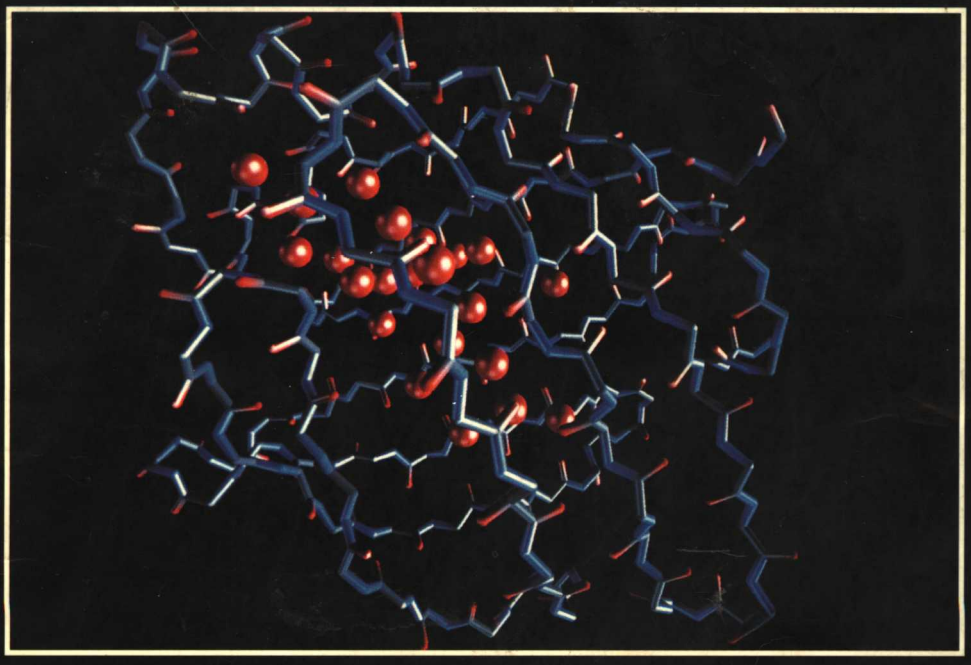
# TECHNIQUES IN PROTEIN CHEMISTRY III



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Edited by Ruth Hogue Angeletti

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# Foreword

My first graduate student learned quickly that if you want to draw a huge crowd around your poster at a meeting, don't show your latest results from an investigation of a biological problem, but present a hot new technique applicable to many problems. Advances in understanding biological systems draw the specialists, admittedly the most important people in your field; but making it possible for anyone to do something more quickly, at higher sensitivity and accuracy, and with less input of supplies and labor draws everyone.

I suppose that this is the wrong lesson for a graduate student to learn; *solving the problem* is the objective after all. But we are always pressing the limits of our assays, and each advance in sensitivity and speed opens new fields of research which were previously inaccessible. Thus, scientists will always be looking for better ways of doing things, will always cite useful technique papers more than those advancing their field of research (Oliver Lowry, where would we be without you?), and will always be interested in books reporting the latest in methodology.

This is the fourth volume resulting from the Protein Society symposia. The number of copies sold and the desire of investigators to contribute to these volumes (our editors always comment on the willingness of the authors to submit their manuscripts *on time!*) are indeed impressive. The Protein Society has always devoted a lot of attention at its meetings to new methodologies, and the Fifth Symposium in Baltimore, June 22–26, 1991, from which this book was compiled, was no exception. There were more posters than ever at the symposium, and their quality was outstanding. We are grateful for that continuing interest and will commit ourselves to fostering it in any way possible.

Special thanks are due Ruth Hogue Angeletti and her associates for editing this volume. Their diligence and the top quality work presented at the Fifth Symposium make this a most useful and informative book.

*Mark Hermodson*
President
The Protein Society

# Preface

The pleasant surprise in editing this volume has been the enthusiastic cooperation of the members of the Protein Society. Without this support, it would have been impossible to produce a book whose ideas are still fresh and current at publication. This active participation indicates to me that the Techniques in Protein Chemistry volumes have truly become our forum for exchanging new methods and research approaches and reflect the spirited exchanges of the poster sessions.

My special appreciation is due those who made this book possible: the associate editors John Crabb, Steve Carr, Phil Andrews, and Ira Ropson; the special reviewers Al Smith and Ed Nieves; the staff of Academic Press; the presidents of the society Finn Wold and Mark Hermodson; my secretary Elyse Rizzo for her organization and attention to detail; Jim Sacchettini, who created the cover graphics; and the authors, who did the real work while tolerating my gentle, but persistent nagging. I hope each volume published becomes used and "dogeared."

*Ruth Hogue Angeletti*

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# Protein and Peptide Recovery from PVDF Membranes

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## I. Introduction

Electroblotting proteins from gels onto solid supports is a widely utilized technique for the microisolation of proteins. Several electroblotting membranes have been developed that allow direct sequence analysis (1-3). Polyvinylidene difluoride (PVDF) is the most frequently utilized support for sequence analysis of electroblotted proteins (4). A limitation of this technique is that N-terminal blocked proteins must be chemically cleaved or proteolytically digested to generate internal peptide fragments which can then be sequenced. *In situ* digestions on PVDF (5-8) and nitrocellulose membranes (9) have been described; however, these methods only result in partial digestion of the membrane-bound proteins. Methods that directly extract protein from PVDF have been developed (10,11), but are based on solutions containing detergents which can limit subsequent proteolytic digestions or interfere with HPLC separations. We have developed a simple extraction procedure using the solvent dimethyl sulfoxide (DMSO) that allows recovery of intact proteins from PVDF membranes. The DMSO can be easily removed, allowing the extract to be analyzed by protein sequencing, amino acid analysis, or by other biochemical analysis.

The extracted protein can be cleaved or digested and the resulting fragments can either be separated by HPLC or by tricine SDS-gels (12) followed by a second round of electroblotting. An additional advantage of this technique is that the protein may be directly digested in the extraction solvent after addition of an appropriate buffer.

## II. Materials and Methods

*Materials.* Proteins were purchased from Sigma, with the exception of human growth hormone (hGH) which was obtained from Genentech, Inc. Lys-C was from Wako chemicals, and cyanogen bromide was from Pierce. Dimethyl sulfoxide (spectrometric grade) was from Aldrich. PVDF (Immobilon-P) membranes were obtained from Millipore and ProBlott membranes were from Applied Biosystems.

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*Electroblotting and elution.* SDS-PAGE was done as described by Laemmli (13) using a BioRad minigel apparatus. Tricine minigels were obtained from Novex. Electroblotting was done in a BioRad transfer tank using 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 20% methanol, pH 11.0 for 1 h at 250 mA (4). Coomassie stained bands from electroblotted proteins were extracted with 200 µl DMSO in a micro Eppendorf tube on a VWR vortexer-2 using a medium setting for 2 h. The DMSO was removed and another 200 µl of DMSO was added. The extraction was continued for an additional 2 h. The extracts were then combined and used for further experiments. Extractions were also performed using a single addition of 200 µl of DMSO, vortexed for 17h.

*Fragmentation.* Extracted proteins were dried in a Speed-Vac to remove DMSO and were cleaved with cyanogen bromide in 0.1 N HCl at 45°C for 3 h (14). Blots were prewetted with 5 µl of methanol prior to the addition of 0.1N HCl and cyanogen bromide.

Protease digestions were performed on electroblot extracts after partial removal of DMSO by Speed-Vac evaporation. Ammonium bicarbonate (0.1 M) was added to the residual DMSO (20-50 µl) resulting in a final solution of 200-500 µl of 10% DMSO. Digestion with Lys-C was carried out at 37°C for 17 h. with an enzyme to substrate ratio of 1:20 (w/w).

Electroblots were first treated with a modified methanol-chloroform precipitation (15) prior to DMSO elution in order to remove Coomassie blue and HPLC artifact peaks. Blots (wet) were placed in 100 µl of water in an Eppendorf tube and 400 µl of methanol was added. The solution was vortexed for 2 min. and 100 µl of chloroform was added. The solution was vortexed again for 2 min. and the liquid was removed. The membrane was then extracted with DMSO. This method effectively removed the Coomassie blue and minimized artifact peaks in the HPLC chromatograms.

*Peptide separation.* Peptides were purified by reversed-phase HPLC using a Synchropak 4000 Å C4 column (2 x 100 mm) on a Hewlett-Packard 1090 M liquid chromatograph equipped with diode array detector. Peaks were detected at 214 nm after elution with a linear gradient of 0-70% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 200 µl per minute.

*Protein sequencing.* Automated Edman degradation was performed with Applied Biosystems models 470A and 473A protein sequencers equipped with on-line PTH analyzers. Sequence interpretation was performed on a VAX 8750 as described (16).

*Amino acid analysis.* Extracted proteins were dried in a Speed-Vac to completely remove DMSO. Residual DMSO can result in the oxidation of some amino acids including methionine and cysteine. Blots were prewetted with 5 µl of methanol prior to hydrolysis. Proteins were hydrolyzed in the gas phase for 24 h with 300 µl 6N HCl containing 0.1% phenol at 110°C under vacuum using a Millipore Picotag workstation. Hydrolyzed blots were extracted twice with 100 µl of 6 N HCl. The extracts were dried and reconstituted with Beckman sample buffer and hydrolysates were analyzed on a Beckman Model 6300 amino acid analyzer.