METHODS IN MOLECULAR BIOLOGY™

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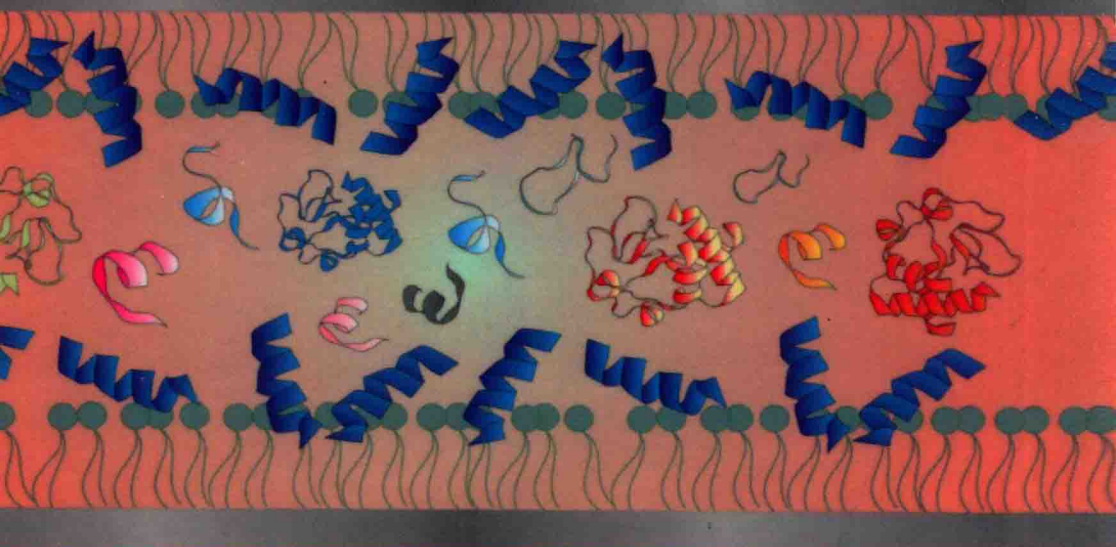
HPLC of Peptides and Proteins

Methods and Protocols

Edited by

Marie-Isabel Aguilar



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Edited by

Marie-Isabel Aguilar

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Preface

The introduction of high-performance liquid chromatography (HPLC) to the analysis of peptides and proteins some 25 years ago revolutionized the biological sciences by enabling the rapid and sensitive analysis of peptide and protein structure through the exquisite speed, sensitivity, and resolution that can be easily obtained. Today, HPLC in its various modes has become the pivotal technique in the characterization of peptides and proteins and currently plays a critical role in both our understanding of biological processes and in the development of peptide- and protein-based pharmaceuticals.

The number of applications of HPLC in peptide and protein purification continues to expand at an extremely rapid rate. Solid-phase peptide synthesis and recombinant DNA techniques have allowed the production of large quantities of peptides and proteins that need to be highly purified. HPLC techniques are also used extensively in the isolation and characterization of novel proteins that will become increasingly important in the postgenomic age. The design of multidimensional purification schemes to achieve high levels of product purity further demonstrates the power of HPLC techniques not only in the characterization of cellular events, but also in the production of peptide- and protein-based therapeutics. HPLC continues to be at the heart of the analytical techniques with which scientists in both academia and in industry must arm themselves to be able to fully characterize the identity, purity, and potency of peptides and proteins.

The aim of *HPLC of Peptides and Proteins: Methods and Protocols* is to provide the beginner with a sufficiency of the practical information needed to develop separation and analytical protocols for peptide and protein analysis. This volume opens with an overview of the basic theory and general methodology of HPLC, with particular reference to the key separation parameters that can be manipulated to achieve high resolution. Each of the commonly used HPLC techniques are covered in Chapters 2–9, whereas methods for capillary to large-scale preparative isolation are described in Chapters 10–15. Chapters 16–27 provide those already experienced in HPLC with a number of specific applications, as in case studies to illustrate the analytical approaches to a particular separation or assay challenge, with examples drawn from contemporary fields in biochemistry and biotechnology. These applications include proteolytic mapping, posttranslational modifications, neuropeptide processing, glycopeptides and glycoproteins,

MHC-binding peptides, toxins/venoms, membrane proteins, antibodies, combinatorial and proteome analysis, and enzymatic activity.

HPLC of Peptides and Proteins: Methods and Protocols will be a valuable resource for a wide range of scientists, including biochemists, molecular biologists, pharmacologists, and microbiologists, who work with peptides and/or proteins in both academic and biotechnology laboratories.

Finally, I would like to thank all of the authors for their enthusiastic participation and excellent contributions.

Marie-Isabel Aguilar

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Contents

I

MODES OF HPLC

1

HPLC of Peptides and Proteins

Basic Theory and Methodology

Marie-Isabel Aguilar

1. Introduction

High-performance liquid chromatography (HPLC) is now firmly established as the premier technique for the analysis and purification of a wide range of molecules. In particular, HPLC in its various modes has become the central technique in the characterization of peptides and proteins and has, therefore, played a critical role in the rapid advances in the biological and biomedical sciences over the last 10 years.

The enormous success of HPLC can be attributed to a number of inherent features associated with reproducibility, ease of selectivity manipulation, and generally high recoveries. The most significant feature is the excellent resolution that can be achieved under a wide range of conditions for very closely related molecules, as well as structurally quite distinct molecules. This arises from the fact that all interactive modes of chromatography are based on recognition forces that can be subtly manipulated through changes in the elution conditions that are specific for the particular mode of chromatography. Peptides and proteins interact with the chromatographic surface in an orientation-specific manner, in which their retention time is determined by the molecular composition of specific contact regions. For larger polypeptides and proteins that adopt a significant degree of secondary and tertiary structure, the chromatographic contact region comprises a small proportion of the total molecular surface. Hence, the unique orientation of a peptide or protein at a particular stationary phase surface forms the basis of the exquisite selectivity that can be achieved with HPLC techniques. All biological processes depend on specific

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interactions between molecules and affinity chromatography exploits these specific interactions to allow the purification of a biomolecule on the basis of its biological function or individual chemical structure. In contrast reversed phase HPLC, ion-exchange and hydrophobic interaction chromatography separate peptides and proteins on the basis of differences in surface hydrophobicity or surface charge. These techniques therefore allow the separation of complex mixtures whereas affinity chromatography normally results in the purification of one or a small number of closely related components of a mixture.

Reversed-phase chromatography (RPC) is arguably the most commonly used mode of separation for peptides, although ion-exchange (IEC) and size exclusion (SEC) chromatography also find application. The three-dimensional structure of proteins can be sensitive to the often harsh conditions employed in RPC, and as a consequence, RPC is employed less for the isolation of proteins where it is important to recover the protein in a biologically active form. IEC, SEC, and affinity chromatography are therefore the most commonly used modes for proteins, but RPC and hydrophobic interaction (HIC) chromatography are also employed.

HPLC is extremely versatile for the isolation of peptides and proteins from a wide variety of synthetic or biological sources. The number of applications of HPLC in peptide and protein purification continue to expand at an extremely rapid rate. Solid-phase peptide synthesis and recombinant DNA techniques have allowed the production of large quantities of peptides and proteins which need to be highly purified. The design of multidimensional purification schemes to achieve high levels of product purity further highlight the power of HPLC techniques in the analysis and isolation of peptide and proteins samples. The complexity of the mixture to be chromatographed depends on the nature of the source and the degree of preliminary clean-up that can be performed. In the case of synthetic peptides, RPC is generally employed both for the initial analysis and the final large scale purification. The isolation of proteins from a biological cocktail however, often requires a combination of techniques to produce a homogenous sample. HPLC techniques are then introduced at the later stages following initial precipitation, clarification and preliminary separations using soft gel. Purification protocols therefore need to be tailored to the specific target molecule. The key factor that underpins the development of a successful separation protocol is the ability to manipulate the retention of the target molecule so that it can be resolved from other contaminating components. This chapter thus provides an outline of the general theory of chromatography and the factors that control both the retention time and peakwidth of solutes undergoing separation in terms of the parameters that control resolution. This information can then be used to understand the approaches used to perform