where CGMP's for dosage form drugs and BPC's are parallel." The guidelines goes on to say that "in most other cases it is neither feasible nor required to apply rigid controls during the early processing steps....At some logical point in the process, usually well before the final step, appropriate CGMP's should be imposed and maintained throughout the rest of the process." A useful interpretation of these guidelines has been done recently by Moore (8).

In 1991 the FDA issued a very helpful guideline on practices and procedures for the preparation of investigational new drug products that constitute acceptable means of complying with the CGMPs (9). The FDA recognizes that manufacturing procedures and specifications will change as the trials of a new drug advance. However, when drugs are produced for clinical trials in humans or animals, compliance with the CGMPs is *required*. According to this guideline, this means that "the drug product must be produced in a qualified facility, using laboratory and other equipment that have been qualified, and the processes must be validated." In contrast, the CGMP regulations do not apply for the preparation of drugs used for preclinical experimentation (such as toxicity studies on laboratory animals). Furthermore, like drugs approved for marketing, investigational drugs have always been subject to the FDA's inspectional activities.

Each company that is developing processes for protein drug products must carefully design its process development laboratories and pilot plants to be capable of adhering to the CGMPs when required. It is highly desirable that some labs be designated as CGMP labs and others as non-CGMP labs. This type of designation can be done for pilot plants also. However, some smaller firms have only one pilot plant, and for this situation it is not a good idea to be switching back and forth between CGMP and non-CGMP use. It is better to stick with CGMP operation entirely to avoid confusion and to make sure that CGMP procedures are followed when they are supposed to be. Likewise a lab should not be switched back and forth between CGMP and non-CGMP use.

IV. PROJECT TEAMS

Bringing a new protein to market is often extremely complex, involving a number of professionals from different disciplines who are involved in tasks that frequently must occur simultaneously. There is almost always pressure to bring the product to market as soon as possible. Competition with other companies is often intense. Furthermore, more time will be left on the product's patent life if the time required to reach the market is relatively short.

A good way to coordinate the various activities in product development is by a project team. For example, the author was on a project team in Phillips' Biotechnology Division for a new peptide with a molecular biologist, a microbiologist, an analytical chemist, and a marketing professional. This group met often to articulate goals, plan strategy, and discuss results obtained by group members. The project at Biogen on human gamma interferon for use as a pharmaceutical had a team of a group of individuals representing the laboratory research, regulatory affairs, quality control and quality assurance, clinical research, process development, and marketing functions of the company (10). This group met both as a complete team and also in smaller groups.

Since purification usually cannot begin until the protein has been synthesized, the question may arise as to when the purification professional should become involved in the process development effort. Strong arguments have been made that the scientists and engineers involved in purification scale-up should take an active part in the decision making from the *start of the process development* (11–13). One example involving recombinant products is the choice of the expression system. This choice probably will have more impact upon the purification system than any other single factor. Purification scientists and engineers can contribute to this decision by evaluating the following factors: equipment available, characterization of the protein or peptide, and consideration of the experience with the group on the various types of protein purification.

V. PROCESS SYNTHESIS

The creation of a processing scheme to purify a protein is called process synthesis. A common approach to process synthesis is the use of rules of thumb, or heuristics, in making the decisions on which separation steps to use and the order in which to use them. The most important heuristics for protein purification have been identified by Prokopakis and Asenjo (14) and Asenjo and Patrick (15) as follows:

1. Choose separation processes based on different physical, chemical, or biochemical properties.
2. Separate the most plentiful impurities first.
3. Choose those processes that will exploit differences in the physicochemical properties of the products and impurities in the most economical manner.
4. Use a high-resolution step as soon as possible.
5. Do the most expensive step last.

Prokopakis and Asenjo rate rules 1 and 3 as the most important and point out that these rules rely heavily on property information that is incomplete and only qualitative most of the time.

Because each protein is different, it is often worthwhile to focus on a property or properties that make the protein unique. An example is the Phillips process for the purification of alcohol oxidase, which the author helped scale up (16). This process was based on the discovery by a biochemist at Phillips, T. R. Hopkins, that alcohol oxidase could be crystallized when the ionic strength was lowered to a certain point (17). The scaled-up process was very simple and consisted of cell lysis, removal of cell debris by crossflow microfiltration, concentration by ultrafiltration, and crystallization by lowering the pH.

One tool that can be very useful in the synthesis of protein purification processes is computer simulation of the process. Software to do this simulation specifically for bioprocesses is now commercially available (for example, by Intelligen, Inc., Westfield, New Jersey). It may be helpful to do this simulation with available laboratory data before the development of the process has proceeded very far. Later on in the development of the process, the economics of the process can be evaluated conveniently using the simulation program to determine the feasibility of the process and to pinpoint those steps in the process that are most in need of improvement. The computer simulation and economic evaluation of the purification process for porcine growth hormone have been well documented (18).

The use of artificial intelligence in the synthesis of protein purification has been a subject of research in the last few years. It is likely that artificial intelligence will become well enough developed in the near future to be used routinely as an aid in the synthesis of protein purification processes. Progress in this field has been reviewed by Prokopakis and Asenjo (14).

VI. PRACTICAL CONSIDERATIONS IN THE SCALE-UP OF INDIVIDUAL STEPS

In scaling up individual purification process steps, four points especially stand out as worth remembering and adhering to, based on the author's experience:

1. Do careful material balances.
2. Pay attention to the biochemistry involved.
3. Initially look at wide ranges of variables and examine narrower ranges as time permits.
4. Be prepared to do engineering analysis.

An elaboration of these points follows.

A common tendency of those working in protein purification is to follow only the main product stream in terms of specific activity (desired protein activity/total protein weight) and total protein concentration. A better approach is to analyze *each stream* in a given step for the desired protein (and for the impurities, if possible). Closure of the material balance should always be checked. Failure of the material balance to close can mean that product inactivation is occurring, which could lead to a study of ways to prevent this from happening or to minimize the inactivation. Because of assay errors, it is often desirable to obtain an average material balance for a number of runs using the same procedure.

Good material balances are possible only if a good quantitative assay is being used. Ideally, the development of a reliable quantitative assay should *precede* the start of the scale-up work.

Careful attention should always be paid to the biochemistry of the process. Often, the process times become longer and different materials are used as the process is scaled up. Both of these factors can lead to unwanted biochemical reactions that can reduce yield and purity. Longer processing times can give more proteolytic degradation, especially if the process has to be partially carried out at a higher temperature than at the previous scale of operation, which is often the case. Different processing materials (such as column packings, materials of construction, or chemicals used in the process) can give reactions that bind or denature the product or convert it to undesired by-products. When these undesired events occur, it is often a good idea to go back to the laboratory and simulate what is happening at the larger scale.

Looking at a wide range of variables is a good idea at the start of the process development and can sometimes lead to unexpected results that possibly can be patented. One should always keep in mind that each protein is different and has the potential to behave in a manner very different from the norm.

Engineering analysis should never be overlooked as a tool to understand what is going on. This can take a myriad of forms, from the simple calcula-

tion of a Reynolds number, which can differentiate laminar from turbulent flow, to more complex analysis. An example of more complex analysis is in the scale-up from laboratory scale to pilot plant scale of a whole broth adsorption process that was done by the author (19). In this process the whole broth passes through a series of columns where the adsorbent resin is mixed. Mass transfer correlations for the lab and pilot plant columns were found to be widely different. This led to the experimental determination of the residence time distribution for the pilot plant column. The result was that the mixing in the column was far from ideal. The mixing was well modeled by a plug flow region in series with a back-mixed region connected to a stagnant zone. The process was successfully modeled on a computer when the mass transfer correlation was corrected for nonideal mixing.

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