CRC Handbook of Immunoblotting of Proteins

Volume II

Experimental and Clinical Applications

Editors

Ole J. Bjerrum

Niels H. H. Heegaard

CRC Handbook of Immunoblotting of Proteins

Volume II

Experimental and Clinical Applications

Editors

Ole J. Bjerrum, Ph.D., M.D.
Associate Professor
The Protein Laboratory
University of Copenhagen
Copenhagen, Denmark

Niels H. H. Heegaard, M.D.
The Protein Laboratory
University of Copenhagen
Copenhagen, Denmark

CRC Press, Inc.
Boca Raton, Florida

PREFACE

The development of the immunoblotting technique has been an event of major importance in protein chemistry. The combination of the high resolving powers of electrophoretic separation techniques with the specificity of antibody detection has brought a new dimension into the molecular characterization of proteins.

In spite of a landslide of methodological papers and reviews, no books devoted to immunoblotting of proteins from a practical point of view have been published until now. We have, therefore, found it expedient to try to collect the sum of our present knowledge about applications and techniques of immunoblotting in this monograph.

Volume I focuses on technical descriptions (Sections 1 to 7). Volume II focuses on experimental applications (Section 8), and Clinical applications (Section 9). Volume I is built up like the chronological progress of an immunoblotting experiment, starting with chapters about separation methods and ending with chapters on detection principles and artifacts. Much attention is given to detailed descriptions and recipes, so that the book can be of direct use for bench work in the laboratory. In the application chapters (Volume II), we have tried to select some representative topics. A chapter on future aspects closes the book.

The volumes deal with immunoblotting but not with DNA, RNA, or ligand blotting. There are two exceptions: a chapter on lectin blotting and a chapter on cell blotting, which have been included to exemplify the versatility of the technique. No further attempts to discuss the heterogenous group of ligand blotting have been made.

Immunoblotting is now an established technique and part of the standard technology of biological sciences, but it has been used in such a diversity of connections that no "authorized" version of the procedure exists. We have chosen to let specialists in different fields write about their own part of the spectrum. The inclusion of so many authors, all describing their personal variant of immunoblotting, we think especially increases the value of the book despite some unavoidable repetitions. This approach should give the researcher seeking a solution to a particular problem in immunoblotting a chance to find useful information. We also hope that the combination of techniques and applications in one handbook, besides illustrating the applicability of the immunoblotting technique, will be a source of inspiration for researchers working in other fields. Therefore, abbreviations have been avoided as much as possible to make the chapters appear intelligible and readable.

We have the sole responsibility for the edition of the chapters, as no editorial board has been involved. We are grateful to our fellow authors for their fine contributions and for granting us editorial license to obtain, we hope, uniformity in style and composition.

THE EDITORS

Ole J. Bjerrum, Ph.D., is Director, Research and Development, Department of Immunotechnology, Novo Industry A/S, Bagsvaerd, Denmark.

Dr. Bjerrum was born 1944 in Copenhagen and obtained his medical degree from the University of Copenhagen in 1969. After finishing his medical internship at the University Hospital, he studied protein chemistry at the Institute of Biochemistry, University of Uppsala, Sweden. At the Protein Laboratory, University of Copenhagen, he was appointed in 1971 as assistant professor and in 1974 as associate professor. Dr. Bjerrum has been director of the institution for 4 years. The Ph.D. degree from University of Copenhagen was obtained in 1978. In 1980-81 Dr. Bjerrum spent a year as visiting professor at the Department of Biochemistry and Molecular Biology, Northwestern University, Illinois. In 1987 he moved to Novo Industry A/S.

Dr. Bjerrum is a member of The Danish Academy of Natural Sciences, Danish Biochemical Society (vice-president 1983-84 and president 1984-86), Scandinavian Electrophoresis Society (council member since 1986), and International Electrophoresis Society (council member since 1986). He is on the editorial boards for *Journal of Biochemical and Biophysical Methods* and for *Electrophoresis*.

Dr. Bjerrum has presented over 30 invited lectures at International and National Meetings, approximately 40 guest lectures at Universities and Institutes, and organized 10 international training courses and workshops. He has published more than 110 research papers and edited handbooks on immuno-biochemical methodology. His current major research interest is the structure and function of membrane proteins and immunoassay technology.

Niels H. H. Heegaard, M.D., was born 1959 in Copenhagen and obtained the medical degree from the University of Copenhagen in 1986.

He is now appointed to the University Hospital for his clinical internship. Simultaneously, he is doing research at the Protein Laboratory, University of Copenhagen, where he has been associated since 1981.

He has presented lectures at International Meetings, acted as a referee for International Journals, and participated in arrangement of and teaching of postgraduate courses in membrane protein chemistry. The focus of his current research is on methodology development, and on the influence of ageing processes and hematological disorders on red cell membrane proteins.

CONTRIBUTORS — VOLUME II

Hans Andersen
Institute of Medical Microbiology
University of Aarhus
Aarhus, Denmark

Svend Birkelund, M.D.
Institute of Medical Microbiology
University of Aarhus
Aarhus, Denmark

Ole J. Bjerrum, Ph.D.
Protein Laboratory
University of Copenhagen
Copenhagen, Denmark

Martin J. Blaser, M.D.
Infectious Disease Section
VA Medical Center
Denver, Colorado

Robert A. Childs, Ph.D.
Immunochemistry Research Group
MRC Clinical Research Centre, Harrow
Middlesex, England

Ten Feizi, M.D.
Immunochemistry Research Group
MRC Clinical Research Centre, Harrow
Middlesex, England

Edward I. Ginns, M.D., Ph.D.
Molecular Neurogenetics Unit
Clinical Neuroscience Branch
National Institute of Mental Health
Bethesda, Maryland

Terence J. Hadley, M.D.
Division of Haematology
School of Medicine
University of Louisville
Louisville, Kentucky

Peter Halken
Protein Laboratory
University of Copenhagen
Copenhagen, Denmark

Søren Naaby Hansen
Protein Laboratory
University of Copenhagen
Copenhagen, Denmark

Edward G. Hayman
Cancer Research Center
La Jolla Cancer Research Foundation
La Jolla, California

Niels H. H. Heegard, M.D.
Protein Laboratory
University of Copenhagen
Copenhagen, Denmark

Peter M. H. Heegaard, Ph.D.
Protein Laboratory
University of Copenhagen
Copenhagen, Denmark

Gunilla Høyer-Hansen
Department of Physiology
Carlsberg Laboratory
Copenhagen, Denmark

Henrik Ipsen
Protein Laboratory
University of Copenhagen
Copenhagen, Denmark

Peter Jackson, Ph.D.
Department of Clinical Biochemistry
University of Cambridge
Addenbrooke's Hospital
Cambridge, England

Jørgen Nedergaard Larsen
Protein Laboratory
University of Copenhagen
Copenhagen, Denmark

Edgar Lauritzen, M. D.
AIDS Laboratory
Rubella Department
Statens Seruminstitut
Copenhagen, Denmark

Bjarne, Ørskov Lindhardt, M.D.
Laboratory of Tumor Virology
The Fibiger Institute
Danish Cancer Society
Copenhagen, Denmark

John Paul Luzio, Ph.D.
Department of Clinical Biochemistry
University of Cambridge
Addenbrooke's Hospital
Cambridge, England

Jeffrey Lyon, Ph.D.
Department of Immunology
Walter Reed Army Research Institute
Walter Reed Army Medical Center
Washington, D.C.

J. B. Olmsted, Ph.D.
Department of Biology
River Campus
University of Rochester
Rochester, New York

Michael G. Pappas, Ph.D.
Covalent Technology Corporation
Ann Arbor, Michigan

Ib Rode Pedersen, Ph.D.
Institute of Medical Microbiology
University of Copenhagen
Copenhagen, Denmark

Guillermo I. Perez-Perez, D.Sc.
Infectious Disease Section
University of Colorado
Denver, Colorado

Errki Ruoslahti
Cancer Research Center
La Jolla Cancer Research Foundation
La Jolla, California

James L. Weber, Ph.D.
Marshfield Medical Research
Foundation
Marshfield, Wisconsin

TABLE OF CONTENTS — VOLUME II

SECTION 8: EXPERIMENTAL APPLICATIONS

8.1 CHARACTERIZATION OF MONOCLONAL ANTIBODIES

Gunilla Høyer-Hansen

INTRODUCTION

The immunoblotting technique is a versatile tool for the characterization of monoclonal antibodies. It is possible to determine not only the antigen and epitope specificity of the antibody, but also its affinity and titer. Even with a crude antigen preparation, a thorough study of a monoclonal antibody can be made. This is particularly important when the antigen is difficult to purify (e.g., membrane proteins).

Photosynthesis is the process by which solar energy is converted to chemical energy by green plants. Many of the proteins involved in these reactions are integral membrane proteins, which are difficult to purify without a concomitant loss of activity. However, various detergents make it possible to produce partially purified preparations that consist of a few polypeptides with retained activity.[1-9] An example of such a membrane preparation is a photosystem I (PSI) particle from barley containing only three Coomassie stainable polypeptides in SDS-PAGE.[1] These are the reaction center protein of PSI, chlorophyll *a*-protein 1 (Chl_a-P1), and two putative iron-sulphur center proteins with apparent molecular weights of 18,300 and 15,200.

Such a PSI particle preparation was used as the antigen for the production of monoclonal antibodies. Twelve hybridoma lines secreting antibodies against Chl_a-P1 and thirteen secreting antibodies against the 15.2 kD polypeptide were obtained from one fusion.[10,11] Some of these monoclonal antibodies have been characterized by immunoblotting with respect to specificity, affinity, and titer.

MATERIALS AND METHODS

Antigen Preparations

Chloroplast membranes were isolated from 7-days-old barley seedlings using differential centrifugation and flotation.[12] The PSI preparation was isolated from chloroplast membranes using the zwitterionic detergent Empigen BB (Marchon, Albright & Wilson Ltd., Whitehaven, England).[1] Protein was determined using bovine serum albumin as a standard.[13]

Antibodies

The monoclonal antibodies were obtained from a somatic cell fusion[14] between spleen cells from a mouse injected with barley PSI particle and the myeloma line Sp2/0-Ag14.[14,15] The initially isolated hybridomas were cloned by limited dilution,[16] and antibodies were collected from the tissue culture media (hybridoma supernatant) every 48 hr. When the antibodies were not purified, the amount of monoclonal antibody was estimated by multiplying the hybridoma cell concentration by 0.05 ng, which is the amount of antibody that one hybridoma cell secretes during 48 hr.[16,17] The concentration of antibodies in the hybridoma supernatants varied from 20 to 30 μg/mℓ. Antibodies of the IgG class were purified by chromatography on Protein A-Sepharose CL-4B (Pharmacia) and those of the IgM class by ammonium sulphate precipitation followed by chromatography on Sephacryl S-300 (Pharmacia).[18] The class of secreted antibodies was determined by Ouchterlony's double diffusion test.[18]

Blotting Procedure

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed either in a discontinuous buffer system with an acrylamide gradient from 11 to 15%[19] or, for the epitope mapping experiments, in a 10 to 20% acrylamide gradient containing 6 *M* urea.[20] Immediately after completion of the electrophoresis, the polypeptides were transferred from the SDS-gel to nitrocellulose filters (Millipore HAHY pore size 0.45 μm) using 125 m*M* Tris, 192 m*M* glycine pH 8.9, 20% (v/v) methanol as the transfer buffer.[10,11] The electrotransfer was performed for 2.5 hr at 2 A and 60 V in a water-cooled chromatography chamber with electrodes (20 × 19 cm^2) of acid-hardened stainless steel (distance between electrodes: 9 cm). Immediately after transfer, the nitrocellulose filters were cut into strips and stored in the dark at room temperature. Reference strips were stained with Amido black (0.1% in 45% methanol/10% acetic acid) and destained in methanol/water/acetic acid (2/2/0.4 v/v). SDS-gels were stained with Coomassie blue. For calibration of molecular weight and estimation of transfer efficiency,[10] the following proteins were used: catalase, ovalbumin, chymotrypsinogen A, and cytochrome *c* (Combithek, Boehringer Mannheim).

Immunoblotting Assay

Nitrocellulose strips were incubated while shaking for 1 hr at 37°C in 10% (v/v) newborn calf serum in phosphate buffered saline (PBS), pH 7.2. They were then washed with three changes of PBS and incubated for 2 hr at 37°C with monoclonal antibody either in tissue culture media or, if purified, in 10% (v/v) newborn calf serum in PBS. After washing five times in 1% (w/v) bovine serum albumin in PBS, the strips were incubated for 1 hr with peroxidase conjugated rabbit immunoglobulins against mouse immunoglobulins (Dako code P 161, Dakopatts) diluted 500-fold with 10% (v/v) newborn calf serum in PBS. The strips were washed five times with PBS before the color was developed with 3-amino-9-ethylcarbazole[21] (cf. Volume I, Appendix, Chapter 1). After drying, the strips were photographed.

RESULTS

Antibody Specificity

The hybridoma technique[14,22] necessitates a fast and reliable assay for the initial screening of the specificity of the antibodies secreted by the hybridoma clones. Immunoblotting is the assay of choice, since the antigen does not need to be purified for specificity determination.[10] However, when SDS-PAGE is used for separation of the antigen preparation, it is likely that only primary sequence epitopes are detected by the immunoblotting assay.

After a fusion only between 5 to 20% of the hybridomas secrete antibodies reacting with the injected antigen.[10,23] In the fusion reported here, 6.5% of the hybridomas secreted antibodies to the primary sequence epitopes of the PSI particle polypeptides. The rest produced antibodies not detectable by immunoblotting assays or no antibodies at all. The amount of work is drastically reduced when the hybridomas secreting antibodies of interest have been found. Figure 1 shows an example of an initial specificity screening. Providing the electrotransfer of the antigen has been optimized, 0.01 to 1.5 mℓ hybridoma supernatant is enough to obtain a clear reaction with 0.5-cm-wide strips (Figure 1). Only a few of the clones secreted antibodies that reacted with one polypeptide and in this case three clones, numbers 1 and 9 (15.2 kD polypeptide) and number 14 (Chl_a-P1) were of interest. The stronger intensity of the former bands as compared to the latter band is due to the high transfer efficiency of the 15.2 kD polypeptide in contrast to Chl_a-P1.[10] These hybridomas were later cloned by limited dilution,[16] which means that the hybridoma suspension is diluted in such a way that no more than one cell is placed in the cups of the microtiter plate. This ensures that the antibodies are secreted from a single clone and react with only one epitope.

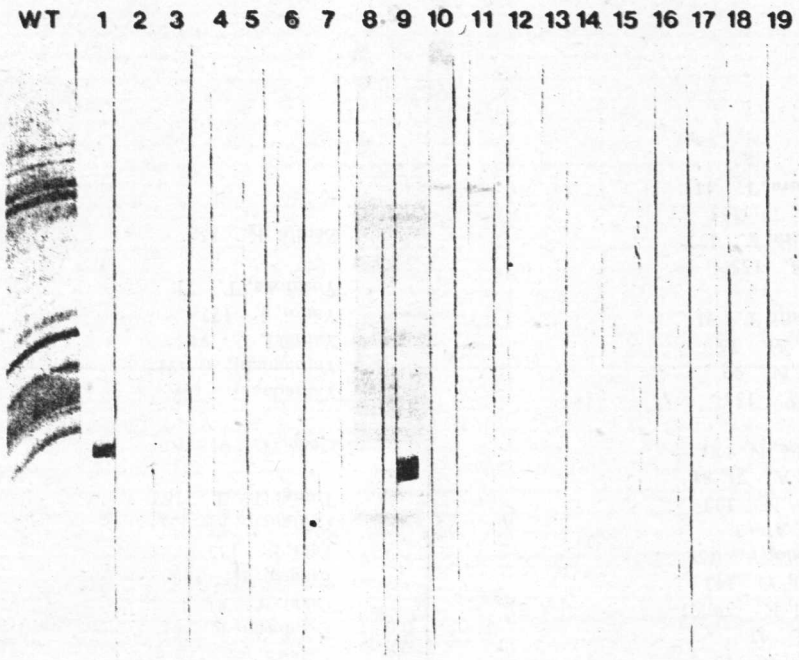

FIGURE 1. Screening for antibody specificity. Thylakoid membrane proteins (~100 μg chl) were separated by 11 to 15% SDS-PAGE and electroblotted. The transfer efficiency was checked by staining a strip of the nitrocellulose filter with Amido black (WT). The rest of the filter was cut into strips, 0.5 cm × 15.3 cm. Each of these was probed with 1 to 1.5 ml hybridoma supernatant from newly formed clones (total incubation volume 1.5 ml). Of the 19 clones tested, only 3 clones were chosen: numbers 1 and 9 (CMp15.2) and number 14 (CMpChl$_a$-P1).

The epitope is frequently unique to one antigen. However, some monoclonal antibodies react with two polypeptides (Figure 2) although with unequal intensity.[10,18,24] Recently, monoclonal antibodies, which react with groups of polypeptides, have also been found. This is illustrated by the monoclonal antibodies to the nordeins, the major storage proteins of the barley grain,[25] and it was also found that the five polypeptides of the light-harvesting complex of PSI have at least one common epitope.[26]

After the antibody class has been determined,[18] the monoclonal antibodies can be purified by column chromatography on either Protein A-Sepharose CL-4B or Sephacryl S-300.[18] The antibody containing fractions are found by immunoblotting assays of the collected fractions. The peak fraction(s) is determined by visually comparing the intensity of the immunostained bands in the assay.

Epitope Specificity

A protein contains more than one epitope, and since each monoclonal antibody reacts with only one of these, it is important to determine which one. This is easily done with immunoblotting assays. A fixed concentration of antigen is electroblotted and assayed with saturating amounts of monoclonal antibodies. Figure 3 shows that the staining intensity of the 15.2 kD band was the same when incubated with 150 μg of CMp15.2:1, or 150 μg of CMp15.2:1 plus 150 μg of CMp15.2:2. However, when 150 μg of CMp15.2:1 was added with 150 μg of CMp15.2:3, the intensity of the 15.2 kD band was markedly increased (Figure 3, lane 3).[27] This shows that CMp15.2:1 and CMp15.2:2 both recognize the same epitope and that CMp15.2:3 reacts with a different epitope on the 15.2 kD polypeptide. In

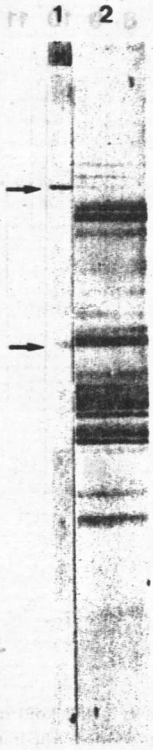

FIGURE 2. Monoclonal antibody reacting with two polypeptides. Thylakoid membrane polypeptides were the antigens and the antibody was a monoclonal IgM reacting with both a 68 and 31 kD polypeptide (arrows). (1) Immunoblot (conditions as in Figure 1). (2) Nitrocellulose filter stained with Amido black.

Figure 4 it is demonstrated that three monoclonal antibodies to Chl_a-P1 reacted with three different epitopes on the antigen.

The total number of primary sequence epitopes on a small protein ($M_r \sim 10{,}000$) can be estimated by comparing the band intensity obtained with a polyclonal antibody, presumably reacting with all epitopes, to that obtained with a single monoclonal antibody. This is exemplified by comparing the band intensity using affinity purified polyclonal rabbit antibody (100 μg) and monoclonal antibody (100 μg) against the chymotrypsin inhibitor 2 of barley (9000) (Figure 5).[28,29] The immunostaining of the polypeptide band, using the polyclonal antibody, is visually estimated to be three to four times stronger than with the monoclonal antibody. Assuming that the affinity of the secondary antibodies as well as the coupling to peroxidase are the same, this suggests that there are in the magnitude of three to four primary sequence epitopes on the barley chymotrypsin inhibitor 2.

Antibody Affinity and Titer

The antibody affinity is found by blotting decreasing amounts of antigen and then assaying with a fixed amount of the antibodies (Figure 6). In the assays shown here, six different antibodies have been tested. $CMpChl_a$-P1:3 and 7 are of the IgM class, while the others are IgGs. Only $CMpChl_a$-P1:1 showed reaction when as little as 1 μg of PSI particle had been applied on the gel. Thus this is the preferred antibody for immunoprecipitation experiments.

A polyclonal antibody titer is the reciprocal of the highest serum dilution giving an unequivocal positive reaction. A monoclonal antibody titer can be defined as the reciprocal

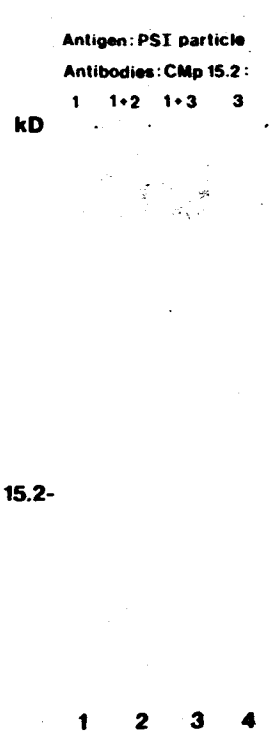

FIGURE 3. Reaction at different epitopes of the 15.2 kD polypeptide subunit. One microgram of photosystem I particle protein was loaded in each slot and separated on an 11 to 15% SDS-polyacrylamide gel and electroblotted. The nitrocellulose strips were probed with three different monoclonal antibodies against the 15.2 kD polypeptide subunit of PSI. The strips were 1 cm × 15.3 cm and the incubation volume 5 *ml*. Lane 1: 150 μg CMp15.2:1; lane 2: 150 μg CMp15.2:1 and 150 μg CMp15.2:2; lane 3: 150 μg CMp15.2:1 and 150 μg CMp15.2:3, and lane 4: 150 μg CMp15.2:3.

of the highest dilution of hybridoma supernatant if this is collected at constant intervals (e.g., every 48 hr). This value is important for deciding the appropriate amount of monoclonal antibody to use in antibody and immunoprecipitation procedures. The monoclonal antibody titer can be determined with an immunoblotting assay using a fixed antigen concentration. In Figure 7 increasing amounts of CMp15.2:1 were added to nitrocellulose strips with immobilized thylakoid polypeptides. This antibody has a very great affinity for its antigen, and the hybridoma supernatant can be diluted 1500 times and still give a weak positive reaction. An unequivocal positive reaction is obtained with a 750 times dilution, thus the titer for CMp15.2:1 is 750. This corresponds to only about 0.015 μg antibody per milliliter.

Epitope Mapping

The availability of monoclonal antibodies has made epitope mapping possible (see Chapter 8.2) and has been employed to characterize polypeptide fragments of Chl_a-P1. Although CMpChl_a-P1:1, 2, and 3 react with different epitopes on the Chl_a-P1 (Figure 4), they all react with V8 protease fragments of Chl_a-P1 of very similar, if not identical, apparent molecular weights (Figure 8). This implies that a portion of the Chl_a-P1, containing the three epitopes, is exposed at the thylakoid membrane surface and stimulates the major antibody response.

FIGURE 4. Reaction at different epitopes of the Chl$_a$-P1. Ten micrograms of PSI particle protein were loaded in each slot in this case because of the very poor transfer efficiency of Chl$_a$-AP1. After electroblotting, the nitrocellulose strips were probed with three different monoclonal antibodies to Chl$_a$-P1. Conditions were as in Figure 3. Lane 1: 150 μg CMpChl$_a$-P1:1; lane 2: 150 μg CMpChl$_a$-P1:1 and 150 μg CMpChl$_a$-P1:2; and lane 3: 150 μg of each of CMpChl$_a$-P1:1, 2, and 3.

DISCUSSION

For the characterization of monoclonal antibodies, two techniques are used more frequently than others, namely the enzyme-linked immunoadsorbent (ELISA or EIA)[30-34] and the immunoblotting assay. To determine the antibody specificity with an ELISA, a pure antigen is needed. Using an immunoblotting assay, the antibody specificity can be determined with a crude preparation, where the antigen constitutes approximately 1% of the total protein. However, the antigen preparations are with few exceptions separated by SDS-PAGE, where the proteins are more or less denatured, sparing only primary sequence epitopes. When ELISA is employed, the antigen can be immobilized in a native state and antibodies to tertiary structure epitopes can thus be found. The method chosen for the antibody screening depends on the problem to be solved with the aid of the antibodies. For immunogold labeling of proteins in membranes,[31] an antibody that recognizes a tertiary structure epitope might be more useful. In contrast, antibodies to primary sequence epitopes are used for immunoprecipitation of in vitro translated membrane polypeptides.[27]

The ELISA has been used to determine the epitope specificity of monoclonal antibodies to the β-subunit of *Escherichia coli* tryptophan synthase.[33,34] In these studies, highly purified β_2 and its F_1 and F_2 fragments were used as antigens. A single or two different monoclonal antibodies were applied. Antibodies that recognize different epitopes produce a more intensive color reaction than a pair of antibodies that recognize the same epitope. The principle

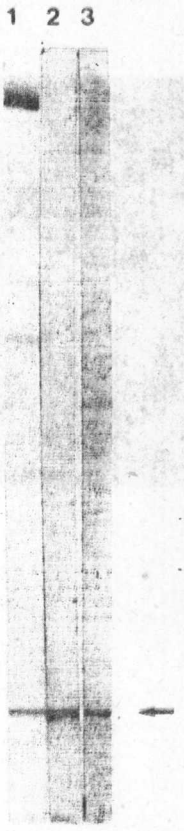

FIGURE 5. Estimate of total number of epitopes on a small protein. A crude extfact of barley endosperm protein was separated by 11 to 15% SDS-PAGE. Lane 1: nitrocellulose strip stained with Amido black. Lane 2: filter strip reacted with 100 μg affinity purified rabbit polyclonal antibody to the barley chymotrypsin inhibitor 2. Lane 3: filter strip probed with 100 μg purified monoclonal IgG to the same antigen. Arrow indicates the position of chymotrypsin inhibitor 2. Immunoblotting conditions were as in Figure 1. (Courtesy of U. Rasmussen).

is the same as the epitope specificity assay with immunoblotting, but in the latter, the antigen can be a crude protein mixture (Figures 3 and 4).

If both polyclonal and monoclonal antibodies of the IgG class are available, the epitope specificity assay can be employed to calculate the total number of primary sequence epitopes on a given antigen. Employing a fixed antigen concentration and saturating amounts of antibody and ^{125}I-Protein A, the number of epitopes is calculated by dividing the counts per minute bound using the polyclonal antibody with the counts per minute bound using a monoclonal antibody.

If monoclonal antibodies to more than one epitope on a protein are available, epitope mapping (see Chapter 8.2) is a quick way of obtaining information about the antigen. This is elegantly demonstrated by the work on Avena phytochrome.[32] This protein exists in two forms: Pr, the 660 nm light-absorbing form with a molecular weight of 114,000, and Pfr, the 730 nm light-absorbing form of molecular weight 124,000. The two forms of phytochrome are cut by endogenous proteases, which produce 9 fragments of the Pr form and 12 of the Pfr form. Three of the latter were assumed to be proteolytic products from contaminating phytochrome in the Pr form. The fragments were blotted onto nitrocellulose filters and

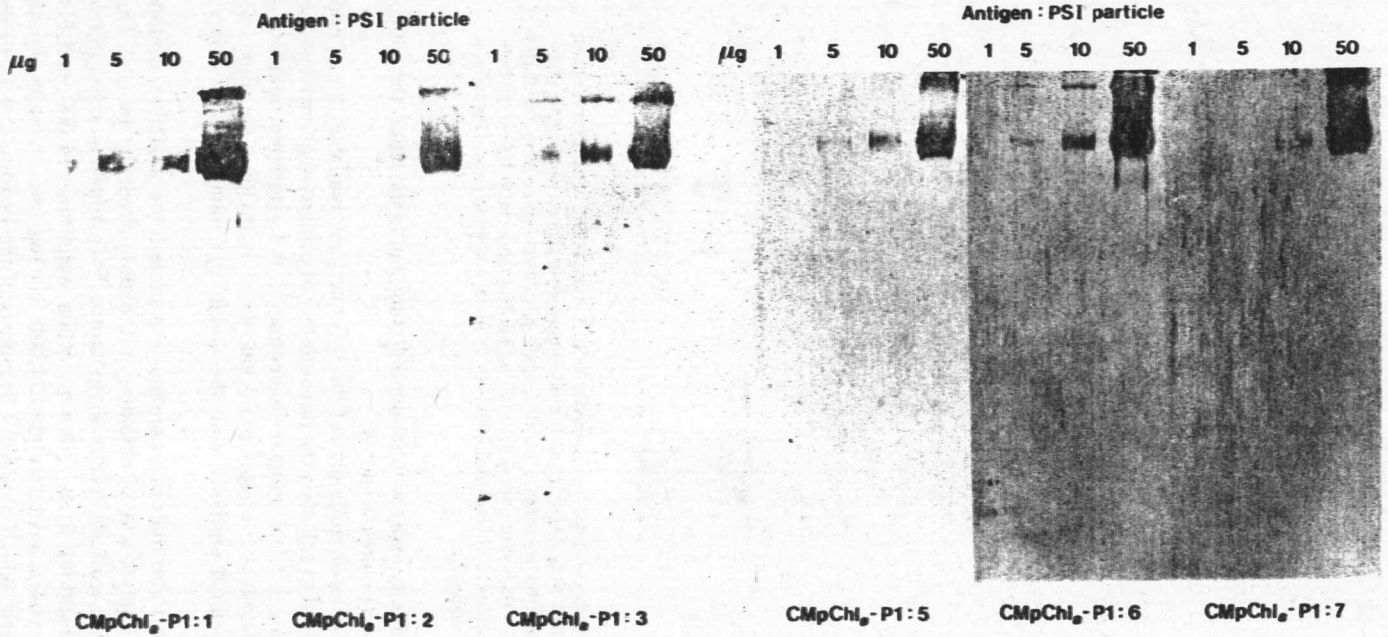

FIGURE 6. Affinity test of different monoclonal antibodies to the Chl$_a$-P1. Photosystem I particle protein was applied to the gels in the amounts stated on the figure. The nitrocellulose filters (4.5 cm × 15.3 cm) were probed with 350 μg of six different monoclonal antibodies to the Chl$_a$-P1 in a final volume of 12 mℓ. The antibody that reacts with the smallest amount of antigen, i.e., CMpChl$_a$-P1:1, has the highest affinity.

FIGURE 7. Monoclonal antibody titer of CMp15.2:1. Thylakoid membrane proteins (100 μg/cm) were separated on an 11 to 15% SDS-polyacrylamide gel and electroblotted. The nitrocellulose strips were probed with the indicated dilutions in 10% newborn calf serum in PBS of hybridoma supernatant from CMp15.2:1, using the same conditions as in Figure 1. Under the conditions chosen, the monoclonal antibody titer for CMp15.2:1 is 750. Arrow indicates the position of the 15.2 kD polypeptide band.

probed with 46 different monoclonal antibodies. The antibodies bound to three distinct domains of the phytochrome. Sixty-five percent of the antibodies bound to the 6 kD amino terminus of the 124 kD phytochrome. Another 25% of the antibodies bound to the chromophoric segments of the polypeptide. The last 10% of the antibodies reacted with the nonchromophoric side of the molecule.[32]

The phytochrome, like other plant membrane proteins, is N-terminally blocked, and therefore repetitive Edman degradation will not yield an amino acid sequence. However, cleaving the protein with a proteolytic enzyme and then affinity purifying the individual fragments provides a solution. A prerequisite for such a procedure is the availability of monoclonal antibodies to different epitopes on the protein.

To conclude, the immunoblotting assay can be used to determine the antigen and epitope specificity of a monoclonal antibody as well as its affinity and titer. All of this information is necessary for the proper use of one of the most powerful tools in molecular biology and protein chemistry: the monoclonal antibody.