Analytical Biotechnology

Capillary Electrophoresis & Chromatography

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Capillary Electrophoresis and Chromatography

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Foreword

The ACS SYMPOSIUM SERIES was founded in 1974 to provide a medium for publishing symposia quickly in book form. The format of the Series parallels that of the continuing ADVANCES IN CHEMISTRY SERIES except that, in order to save time, the papers are not typeset but are reproduced as they are submitted by the authors in camera-ready form. Papers are reviewed under the supervision of the Editors with the assistance of the Series Advisory Board and are selected to maintain the integrity of the symposia; however, verbatim reproductions of previously published papers are not accepted. Both reviews and reports of research are acceptable, because symposia may embrace both types of presentation.

Preface

ADVANCES IN BIOTECHNOLOGY HAVE BROUGHT ABOUT novel and efficient means for industrial production of therapeutic proteins and other biological substances. As a corollary to these accomplishments, the need has arisen for rapid analytical techniques with high resolution and sensitivity to facilitate research and development, process monitoring, and quality control in biotechnology. Such applications have provided the major driving force for the rapid growth of new methodologies for biopolymer analysis in the past few years.

In fact, we have witnessed the birth of new analytical methods that play an essential role in biotechnology. Further progress in life sciences also depends on the introduction of increasingly sophisticated analytical tools for the separation, characterization, and quantitative assay of complex biological molecules. Consequently, the concepts, methods, and strategies of analytical biotechnology will inevitably be adopted by the life sciences.

In the manufacture of proteinaceous drugs, the purity of the final product is of paramount importance. Purity, however, is by no means an absolute term; it depends on the method used for its measurement. Since traditional chromatographic methods and slab gel electrophoresis have been the main tools for biopolymer analysis, the commonly used but utterly vague terms *chromatographically* or *electrophoretically* pure demonstrate the role of available techniques not only in the measurement but also in the definition of purity.

In the past decade, we have benefited from major improvements in the sensitivity and efficiency of both chromatography and electrophoresis. High-performance liquid chromatography (HPLC) has become firmly ensconced as a powerful method for protein analysis. Ironically, reversed-phase chromatography, which employs denaturing conditions, has found a particularly wide application, at least when only analytical information is sought. This is the case in most routine analytical work required for many biotechnological applications.

The advantages of HPLC over classical chromatographic methods stem from the employment of a precision instrument that utilizes high-performance columns with concomitantly high analytical speed and resolution and affords total control over the chromatographic process and sensitivity of analysis. In a way, the recent emergence of capillary electrophoresis (CE) follows the same patterns: electrophoresis, a well-established and widely used method of biopolymer analysis, is carried out

by a suitable instrument that exhibits some of the major features of a liquid chromatograph. Although numerous technical problems are yet to be solved, it is not difficult to anticipate that instrumentation of electrophoresis will bring forth the advantages also germane to HPLC and thus will greatly expand the potential of electrophoretic analysis.

This volume accounts for some of the recent developments in CE and HPLC that are of particular interest in biotechnology. Four chapters dealing with capillary electrophoresis present an introduction to this technique and discuss its application to various analytical problems ranging from the analysis of cyclic nucleotides to quality control in the pharmaceutical industry. Another four chapters encompass recent developments in HPLC and has a sharper focus: analysis of pharmaceutical proteins. This particular area is of vital importance to biotechnology, and recent progress has been quite impressive. Enhancement of analytical performance in terms of speed, resolution, and sensitivity combined with the integration of various techniques has engendered powerful schemata for routine assay of proteins at purity standards unheard of a decade ago. An adjunct chapter elaborates problems associated with the use of HPLC as a detection method in preparative chromatography, an approach that will surely find increasing application in industrial protein purification. Mass spectrometry has found growing employment in the structure determination of peptides on a routine basis, and one chapter deals with the use of this technique. The last contribution in this volume is aimed at the use of the displacement mode of chromatography, which is primarily a preparative technique. Its potential in analytical work, however, is being more and more recognized for the enrichment of trace components.

We thank the authors for their contributions, which made possible the publication of this volume. Analytical biotechnology continues to face new challenges, and its armory of adequate tools for meeting them is far from being complete. The collection of papers presented here should be viewed as a testimony for both the incipient nature and the vast potential of this field, which does not yet warrant the publication of a treatise.

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Chapter 1

High-Resolution Nanotechnique for Separation, Characterization, and Quantitation of Micro- and Macromolecules

Capillary Electrophoresis

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A powerful high-efficiency, high-resolution analytical technique is described for the separation, characterization and quantitation of minute amounts of analytes. This technique, termed capillary electrophoresis, offers the capability of on-line detection, the use of multiple detectors, micropreparative operation and automation.

The determination of minute quantities of micro- and macromolecules is an important problem in biological chemistry and poses a challenge to biological chemists. Attempts to optimize separation and characterization conditions and techniques have always been a major concern to many scientists. Unfortunately, most of the advanced new technologies currently available to biological chemists still require microliter quantities and hardly reach subpicomole sensitivities.

Two of the most powerful separation techniques used today are chromatography and electrophoresis. Although various modes

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of chromatography are used for separation and characterization of macromolecules, quite often the final purity test is performed through electrophoretic analysis. If a single peak is obtained during the chromatographic analysis of proteins and peptides, electrophoresis will probably be used as a confirmatory purity test. The opposite is unusual.

Although the separation modes of the electrophoretic methods practiced today are many (1,2), they are slow, labor-intense, prone to relatively poor reproducibility and have limited quantitative capability. In addition, it has been difficult to accomplish a fully automated operation. On the other hand, the emergence of capillary electrophoresis (CE) gradually has begun to solve problems in which the handling of low nanoliter samples and subfemtomole quantities is necessary. Furthermore, among the major advantages of capillary electrophoresis is that it can be made fully automated, it has high resolution capability, and it can quantitate fully minute amounts of sample to be analyzed. Because a significant amount of information has been reported during the last decade about capillary electrophoresis (for recent reviews see 3-9), we are aiming to update new developments in instrumentation, bonding chemistries of capillaries, and applications on the analysis of proteins and their building-block components.

Although numerous examples of capillary electrophoresis separations of micro- and macromolecules can be cited (3-9), the most troublesome (and probably the application most commonly used), is the separation and analysis of proteins, peptides and amino acids. Table I shows a comprehensive view of the literature regarding the analysis of these substances, which are biologically the most diverse of all biological compounds, serving a vast array of functions.

The need for high-resolution protein separations has become more important due to the recent revolution in molecular biology. Typically, the recovery of an expressed protein from

TABLE I. Analysis of Proteins, Peptides, and Amino Acids by Capillary Electrophoresis

Analyte	Mode of CE	Detection System	Reference
Dansyl amino acids	Open-tubular	Fluorescence	10-14
Fluorescamine derivatized dipeptides	Open-tubular	Fluorescence	10
Leucine enkephalin vasotocin dipeptides	Open-tubular	Mass spectrometry	14
Lysozyme cytochrome c ribonuclease chymotrypsinogen horse myoglobin	Open-tubular	Fluorescence	15
Egg white lysozyme peptides	Open-tubular	Fluorescence	16
Chicken ovalbumin tryptic peptides	Open-tubular	Fluorescence	17
Phenylthiohydantoin amino acids	Open-tubular	UV	18
Human transferrin Human hemoglobin	Packed-tubular	UV	19
Cewl, hhcc, bprA, wsmm, csmm, hhm, dhm, dsmm,cewc, bcca, bmlb, bmla, ceo	Open-tubular	UV	20
Horse myoglobin β-lactoglobulin A β-lactoglobulin B swm, hca, bca	Open-tubular	UV	21

Continued on next page

Table I. *Continued*

Analyte	Mode of CE	Detection System	Reference
D-L amino acids	Packed-tubular	UV	22
Human growth hormone	Packed-tubular	UV	22
α-Lactalbumin β-lactalbumin trypsinogen pepsin	Packed-tubular	UV	23
Rabbit hemoglobin	Packed-tubular	UV	4
OPA-amino acids	Open-tubular	Fluorescence	5
Myoglobin and myoglobin fragments	Open-tubular	UV	23
Synthetic peptides	Open-tubular	UV	24
Lysozyme trypsinogen myoglobin β-lactoglobulin A β-lactoglobulin B	Open-tubular	UV	25
Hirudin (thrombin-specific inhibitor)	Open-tubular	UV	5
Ggqa, ggea, ggda, wa, we, wg, ggra, wgg, wf	Open-tubular	UV	6
Untreated amino-acids, Dipeptides	Open-tubular	Electrochemistry	7,27
L-dihydroxy-phenylalanine	Open-tubular	Electrochemistry	26,27
Dipeptides	Open-tubular	UV	28

Continued on next page

Table I. *Continued*

Analyte	Mode of CE	Detection System	Reference
Phycoerythrin	Open-tubular	UV	29
Enolase β-amylase	Packed-tubular	UV	29
Chicken lysozyme β-lactoglobulin A β-lactoglobulin B rabbit parvalbumin hcc, hhm	Open-tubular	UV	28
Cytochrome c proteins	Open-tubular	UV	28
Neuropeptides	Open-tubular	UV	30-38
Prolyl 4-hydroxylase β-subunit peptides	Open-tubular	Fluorescence	39
Glycine, wsmm, carbonic anhydrase, β-lactoglobulin A, β-lactoglobulin B	Open-tubular	Fluorescence	40
Putrescine	Open-tubular	Fluorescence	41
Monoclonal antibodies	Open-tubular	Fluorescence	42,43

Abbreviations used here: cewl, chicken egg white lysozyme; hhcc, horse heart cytochrome c; bprA, bovine pancreas ribonuclease A; wsmm, whale skeletal muscle myoglobin; esmm, equine skeletal muscle myoglobin; hhm, horse heart myoglobin; dhm, dog heart myoglobin; dsmm, dog skeletal muscle myoglobin; cewc, chiken egg white conalbumin; beca, bovine erythrocytes carbonic anhydrase; bmlA, bovine milk β-lactoglobulin A; bmlB, bovine milk β-lactoglobulin B; ceo, chicken egg ovalbumin; swm, sperm whale myoglobin; hca, human carbonic anhydrase; bca, bovine carbonic anhydrase; hcc, horse cytochrome c.

tissue culture media or fermentation broths is difficult because host cell contaminants and artifacts of the recombinant product must be removed. Artifacts arising from translation errors, improper folding, premature termination, incomplete or incorrect post-translational modification, and chemical or proteolytic degradation during purification all contribute to the production

of polypeptide species with structures similar to the desired native polypeptide. Therefore, a high-resolution method such as capillary electrophoresis would be useful for monitoring biosynthetic fidelity and protein purity during the production of recombinant proteins. For example, it would be useful to separate peptides which differ only in one aminoacid or if the location of the same amino acid in the sequence is different. Similarly, it would be useful in the characterization of closely related proteins, such as isoenzymes and immunoglobulins.

The use of capillary electrophoresis as an analytical tool has been quite successful in the separation of a few small molecular weight proteins and many peptides obtained from commercial sources, most probably highly purified. However, ***in cellulo***, proteins are usually associated with multimolecular complexes which are known to participate in essential cellular processes such as DNA replication, DNA recombination, and protein synthesis. Furthermore, other important biological processes that require protein complexes for activity include cellular motion, catalysis of metabolic reactions, regulation of biochemical processes, transport of micro- and macromolecules, and the structural maintenance of cells and the cellular matrix. In addition, the disruption of normal processes by viral infection produces virus-encoded multimolecular protein complexes, including the partially assembled precursors of the mature virus. Therefore, these complex protein-macromolecules may present a problem (for their separations) when using untreated fused-silica capillaries due to the adsorption of many proteins onto the walls of the capillary. Since the performance of any analytical technique is characterized in terms of accuracy, precision, reproducibility and dynamic range, many changes have to be made to the system in order to optimize the performance of capillary electrophoresis for the analysis of peptides and proteins.

For small peptides, separation efficiencies in excess of one million theoretical plates have been demonstrated (13,20,44).

Separation efficiencies for large proteins are more common in the hundred thousand theoretical plates. In comparison with gas chromatography, supercritical fluid chromatography, and liquid chromatography, capillary electrophoresis is the best separation technique from the point of view of molecular weight range of applicability. In the same column, it is possible to separate species ranging in size from free amino acids to large proteins associated with complex molecular matrices. In addition, from the detection standpoint, high-performance liquid chromatography is proven to provide better concentration sensitivity. On the other hand, capillary electrophoresis can provide better mass sensitivity.

As an instrumental approach to conventional electrophoresis, capillary electrophoresis offers the capability of on-line detection, micropreparative operation and automation (6,8,45-47). In addition, the ***in tandem*** connection of capillary electrophoresis to other spectroscopy techniques, such as mass spectrometry, provides high information content on many components of the simple or complex peptide under study. For example, it has been possible to separate and characterize various dynorphins by capillary electrophoresis-mass spectrometry (33). Therefore, the combination of CE-mass spectrometry (CE-MS) provides a valuable analytical tool useful for the fast identification and structural characterization of peptides. Recently, it has been demonstrated that the use of atmospheric pressure ionization using Ion Spray Liquid Chromatography/ Mass Spectrometry is well suited for CE/MS (48). This approach to CE/MS provides a very effective and straightforward method which allow the feasibility of obtaining CE/MS data for peptides from actual biological extracts, i.e., analysis of neuropeptides from equine cerebral spinal fluid (33).

Peptide mapping studies, generated by the cleavage of a protein into peptide fragments, must be highly reproducible and quantitative. Several electropherograms of protein digests have been obtained when chicken ovalbumin was cleaved by trypsin

(17), β-subunit of prolyl 4-hydroxylase cleaved by *Staphylococcus aureus* strain V8 protease (39), egg white lysozyme by trypsin (16), myoglobin and hemoglobin cleaved by trypsin (48), β-lactoglobulin A cleaved by *Staphylococcus* V8 protease (24), and recombinant interferon by trypsin (see Figure 1). Since capillary electrophoresis can provide high mass sensitivity, ultra-high efficiency and nanoliter sample injection, it also provides an excellent tool for the characterization of proteins when comparing peptide mapping, especially if the amount of material is difficult to obtain. For example, it could be quite useful for the identification of mutations in certain proteins which are characteristic of detrimental diseases (such as genetic diseases), or in the identification of site-specific protein modifications. In addition, it can be used as a quality control measure for recombinant protein products.

Routinely, common chemical and enzymatic techniques are used to obtain protein fragments. Unfortunately, when enzymatic digestion techniques and nanograms quantities of proteins are used, the method become ineffective due to dilution and reduced enzymatic activity. An alternative approach to overcome this problem is the use of proteolytic enzymes immobilized to a solid support and a small-bore reactor column. Using trypsin immobilized to agarose, tryptic digests of less than 100 ng of protein can be reproducible obtained (49).

The major concerns that are general to the use of all capillary electrophoresis systems for the separation of proteins and their building-block components are (a) choosing columns; (b) buffer solution compatibility with the system; and (c) the selection of the hardware.

CAPILLARY COLUMN

The heart of any chromatographic and electrophoretic system is the column. Preparation of capillary columns requires specific modifications, including bonding chemistries. Although one can