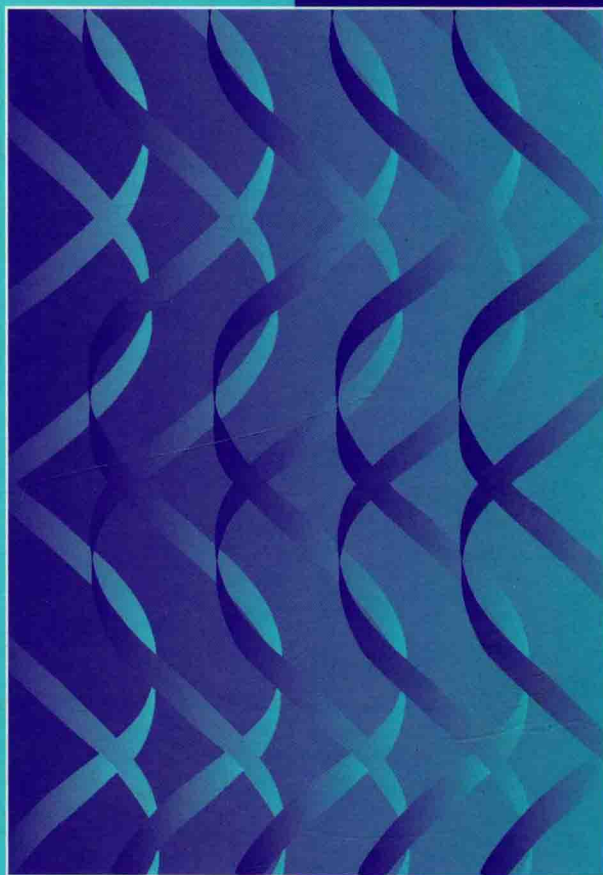


BIOSEPARATION OF PROTEINS

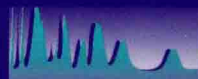
Unfolding/Folding and Validations

Ajit Sadana



VOLUME I

Series Editor **Satinder Ahuja**



SEPARATION SCIENCE AND TECHNOLOGY

BIOSEPARATION OF PROTEINS

Unfolding/Folding and Validations

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BIOSEPARATION OF PROTEINS

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PREFACE

The biotechnology industry is poised for rapid growth and implementation in diverse areas. However, one major constraint is the need for a more complete understanding of protein separation processes. While progress in the technology of cloning genes to attain high and desired levels of expression has been satisfactory, large-scale production and purification of proteins has, until recently, been rather neglected.

Bioseparation stands at the very center of effective biotechnology development. Interest in protein purification has increased rather dramatically in the past few years and has become the focus of intensive research both at academic institutions and in industry. Several papers and a few books on the general area of bioseparations have recently appeared, but no book, and only a few papers, has emphasized the influence of bioseparation processes on protein inactivation. This information, which is scarce and difficult to find in the open literature, is a critical part of the bioseparation framework: One may ask of what use is a bioseparation process if information on the conformational state and the activity of the recovered protein is not presented in any detail? The aim of this book is to provide that information as part of a critical review and synthesis of the literature.

After the introductory chapter, Chapter 2 describes the three basic steps involved in the bioseparation process: cell disruption, initial fractionation, and high-resolution fractionation. The different high-resolution fractionation steps, as described in Chapter 3, are critical for meeting the stringent requirements set for product purity and effectiveness, including those of the different regulatory agencies.

The quality of the separated product is significantly affected by the inactivation of proteins at interfaces during bioseparation. Chapter 4 analyzes these interfacial protein inactivations. Until now, chromatographic processes have been utilized rather heavily during the bioseparation protocol at different stages of separation. Chapter 5 analyzes protein inactivation during chromatographic methods of separation and includes available information on the mechanistic aspects. Other techniques for effectively separating biological products of interest also need to be developed, keeping both the quantitative and the qualitative aspects in mind. Chapter 6 analyzes protein inactivations during novel bioseparation procedures.

During bioseparation, the biological product of interest must adsorb on an interface. Conformational changes accompanied by subsequent activity changes will presumably result and will significantly influence both the quantitative and the qualitative aspects of biological product recovery. Chapter 7 analyzes the influence of protein adsorption and inactivation during bioseparation. The economics of the downstream process plays a significant role in getting a biological product ready for market. This sort of information is not readily available in the open literature but is presented here in Chapter 8.

Some denaturation during bioseparation is unavoidable. Different renaturation techniques, presumably as "corrective" steps to minimize the extent of denaturation of at least some of the biological products recovered, would be extremely helpful. Some of these techniques may also be used to enhance or improve the quality of the product by facilitating the process by which the product attains the required conformational state(s). Chapter 9 analyzes protein refolding strategies and inactivation during bioseparation.

Consistency in the safety, potency, efficacy, and purity of a biological product is the manufacturer's responsibility. This consistency is the basis of governmental regulation and evaluation. Tests for impurities and contaminants are critical in the development and validation of bioseparation processes and in final product testing. Validation is the "assurance that a process is closely followed during a product's manufacture." Validation protocols or strategies provide written documentation that a process is consistently doing what its manufacturer claims it can accomplish. This validation procedure provides assurance that the process is "under control." Because recombinant techniques are used to make quite a few bioproducts, the importance of this step cannot be overemphasized. This validation process and the protocols and strategies involved therein, along with the appropriate governmental and regulatory guidelines, are presented in Chapter 10.

The text is intended for instruction at the graduate level and even at the senior undergraduate level, as well as for the industrial practitioner who, after examining the "science-based" information in the earlier chapters, will appreciate the focus on the monitoring, validation, and economics of bioseparation processes. The generalized treatment will also interest chemical, biochemical, and biomedical engineers, chemists, biochemists, and those in the medical profession who wish to better understand the fundamentals of bioseparation and its influence on protein/enzyme inactivation. Even venture capitalists will find the book of interest. Biotechnology, by its very nature, is an interdisciplinary area that requires diverse expertise. I hope that this book will foster these in-

teractions, facilitate an appreciation of all perspectives, and help in efforts toward improved economics of bioseparation.

This text is unique in that it provides the appropriate background on bioseparation processes, while emphasizing the extent of, mechanisms of, and control of protein inactivation during these processes and their essential validation. Comparisons of protein inactivation during different bioseparation processes provide valuable information for workers in different areas who are interested in bioseparations. Readers may thus consider this a “second-level” book on bioseparations, with “first-level” books providing the fundamentals of bioseparation processes. This second-level examines and analyzes the control and validation of the product (protein) during these bioseparation processes. Second, and presumably “higher-level,” books will be required to pave the way for the emergence and consolidation of protein purification as a discipline rather than as a means to an end.

■ LIST OF EXAMPLES

CHAPTER 1

No examples

CHAPTER 2

- 2.1 Describe briefly some of the advantages of protein excretion from cells (Sherwood *et al.*, 1985).
- 2.2 Briefly present a kinetic analysis of enzymatic lysis and disruption of yeast cell walls (Hunter and Asenjo, 1987a).
- 2.3 Briefly describe the principles of operation of expanded beds for particulate removal from protein solutions (Chase, 1994).
- 2.4 Describe briefly the recovery of proteins utilizing membranes (Martin and Manteuffel, 1988).
- 2.5 Provide an example where ultrafiltration has been used to clarify a fermentation broth for producing antibodies (Duffy *et al.*, 1989).
- 2.6 Briefly describe the purification of the IgG antibody by affinity cross-flow filtration (Weiner *et al.*, 1994).
- 2.7 Briefly analyze the extraction of penicillin G by an emulsion liquid membrane (ELM) process (Lee and Lee, 1992).
- 2.8 Briefly describe the traditional purification process for insulin production (Ladisich and Kohlmann, 1992).

- 2.9 Briefly analyze affinity precipitation using chitosan as a ligand carrier for protein purification (Senstad and Mattiasson, 1989).
- 2.10 Briefly describe an affinity precipitation method for proteins by surfactant-solubilized, ligand-modified phospholipids (Powers *et al.*, 1992).
- 2.11 Briefly analyze the large-scale purification of staphylococcal enterotoxin B using chromatographic procedures (Johansson *et al.*, 1990).
- 2.12 Briefly analyze the use of modified divinylbenzene-polystyrene resins for the separation of aspartame, phenylalanine, aspartic acid, and asparagine (Casillas *et al.*, 1992).
- 2.13 Briefly analyze the final fractionation steps for the recovery of SEB using chromatographic methods (Johansson *et al.*, 1990).
- 2.14 Briefly describe the ultrafast HPLC separation of recombinant DNA-derived proteins (Olson and Gehant, 1992).
- 2.15 Briefly describe the purification and characterization of lamb pre-gastric lipase (D'Souza and Oriel, 1992).

CHAPTER 3

- 3.1 Describe briefly the isolation and purification of carboxylesterase from *Bacillus stearothermophilus* (Owusu and Cowan, 1991).
- 3.2 Briefly analyze the processing steps for obtaining tissue plasminogen activator (tPA) from animal cell and bacterial sources with special attention to the quality of the product recovered (Datar *et al.*, 1993).
- 3.3 Briefly describe the purification of two endo- β -glucanases from the aerobic fungus *Penicillium capsulatum* (Connelly and Coughlan, 1991).
- 3.4 Briefly analyze the purification of pectin methylesterase from *Bacillus subtilis* (Pitkanen *et al.*, 1992).
- 3.5 Briefly analyze the purification of *Clostridium thermocellum* β -glucosidase B using ion exchange, hydrophobic interaction, and hydroxylapatite chromatography (Romaniec *et al.*, 1993).
- 3.6 Briefly analyze the purification of D-xylulokinase from the yeast *Pichia stipitis* NCYC 1541 using adsorption (hydroxylapatite column) chromatography (Flanagan and Waites, 1992).
- 3.7 Briefly analyze the purification of feruloyl/*p*-coumaroyl esterase from the fungus *Penicillium pinophilum* (Castanares *et al.*, 1992).
- 3.8 Briefly analyze the purification of chitinase from *Trichoderma harzianum* (Ulhoa and Peberdy, 1992).
- 3.9 Briefly describe the concerted cluster model of multivalent affinity for heterogeneous adsorption of enzymes (Dowd and Yon, 1995).

- 3.10 Briefly analyze the purification of κ -carrageenase from *Pseudomonas carrageenovora* (Ostgaard *et al.*, 1993).
- 3.11 Briefly analyze the production of blood proteins using the ion-exchange technique (Cueille and Tayot, 1985).
- 3.12 Briefly analyze the large-scale purification and crystallization of lipase from *Geotrichum candidum* (Hedrich *et al.*, 1991).
- 3.13 Briefly analyze the purification and the crystallization of lipase from *Vibrio harveyi* (Lang *et al.*, 1992).
- 3.14 Briefly analyze the purification and crystallization of penicillin (Bienskowski *et al.*, 1988).
- 3.15 Briefly analyze the purification and crystallization of cephalosporin (Bienskowski *et al.*, 1988).
- 3.16 Briefly analyze the purification of β -galactosidase from *Aspergillus fonsecaeus* (Gonzalez and Monsan, 1991).
- 3.17 Briefly analyze the purification of β -glucosidase from the fungus *Neocallimastix frontalis* EB188 (Li and Calza, 1991).
- 3.18 Briefly analyze the separation of peroxidase from soybean hulls by the ARMES technique (Paradkar and Dordick, 1993).

CHAPTER 4

- 4.1 A two-phase system that exhibits potential for bioseparation other than the classical polyethylene glycol (PEG)-dextran system is described (Pathak, *et al.*, 1991).
- 4.2 Present a brief analysis of interfacial transport processes in reversed micellar extraction of proteins (Dugan *et al.*, 1991).
- 4.3 Briefly describe the kinetics and mechanism of shear inactivation of lipase from *C. cylindracea* (Lee and Choo, 1989).
- 4.4 Describe an example where protein adsorption at an air–water interface has been studied by the radiotracer technique. Briefly describe the information that is made available (Hunter *et al.*, 1990).
- 4.5 Briefly describe protein separation by differential drainage from foam (Mohan and Lyddiatt, 1994).
- 4.6 Present an example where proteins are adsorbed on small particles. Also, describe the conformational changes (Tan and Martic, 1990).
- 4.7 Present an analysis of the influence of surface hydrophobicity on the conformational changes of adsorbed fibrinogen (Lu and Park, 1991).
- 4.8 Describe adsorption behavior of different proteins with wide variations in their molecular properties (Kondo and Hagashitani, 1992).
- 4.9 Briefly describe the driving forces involved in the adsorption of the enzyme savinase at solid–liquid interfaces. Also, determine the major driving forces (Duinhoven *et al.*, 1995).
- 4.10 Briefly describe the adsorption of the fungal lipase lipolase at solid–liquid interfaces (Duinhoven *et al.*, 1995).

- 4.11 Briefly compare the adsorption of hen lysozyme (LS2) and milk LAC on colloidal AgI (Galisteo and Norde, 1995).
- 4.12 Describe by appropriate modeling: (1) the principle of the replacement method, and (2) the simulation of adsorption in a well-mixed particle suspension (Cornelius *et al.*, 1992).

CHAPTER 5

- 5.1 Describe a procedure for the HPIEC separation of biopolymers especially suited for applications at high pH and to high-molecular weight samples (Kato *et al.*, 1984).
- 5.2 A method for the separation of mRNAs (van der Mast *et al.*, 1991).
- 5.3 Briefly describe the separation of lipase from *Pichia burtonii* (Sugihara *et al.*, 1995).
- 5.4 Briefly describe a process to separate basic fibroblast growth factor (bFGF) and alkaline phosphatase (PALP) from human placenta (Costa *et al.*, 1993).
- 5.5 Provide an example for the HPLC separation of an enzyme exhibiting microheterogeneity (Wong *et al.*, 1988).
- 5.6 Provide an example of protein separation using conformational differences (Regnier, 1987).
- 5.7 Provide an example of kinetics of denaturation of an enzyme or enzymes on a surface used in RP-HPLC (Benedek *et al.*, 1984).
- 5.8 Provide an analysis of the scale-up of HIC purification of the anti-tumor antibiotic SN-07 (Ishida *et al.*, 1989).
- 5.9 Provide an example for the heparin HPLC separation of proteins (Dyr and Suttmar, 1991).
- 5.10 Provide an example of a large-scale immunoaffinity purification of recombinant soluble human antigen (sCDS) from *E. coli* cells (Wells *et al.*, 1993).
- 5.11 Briefly describe a method to purify α -amylase by immunoaffinity chromatography with a cross-reactive antibody (Katoh and Terasima, 1994).
- 5.12 Describe the separation of enzymes and long-chain fatty acids by CPC (Cazes, 1989).

CHAPTER 6

- 6.1 Describe a thermodynamic analysis of the activity and stability of globular proteins in the interior of reverse micelles (Battistel *et al.*, 1988).

- 6.2 Provide information pertaining to (1) the amount of enzyme/protein recovered (Jolivald *et al.*, 1990); (2) the loss of activity (Sarcar *et al.*, 1992); and (3) the structural changes, if any (Samana *et al.*, 1984) exhibited by enzymes when subjected to the reverse micelle technique.
- 6.3 Provide information concerning the amount of surfactant and solubilizing water required to extract a given amount of protein using reverse micellar systems (Ichikawa *et al.*, 1992).
- 6.4 Describe the influence of temperature on protein desolubilization from reverse micelles (Dekker *et al.*, 1990).
- 6.5 An analysis of the continuous extraction of an enzyme by reverse micelles (Dekker *et al.*, 1986).
- 6.6 Describe the effect of water content and reverse micellar extraction on protein extraction from an aqueous phase into a reverse micellar phase (Hilhorst *et al.*, 1995).
- 6.7 Describe an analysis for the affinity partitioning of glycoproteins in reverse micelles (Paradkar and Dordick, 1991).
- 6.8 Provide an example of a large-scale fermentation and separation of a recombinant protein from *E. coli* (Strandberg *et al.*, 1991).
- 6.9 Describe the two-phase aqueous extraction of enzymes (Kula, 1987).
- 6.10 Briefly describe on-line monitoring of protein activity and concentration during a two-phase aqueous extraction (Papamichael *et al.*, 1991).
- 6.11 Briefly describe the genetically altered charge modification utilized to enhance the electrochemical partitioning of a β -galactosidase and T4 lysozyme in aqueous two-phase systems (Luther and Glatz, 1994).
- 6.12 Describe a theory that helps predict the partitioning of biomolecules in two-phase systems (Diamond and Hsu, 1989).
- 6.13 Describe the partition behavior of the extracellular protein, lipase from *Pseudomonas cepacia* using detergent-based two-phase aqueous systems (Terstappen *et al.*, 1992).
- 6.14 Describe briefly a mathematical model for the metal affinity partitioning of proteins (Suh and Arnold, 1990).

CHAPTER 7

- 7.1 Briefly describe some of the processes that are influenced both in a favorable and in a deleterious manner by protein adsorption. Also, briefly describe some of the effects that primarily control protein adsorption (Haynes *et al.*, 1994).
- 7.2 Describe briefly the adsorption of blood proteins to different surfaces.
- 7.3 Protein adsorption on surfaces indicates quantitative as well as qualitative features (Shirahama *et al.*, 1990).

- 7.4 Provide applications for heterogeneous adsorption of solutes from dilute solutions (Nikitas, 1989).
- 7.5 There is a paradox between concentration dependent adsorption and lack of desorption in pure buffer (Kop *et al.*, 1989).
- 7.6 Briefly describe the competitive adsorption of HSA, IgG, and fibrinogen on silica made hydrophobic by methylation or plasma deposition of hexamethyldisiloxane (HMDSO) using *in situ* ellipsometry and TIRF (Malmsten and Lassen, 1994).
- 7.7 Briefly describe plasma protein adsorption onto glutathione immobilized on gold (Lestellius *et al.*, 1995).
- 7.8 Briefly describe the adsorption of IgG and glucose oxidase (GO_x) to highly oriented pyrolytic graphite (HOPG) as analyzed by AFM (Cullen and Lowe, 1994).
- 7.9 Briefly describe a macroscopic model for a single-component protein adsorption (Al-Malah *et al.*, 1995).
- 7.10 Develop the equations between flowing blood proteins and an artificial surface (Schaaf and Dejardin, 1987).
- 7.11 There are some correlations between blood protein adsorption and surface properties (Grainger *et al.*, 1989).
- 7.12 Describe a technique for measuring protein adsorption wherein protein molecules are not modified by the introduction of some extrinsic label that might affect the adsorption kinetics (Norde and Rouwendal, 1990).

CHAPTER 8

- 8.1 Provide a brief economic analysis for utilizing centrifuges for single- and multiuse facilities (Mahar, 1993).
- 8.2 Describe briefly the changes made by Genentech as the dosage requirements for tPA increased from 1 to 100 mg during clinical trials (Spalding, 1991).
- 8.3 Describe briefly the modifications made by Hoffman-LaRoche during the large-scale processing of α -interferon A.
- 8.4 Demonstrate the applicability of the down-scaling approach for the gel filtration of a polymeric protein mixture that has a molecular weight-size distribution between 30×10^6 and 80×10^3 Da and a mass average molecular weight of 3.98×10^6 (Naveh, 1990).
- 8.5 Provide economic data for the separation of tPA, monoclonal antibodies, and animal growth factors utilizing perfusion chromatography. Present three different strategies for operating chromatographic columns (Fulton *et al.*, 1992).
- 8.6 Provide some reasons why other bioseparation techniques have not been applied on a commercial scale. Consider a particular case, for example, two-phase aqueous systems (Huddleston *et al.*, 1992).

- 8.7 Provide some economic data on a technique that effectively separates relatively large amounts of monoclonal antibodies (Duffy *et al.*, 1989).
- 8.8 Analyze briefly some of the major cost elements in designing immunosorbent columns on a large scale (Desai, 1990).
- 8.9 Briefly present the costs involved in running chromatographic separations on a large scale (Peskin and Rudge, 1992).
- 8.10 Describe briefly the qualitative features of the Porter-Ladisch model (Porter and Ladisch, 1992) for the cost estimation of separation of α -galactosidase from soybean seeds. In other words comment on the relative costs of each purification step.
- 8.11 Present briefly the economics of separation of bioproducts for an *E. coli* based fermentation process (Datar, 1986).
- 8.12 Present briefly the process design and economics for the production of polygalaturonases from *Kluyveromyces marxianus* (Harsha *et al.*, 1993).

CHAPTER 9

- 9.1 Briefly mention some of the nonproteinaceous materials or additives that have been utilized to assist in protein refolding (Zardeneta and Horowitz, 1994).
- 9.2 Briefly present and analyze the different protein purification strategies (protocols) that have been utilized to separate proteins in the denatured state (Knuth and Burgess, 1987).
- 9.3 Briefly describe the effects of mutations on the aggregation of proteins (Wetzel, 1994).
- 9.4 Briefly describe the simulation of a folding pathway (Hinds and Levitt, 1995).
- 9.5 Describe the influence of the reversible and irreversible denaturation of Nase on aggregate formation (Nohara *et al.*, 1994).
- 9.6 Briefly describe the purification and renaturation of recombinant human interleukin-2 (IL-2) (Weir and Sparks, 1987).
- 9.7 Briefly describe the *in vitro* folding of glycoprotein hormone chorionic gonadotropin (Huth *et al.*, 1994).
- 9.8 Briefly show the influence of chaperonins and protein disulfide isomerases on the renaturation of single-chain immunotoxin (Buchner *et al.*, 1992).
- 9.9 Briefly describe the chaperonin-facilitated *in vitro* folding of monomeric mitochondrial rhodanese (Mendoza *et al.*, 1991).
- 9.10 Compare briefly the refolding of proteins by the use of assistants such as detergents, lipids, and micelles with chaperonin-assisted refolding (Zardeneta and Horowitz, 1994).
- 9.11 Briefly analyze cysteine to serine substitution on basic fibroblast growth factor (bFGF) IB formation during *in vitro* refolding (Rinas *et al.*, 1992).

- 9.12 Describe PEG-assisted refolding of three recombinant human proteins (Cleland *et al.*, 1992).
- 9.13 Briefly analyze the antibody-assisted protein refolding (Carlson and Yarmush, 1992).
- 9.14 Briefly analyze protein refolding in reverse micelles (Hagen *et al.*, 1990a).
- 9.15 Briefly describe the influence of environmental conditions on the refolding selectivity of insulin-like growth factor I (Hart *et al.*, 1994).

CHAPTER 10

- 10.1 Describe briefly some of the considerations that must be examined to set the stage for later validation work (Akers *et al.*, 1994).
- 10.2 Briefly describe the procedures involved in the validation of β -Urogastrone (Brewer, 1986).
- 10.3 Explain the concern over the removal of DNA and protein impurities in biopharmaceuticals (Briggs and Panfili, 1991).
- 10.4 Briefly describe the avoidance of unsafe levels of host cell protein contaminants that might lead to toxic or immunologic reactions (Eaton, 1995).
- 10.5 Briefly describe the validation of column-based separation processes (PDA Report, 1992).
- 10.6 Briefly describe the life cycle approach to analytic methods during pharmaceutical product development (Hokanson, 1994).
- 10.7 Briefly describe the validation procedure to purify MAbs from mouse ascites fluid (Mariani and Tarditi, 1992).
- 10.8 Show validation studies in the regeneration of ion-exchange celluloses (Levinson *et al.*, 1995).
- 10.9 Briefly describe some of the important results of cleaning validation and residue limits (Zeller, 1993).
- 10.10 Briefly describe chromatography cleaning validation (Adner and Sofer, 1994).

CONTENTS

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