Enzymes, Reccptors and

Carriers of Biological Membranes

A Laboratory Manual

Edited by

A. Azzi U. Brodbeck P. Zahler

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Preface

This manual follows at a distance of 3 years the previous one entitled *Membrane Proteins,* and, like its predecessor, it is the result of an International Advanced Course sponsored by FEBS, SKMB and SNG, which was held in Bern in September 1983.

The experiments offered to the students in the course had to be largely updated or chosen from new areas of membrane research, because of the substantial and rapid development of the field.

Using the protocols of the course, the participants (graduate students, postdoctoral fellows and also senior scientists), in most cases not at all expert in biomembrane research, were able to repeat all the experiments successfully. Those few protocols which for some reason did not fulfill the role we expected were modified.

These protocols have now been collected in this manual, which we are able to offer to a number of biology, biochemistry and biophysics laboratories, hoping that the selected number of methods which have been successfully used during the Advanced Course may be useful to them. This manual is also intented for teachers of practical classes, who may use it as a textbook and as source of selected references, collected not in the library, but in the laboratory, from the notebooks of the young researchers who have contributed so much to the success of the Course.

Bern, September 1984

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Electrophoretic Transfer of Chloroplast Membrane Proteins from SDS-Gels onto Nitrocellulose and Their Immunological Detection

A. BOSCHETTI, J. KARLEN, and H.P. MICHEL

I. Introduction

The electrophoretic transfer of proteins out of a polyacrylamide slab gel onto a sheet of nitrocellulose was introduced by Towbin et al. (1979). Their work was based on the previously developed technique for the transfer of nucleic acids onto papers or membranes of derivatized cellulose and, together with the technical know-how of several manufacturers, opened a wide new area of experimental procedures in protein analysis (for a review see *Technical Bulletins* of Bio-Rad and of Schleicher and Schüll Inc.).

The aim of the method is to obtain a replica of an electrophoretic separation in such a way that the proteins are immobilized and concentrated at the surface of the nitrocellulose (or another rigid support), and become freely accessible for chemical and immunological reactions.

The method is most often used for the detection of specific proteins. Usually, the very sensitive, indirect radioimmunological assay is applied, where the replica on nitrocellulose is first allowed to react with a specific antiserum and secondly with ^{125}I-protein A or ^{125}I-anti-antiserum. In order to save time in the present experiment, which has to be finished in 1 day, a direct immunofluorescence assay will be used, although due to rather low fluorescent labeling of the antibodies it is less sensitive than the radioimmunoassay. However, by this method, two different proteins can be detected simultaneously when different fluorescent labels are used.

The experiment described here should demonstrate that two groups of distinct proteins of the photosynthetic membranes of green plants can rapidly and easily be detected by the combination of electrophoresis of membrane proteins, transfer onto nitrocellulose and the serological test outlined above.

A rough preparation of thylakoid membranes from the green alga *Chlamydomonas reinhardii* (provided by the course staff) is solubilized with SDS as such, after washing with urea (removal of extrinsic proteins) and treatment with protease (removal of extrinsic proteins, "shortening" of intrinsic proteins). After SDS-electrophoresis of the solubilized samples on polyacrylamide gradient gels, the proteins are transferred to nitrocellulose.

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A duplicate of the gel is stained with Coomassie blue. On the nitrocellulose, the intrinsic light-harvesting chlorophyll a/b proteins (or their degradation products) as well as the extrinsic α- and β-subunits of the ATP-synthase bind their specific antibodies, which were differentially labeled with fluorescein isothiocyanate and tetramethyl-rhodamine isothiocyanate, respectively. The green and red fluorescent bands can be observed under UV-light.

II. Solutions, Reagents, Starting Material

A. Stock Solutions

Acrylamide Solution

- 30g Acrylamide (Serva, 2x cryst.)
- 0.8g N,N'-Methylene-bisacrylamide (Bio-Rad)

made up to 100 ml with H_2O bidest.

Buffer for Separating Gel (Laemmli 1970)	Final conc.
– 364g Tris(hydroxymethyl)aminomethane (Fluka, puriss)	3 M
– 8g SDS (Serva)	0.8 %

made up to 800 ml with H_2O bidest.
brought to pH 8.8 with conc. HCl (Merck, p.a.),
made up to 1000 ml with H_2O.

Buffer for Stacking Gel (Laemmli 1970)	
– 60.6g Tris(hydroxymethyl)aminomethane (Fluka, puriss)	0.5 M
– 4g SDS (Serva)	0.4 %

made up to 800 ml with H_2O bidest.
brought to pH 6.8 with conc. HCl (Merck, p.a.)
made up to 1000 ml with H_2O.

Marker Proteins, FITC-labeled	Mol. wt.
– Lysozyme, grade I (Sigma)	14, 300
– Chymotrypsinogen, cryst. (Serva)	25, 000
– Ovalbumin (Serva)	45, 000
– Bovine serum albumin (Sigma)	67, 000
– Phosphorylase a, rabbit (Sigma)	92, 500

The mixture of the proteins (5 mg/ml of each) was conjugated with fluorescein isothiocyanate according to the procedure given in Hudson and Hay (1980). The labeled proteins are stored at a concentration of about 5 mg/ml in phosphate buffered saline (PBS: 20 mM Na-phosphate, pH 7.2; 0.15 M NaCl) at −70°C.

CF_1-Proteins of Chlamydomonas reinhardii

The CF_1-preparation in 20 mM Tricine, 2 mM EDTA, 1 mM ATP and 5 mM dithiothreitol, isolated according to Younis et al. (1977), was diluted with 1.5 vol. of solubilization buffer (II B) to obtain a final conc. of 2 mg/ml protein.

Tris Buffer	Final conc.
– 121 g Tris(hydroxymethyl)aminomethane (Merck, LAB) made up to 800 ml with H_2O bidest. brought to pH 7.6 with HCl (Merck, p.a.), made up to 1000 ml with H_2O, bidest.	1 M

B. Reagents for Sample Preparation

Urea Buffer	
– 24g Urea (Merck, p.a.)	8 M
– 5 ml Tris buffer made up to 50 ml with H_2O bidest.	0.1 M

Pronase Solution	
– 4 mg Protease from *Strept. griseus*, purified Type XIV (Sigma) dissolved in 2 ml H_2O bidest.	2 mg/ml

Protease Inhibitor	
– 10 mg Phenylmethylsulfonylfluoride (Sigma), dissolved in 1 ml ethanol.	10 mg/ml

Solubilization Buffer	
– 0.4g SDS (Serva)	4 %
– 0.4 ml β-Meracptoethanol (Serva)	4 %
– 1 ml Glycerol (85%, Merck p.a.)	8.5 %

– 2.5 ml Buffer for stacking gel 25 %
made up to 10 ml with H_2O bidest.

C. For Polyacrylamide Gel Electrophoresis

To prepare a gel with a linear acrylamide gradient (8.5 to 17.5%), "heavy", "light" and 3% stacking gel solutions are needed.

	Heavy solution	Light solution	Stacking gel solution
Acrylamide solution	9.33 ml	4.25 ml	1 ml
Buffer for separating gel	2.0 ml	1.88 ml	–
Buffer for stacking gel	–	–	2.5 ml
Glycerol (Merck, p.a.)	0.64 ml	–	–
made up with H_2O to	16 ml	15 ml	20 ml
Just before use add:			
Ammonium persulfate solution 10%, freshly prepared	20 µl	20 µl	75 µl
N, N, N', N'-tetramethyl-ethylene diamine	5 µl	5 µl	7.5 µl

Running Buffer Final conc.

– 15 g Tris(hydroxymethyl)-aminomethane (Merck, LAB) 25 mM
– 72 g Glycine (Merck, for medical purposes) 192 mM
– 5 g SDS (Serva) 0.1 %
made up to 5000 ml with H_2O

Staining/Destaining Solution for PAG

– 2.5 g Coomassie brilliant blue R 250
– 454 ml Ethanol
– 454 ml H_2O
– 92 ml Acetic acid, conc.

The destaining solution contains no Coomassie blue.

D. Transfer on Nitrocellulose

Nitrocellulose BA 85 (Schleicher and Schüll)

Transfer Buffer

– 9 g Tris(hydroxymethyl)aminomethane (Merck, LAB)	25 mM
– 43.2 g Glycine (Merck, for med. purposes)	192 mM
– 600 ml Ethanol	20 %
made up to 3000 ml with H_2O	

E. Immunological Test

BSA Buffer	Final conc.
– 2.25 g NaCl	0.9 %
– 7.5 g Bovine serum albumin (Fluka)	3 %
– 2.5 ml Tris buffer	0.01 M
– 0.05 g NaN_3	0.02 %
made up to 250 ml with H_2O	

Wash Buffer

– 10 ml Tris buffer	0.01 M
– 9 g NaCl	0.9 %
made up to 1000 ml with H_2O	

FITC- and TRITC-labeled Antibodies

Antibodies against light-harvesting chlorophyll a/b (LHCP) apoproteins of *Chlamydomonas* (25 mg/ml IgG) have been labeled with fluorescein isothiocyanate (FITC) according to Hudson and Hay (1980). Antibodies against coupling factor 1 (CF_1) of *Chlamydomonas* (6 mg/ml IgG) have been labeled with N, N, N', N'-tetramethylrhodamine isothiocyanate (TRITC) according to Bergquist and Nilsson (1974) and the unreacted TRITC removed with DEAE-cellulose according to Johnson et al. (1979). The antibodies are kept frozen in small portions at –70°C.

F. Thylakoid Membranes of *Chlamydomonas*

As starting material, thylakoid membranes of the green alga *Chlamydomonas reinhardii* are prepared according to Michel et al. (1981) containing 1 mg/ml chlorophyll in 0.1 M Tris/HCl, pH 7.6.

III. Experimental Procedures

The experiment can be divided into six steps.

A. Preparation of gels
B. Preparation of samples
C. Electrophoresis
D. Transfer onto nitrocellulose
E. Immunological assay
F. Staining of the gel

Since the experiment must be carried out in 1 day during the course, the six above-mentioned steps cannot be done in chronological order. Therefore, one group has to finish the experiment from the day before and start with a new experiment. Hence, the order of steps will be according to Table 1.

Table 1. Time schedule of experiment

10:00	11:00	12:00	13:00	14:00	15:00	16:00	17:00	18:00
Introduction	C. Prep. of stack. gel/Appl. sample	Lunch	Electrophoresis 5 h				D. Transfer on NC/ F. gel staining	Overnight
	E. NC in BSA and Ab		Incubation				E. wash NC	Results
	F. Gel		Destaining					Results
			B. Sample prep. pronase/urea		A. prep. gel			
10:00	11:00	12:00	13:00	14:00	15:00	16:00	17:00	18:00