0273-2289/94/4803—0185$07.80

Electrophoretic Isolation of Membrane Proteins from Acrylamide Gels

HERMANN SCHÄGGER

Zentrum der Biologischen Chemie, Universitätsklinikum Frankfurt, Theodor-Stern-Kai 7, Haus 25B, 60590 Frankfurt am Main, Germany

Received November 18, 1993; Accepted January 6, 1994

ABSTRACT

Protocols for the optimal resolution of membrane and water-soluble proteins in SDS-denatured state (Tricine SDS-PAGE and Blue Tricine SDS-PAGE; Laemmli SDS-PAGE and Blue Laemmli SDS-PAGE) and in the native state (Blue Native PAGE) are presented. The protocols for protein recovery from these gels include techniques of electroelution and electroblotting optimized to the type of the preceding electrophoresis system. Native and denatured proteins thus are obtainable in near quantitative yield in soluble and in immobilized form. These techniques can optionally be performed in the milligram range, e.g., for the use of immunization and N-terminal protein sequencing, or in the analytical range.

Index Entries: Blue Native Electrophoresis; Blue SDS Electrophoresis; membrane proteins; electroelution; electroblotting.

INTRODUCTION

Membrane proteins can be separated within polyacrylamide gels by denaturing techniques using sodium dodecyl sulfate (SDS), as well as by native techniques retaining the structural and functional properties of the proteins. The techniques most useful in our lab for separation and for recovery of the separated proteins are described.

The Tricine SDS-PAGE (*1*) for separation of small proteins and the Laemmli SDS-PAGE for larger proteins (*2*) provide the basic commonly

used SDS techniques. However, since the recovery of membrane proteins from fixed and stained gels is problematic, modified procedures, the Blue Tricine SDS-PAGE and the Blue Laemmli SDS-PAGE (3), are used instead. The migration behavior of proteins is almost identical to that of the basic techniques. A detailed comparison of the advantages of the two techniques is described in (4).

The Blue SDS-PAGE techniques (3), facilitating the quantitative isolation of proteins in the microgram to milligram scale use noncovalent staining by Coomassie dye during electrophoresis. Noncovalent staining during electrophoresis is achieved by halving the SDS concentration in the cathode buffer and adding 25 mg of Coomassie dye/L. Under these conditions, Coomassie can compete with SDS for binding to the unfolded proteins. Proteins migrate as blue bands through the gel. The bands are cut out and electroeluted almost quantitatively, since any fixation step prior to electroelution is avoided. Conventional fixation and staining of proteins after SDS-PAGE would allow a more sensitive detection, but would result in bad yields of extracted membrane proteins.

Electroelution and electroblotting of SDS-denatured membrane proteins are valuable techniques for isolation of proteins, e.g., for immunization and for N-terminal sequencing of proteins and protein fragments. The techniques described here circumvent extraction and transfer problems with large-membrane proteins, as well as problems with small protein fragments that usually do not bind to PVDF membranes during electroblotting.

Blue Native PAGE (BN-PAGE) is a novel technique (4–6) that is suitable for:

1. Isolation of 1–100 μg quantities of native membrane proteins directly from biological membranes; optimal resolution is achieved in the 10^5–10^6 dalton molecular-mass range;
2. Final purification of milligram amounts of partially purified membrane proteins; and
3. Analysis of molecular mass, oligomeric state, and degree of homogeneity by using microgram amounts of protein.

BN-PAGE makes use of Coomassie dye binding to proteins similar to Blue SDS-PAGE, but Coomassie dye here competes with neutral, nondissociating detergents instead of the denaturing SDS and binds only to the protein surface. Coomassie dye has four main functions in BN-PAGE:

1. Coomassie dye stains proteins during electrophoresis and allows detection of enzymatically active enzymes;
2. Coomassie dye expands the applicability to the range of basic proteins, because the binding of the anionic dye induces a charge shift on the proteins; all proteins binding the Coomassie dye, even basic ones, will migrate to the anode at the running pH 7.5;

3. Coomassie dye reduces the aggregation of membrane proteins, because negatively charged proteins and complexes will repel each other; and
4. Coomassie dye minimizes protein denaturation, because the bound dye maintains membrane proteins solubilized in the absence of detergents; membrane proteins thus are converted to water-soluble proteins, and separation is achieved in detergent-free gels.

Native electroelution of membrane proteins from BN-PAGE allows recovery of native proteins, e.g., for functional studies and for immunization, and production of antibodies. Native proteins separated by BN-PAGE can also be processed under denaturing conditions by a second-dimension SDS-PAGE. This special type of 2D electrophoresis allows identification of the protein subunits that are assembled to a multiprotein complex. This is in contrast to the 2D techniques using denaturing isoelectric focusing in the presence of urea in the first dimension.

MATERIALS

Tricine SDS-PAGE and Blue Tricine SDS-PAGE

1. Vertical electrophoresis apparatus according to Studier (*7*).
2. SDS p.A. 20% (w/v).
3. Urea p.A.
4. Glycerol p.A.
5. TEMED (*N,N,N′,N′*-tetramethyl-ethylenediamine).
6. Ammonium peroxodisulfate p.A.
7. Tris (≥99%).
8. Mercaptoethanol.
9. Serva Blue G (highly pure Coomassie Blue G 250) from Serva, Heidelberg, Germany.
10. Acrylamide and *bis*-acrylamide (the commercially available 2× cryst. products)
11. AB-mix S (500 mL); *see* Note 1: (49.5% T, 3% C standard acrylamide-*bis*-acrylamide stock) 240 g acrylamide, 7.5 g *bis*-acrylamide dissolved in water to a final vol of 500 mL (%T: total concentration of both monomers, %C: percentage of cross-linker to total monomer). Store at 7°C. Crystallization may occur at lower temperature!
12. AB-mix H (100 mL): (49.5% T, 6% C stock with high cross-linker concentration) 46.5 g acrylamide, 3 g *bis*-acrylamide dissolved in water to a final vol of 100 mL. Store at 7°C.

13. Sample buffer (4X) (50 mL): 30 mL Na-SDS (20%), 15 g glycerol, 0.91 g Tris; adjust pH to 6.8–7.0 with HCl, add water to a volume of 47 mL, dissolve 40 mg Serva Blue G, and add 3 mL mercaptoethanol. Store at room temperature. (Without addition of mercaptoethanol, SDS precipitates at $<20°C$).
14. Tricine ($\geq 99\%$).
15. Cathode buffer (Tricine-SDS-PAGE): 100 m*M* Tris, 100 m*M* Tricine, 0.1% Na-SDS. No correction of pH (around 8.25).
16. Cathode buffer (Blue Tricine SDS-PAGE): 100 m*M* Tris, 100 m*M* Tricine, 0.05% Na-SDS, 25 mg Serva Blue G/L. No correction of pH (around 8.25).
17. Anode buffer: 100 m*M* Tris. Adjust to pH 8.9 with HCl.
18. Gel buffer (3X): 0.3% Na-SDS, 3*M* Tris, 1*M* HCl, pH 8.45 (identical for sample and separating gel).

Unless otherwise indicated, pH was adjusted at room temperature, and solutions were kept at room temperature.

Laemmli SDS-PAGE and Blue Laemmli SDS-PAGE

1–13. *See* 1–13 under Tricine SDS-PAGE and Blue Tricine SDS-PAGE.

14. Glycine (analytical grade).
15. Cathode buffer (Laemmli): 25 m*M* Tris, 192 m*M* glycine, 0.1% Na-SDS.
16. Cathode buffer (Blue Laemmli): 25 m*M* Tris, 192 m*M* glycine, 0.05% Na-SDS, 25 mg Serva Blue G/L.
17. Anode buffer: 25 m*M* Tris, 192 m*M* glycine, 0.1% Na-SDS.
18. Sample gel buffer (4X): 0.5*M* Tris, 0.4% Na-SDS. Adjust pH 6.8 with HCl.
19. Separating gel buffer (4X): 1.5*M* Tris, 0.4% Na-SDS. Adjust pH 8.6 with HCl.

Unless otherwise indicated, pH was adjusted at room temperature, and solutions were kept at room temperature.

Electroelution of Denatured Proteins after Blue SDS-PAGE

1. Elutor/concentrator made according to Hunkapiller et al. (*8*). A similar apparatus is available from CBS Scientific Co., Del Mar, CA.
2. Dialysis membranes with a cutoff value of 2 kDa (e.g., from Reichelt GmbH, Heidelberg, Germany) for sealing both arms of the elutor vessel.
3. Electroelution buffer A (without SDS): 50 m*M* NH_4HCO_3.
4. Electroelution buffer B: 0.1% SDS, 100 m*M* NH_4CO_3.

Electroblotting of Denatured Proteins after Conventional or Blue SDS-PAGE

1. Semidry blotting apparatus, e.g., Sartoblott II-S (Sartorius, Göttingen, Germany).
2. Filter papers for soaking with electrode buffers. The less sheets required for a 3-mm stack, the less trapped air bubbles will occur.
3. Inert PVDF (polyvinyliden difluoride) membranes.
4. Methanol (analytical grade).
5. Cathode buffer: 300 m*M* aminocaproic acid, 30 m*M* Tris. No correction of pH, which is around 8.7.
6. Anode buffer: 300 m*M* Tris, 100 m*M* Tricine. No correction of pH that is around 8.7.

Buffers 5. and 6. are stored at 4°C.

Blue Native Electropohoresis (BN-PAGE)

1. Use a vertical apparatus according to Studier (*7*).
2. AB-mix S: *see* 11.
3. Dodecyl-maltoside (dodecyl-β-D-maltoside) 10% (w/v) from Boehringer, Mannheim, Germany.
4. 6-Aminocaproic acid (2*M;* 1 L): Fluka, Buchs, Switzerland.
5. Serva blue G (*see* 9 under Tricine SDS-PAGE and Blue Tricine SDS-PAGE).
6. Tricine (1*M;* 1 L).
7. *Bis*-tris (1*M;* 1 L).
8. *Bis*-tris-HCl (1*M*, pH 7.0 at 4°C): About 53 mL of HCL (5*M*) are required for neutralization of 209.2 g *Bis*-tris in a final volume of 1 L.
9. HCl (5*M*): Add 400 mL of 32% HCl to 400 mL of water in a 1-L plastic bottle (cooled on ice) with stirring.
10. Solubilization buffer (100 mL): Aminocaproic acid (750 m*M*), *Bis*-tris/HCl (50 m*M*), pH 7.0.
11. 5% Serva Blue G: 500 mg Serva Blue G in 10 mL 750 m*M* aminocaproic acid.
12. Gel buffer 3X (triple concentrated, 500 mL): 150 m*M* *Bis*-tris-HCl, 1.5*M* aminocaproic acid, pH 7.0 (4°C).
13. Cathode buffer B: 50 m*M* Tricine, 15 m*M* *Bis*-tris/HCl, pH 7.0 (4°C) plus 0.02% Serva blue G (1 g/5 L). Dissolve the dye by stirring for several hours at room temperature. Keep it at room temperature to avoid formation of large micelles that would not enter the sample gel. We used exclusively Serva Blue G, which is highly pure Coomassie blue G 250.

14. Cathode buffer B/10: Dilute 1 vol of cathode buffer B by 9 vol of the same buffer without dye (electroblotting buffer). This buffer with low dye concentration is stored at 4°C.
15. Anode buffer: 50 m*M* *Bis*-tris/HCl, pH 7.0 (4°C).

Electroelution of Native Proteins

1. Use the same elutor/concentrator and the same dialysis membranes as for electroelution of SDS-denatured proteins (*see* 1 and 2 under Electroelution of Denatured Proteins After Blue SDS-PAGE).
2. Electroelution buffer: 25 m*M* Tricine, 7.5 m*M* *Bis*-tris-HCl, pH 7.0 at 4°C (diluted electroblotting buffer). Store at 4°C.

Electroblotting of Native Proteins

1–4. *See* 1–4 under Electroblotting of Denatured Proteins After Conventional or Blue SDS-PAGE.
5. Electroblotting buffer: 50 m*M* Tricine, 15 m*M* *Bis*-tris/HCl, pH 7.0 at 4°C.

Unless otherwise stated, all buffers for native techniques (5–7 under Blue Native Electrophoresis) were stored at 4°C. The volumes suggested are suitable for labs continuously using BN-PAGE.

METHODS

Colorless and Blue SDS-PAGE Techniques

1. Select the optimal electrophoresis system from the four alternatives (Colorless or Blue Tricine SDS-PAGE; Colorless or Blue Laemmli SDS-PAGE) according to Notes 2 and 3, and the optimal gel type from Table 1.
2. Cast gels for (Blue) Laemmli SDS-PAGE according to Table 2 or for (Blue) Tricine SDS-PAGE according to Table 3 (*see* Notes 4 and 5).
3. Prepare the samples, sediments, or solutions/suspensions by incubation in SDS. Sediments: First add an appropriate volume of water and vortex. Suspensions are solubilized much faster by SDS than sediments. Then add 1 vol of sample buffer (4X) to 3 vol of sample. Solutions and suspensions: Add 1 vol of sample buffer (4X) to 3 vol of sample. Then incubate for 30 min at 40°C. Do not incubate at higher temperature. Membrane proteins might aggregate and fail to enter the gel.
4. Consider loading and detection limits (*see* Note 6).
5. Mount the gel, and fill in the appropriate electrode buffers.

Table 1
Gels and Methods for Optimal Protein Separation

Method	M_r optimum, kDa	Total range, kDa	Acrylamide concentration
Laemmli SDS-PAGE	50–100	40–>>200	8%
(*1*)	40–80	30–>200	10%
	30–60	20–>200	12%
Tricine SDS-PAGE	5–50	2–100	10%
(*2*)	2–30	1–70	16.5%
	1–20	1–45	16.5%*

*Acrylamide-*bis*-acrylamide stock solution with high percentage of crosslinker (AB-mix H) is used for optimal resolution of protein fragments instead of the standard AB-mix S. The separation characteristics described for the original colorless electrophoresis systems (*1,2*) are also valid for the blue modifications (*3*) with almost unchanged migration behavior of proteins.

Table 2
Composition of Gels for (Blue) Laemmli SDS-PAGE

	4% Sample gel	10% Separating gel
Sample gel buffer (4x)	2 mL	—
Sep. gel buffer (4x)	—	8 mL
AB-mix S	0.65 mL	6.4 mL
Glycerol	0.8 g	3.2 g
Add water		
to a final volume of	8 mL	32 mL
Polymerization by:		
APS (10%)*	60 µL	150 µL
TEMED*	6 µL	15 µL

*APS (10%): ammonium persulfate 10% (w/v), freshly prepared; TEMED: Tetramethylethylenediamine; glycerol is added also to the sample gel to facilitate the overlay by water if no comb is used with preparative gels. The volumes are sufficient for one 14 × 14 × 0.16 cm gel.

6. Apply the samples dissolved in a small volume. In (Blue) Laemmli SDS-PAGE, the sample volumes applied can be relatively large. The height of the applied sample can exceed 1 cm. In (Blue) Tricine SDS-PAGE, the applied sample volume has to be lower, because stacking of small proteins is more difficult. The height of the applied sample optimally is in the 1–5 mm range.
7. Start electrophoresis, performed at room temperature, at low voltage (30 V) until the sample is completely within the 4% sample gel. Then raise voltage to the values given in Table 4 (*see* Note 7).

Table 3
Composition of Gels for (Blue) Tricine SDS-PAGE*

	4% Sample gel	Separating gels 10%	Separating gels 16.5%
Gel buffer (3x)	2 mL	10 mL	10 mL
AB-mix S	0.5	6.1 mL	10 mL
Glycerol	0.6 g	3 g	3 g
Add water to a final volume of	6 mL	30 mL	30 mL
Polymerization by:			
APS (10%)	50 μL	150 μL	100 μL
TEMED	5 μL	15 μL	10 μL

*For high-resolution separation of protein fragments, AB-mix S can be replaced by AB-mix H in the 16.5% separating gel. If separation of the subunits of a multiprotein complex requires urea, the glycerol in the separating gels can be replaced by 10.8 g urea (6*M*). Separation of small proteins in many cases is improved by use of urea. However, molecular mass determination is less reliable, and dimerization and oligomerization of proteins may occur.

Table 4
Maximal Voltage Settings in (Blue) Tricine SDS-PAGE

	Separation gel		Voltage setting		
	Length thickness,		Initial*	Final†	Time,
Gel type	cm	mm	V	V	h
10%	12	0.7	180	250	4
10%	12	1.2	130	180	6
16.5%	12	0.7	200	300	6

*Initial voltage settings (after sample penetration at 30 V) are the **maximal** settings for the given gel length and thickness. With shorter as well as thicker gels, voltage has to be reduced to avoid cracking of glass plates. **Use lower settings for first trials! Gels may warm up to about 40°C.**

†After more than half of the run, voltage can be **gradually** raised to the indicated values.

Electroelution of Proteins from Blue SDS Gels

1. Use H-shaped electroelutor/concentrator vessels made according to Hunkapiller et al. (*8*) (cf 1 under Electroelution of Denatured Proteins after Blue SDS-PAGE), cut pieces of appropriate size out from thick-walled, mechanically stable dialysis membranes (cf 2 under Electroelution of Denatured Proteins after Blue SDS-PAGE), allow them to swell in water, and seal the lower ends of both vertical tubes with these membranes. Dialysis membranes with low cutoff values,

e.g., 2 kDa, usually are more stable than those with higher cutoff values. Therefore, they are used for small as well as large proteins.

2. Dip the ends into the anodic and cathodic sides of the electrophoresis tank filled with electroelution buffer B (containing 0.1% SDS), and remove any air bubbles below the membranes.
3. Excise visible blue bands from the blue SDS gel.
4. Squeeze large pieces of gel through a syringe directly into the cathodic arm of the electroelutor vessel, or slice pieces with razor blades. By the latter procedure, less linear polymerized acrylamide accumulates in the electroeluate.
5. Cover the crushed gel then with 0.1% SDS solution (without NH_4HCO_3), and incubate for 10 min.
6. Overlay with electroelution buffer A, and fill the anodic arm and the connecting horizontal tube with this buffer A containing no SDS.
7. Extract the proteins at 40 V overnight. Extraction at maximally 70 V (considerable heating) takes several hours.
8. The proteins collect together with SDS and Coomassie dye in a small volume (*see* Note 8).
9. Remove the colorless supernatant as completely as possible.
10. Recover the blue protein solution by using a Pasteur pipet equipped with a soft silicon tubing.
11. If protein concentration is required, lyophilize the sample. This simultaneously will remove most of the NH_4HCO_3.

Electroelution of proteins from Blue Laemmli SDS-PAGE and Blue Tricine SDS-PAGE is performed at room temperature. Extraction of large membrane proteins as well as of 1-kDa fragments (retained by a membrane with a cutoff value of 2 kDa!) is near quantitative.

Electroblotting of Colorless or Blue SDS Gels (*see* Notes 9 and 10)

1. Invert either the polarization or the order within the following assembly if the lower electrode is the anode in your apparatus.
2. Soak a 3-mm stack of papers with electroblotting cathode buffer, and put it on the lower electrode (cathode).
3. Deposit then the gel without prior incubation in any buffer.
4. Cover the gel with a PVDF membrane (briefly wetted with methanol p.A. and equilibrated in electroblotting anode buffer until it begins to sink).
5. Put a 3-mm stack of papers soaked with electroblotting anode buffer on top.
6. Complete the assembly by mounting the anode and placing a 5-kg load on top.

Table 5
Gradient Gels for Blue Native Electrophoresis:
Composition and Polymerization Conditions*

	Sample gel	Gradient separation gel	
	4% T	5% T	13% T
AB-mix	0.5 mL	1.8 mL	4 mL
Gel buffer (3x)	2.0 mL	6.0 mL	5 mL
Glycerol	—	—	3 g
APS (10%)	50 μL	100 μL	70 μL
TEMED	5 μL	10 μL	7 μL
Total volume	6 mL	18 mL	15 mL

*The volumes given are those for a 14 × 14 × 0.16 cm gel. Linear gradients were cast at 4°C and maintained at room temperature for polymerization. APS (10%): freshly prepared ammonium persulfate solution (100 mg/mL); TEMED: Tetramethyl-ethylenediamine.

7. Transfer proteins to the membranes at maximally 15 V. The time required for 0.7-mm gels is 1.5 h for 10% gels and 3 h for 16.5% gels.
8. Stain wet PVDF membranes for 5 min by 0.025% Serva Blue G dissolved in 25% methanol, p.A., and 10% acetic acid, p.A., and destain 3 × 5 min by the same solution without dye.
9. Incubate 10 min in distilled water, and let dry before N-terminal protein sequencing.

General Steps of Blue Native Electrophoresis

1. Select the appropriate gel type according to the size of the protein of interest (*see* Note 11), and cast gels as summarized in Table 5.
2. Select the appropriate path of BN-PAGE according to the sample origin as outlined in Fig. 1. The path describing the processing of partially purified proteins includes membrane and water-soluble proteins.
3. Prepare homogeneously dissolved membrane protein samples either directly from biological membranes according to the BN-PAGE Starting from Biological Membranes (*see also* Note 12) or use partially purified, homogeneously dissolved membrane protein preparations (*see* BN-PAGE for Final Purification of Partially Purified Proteins).

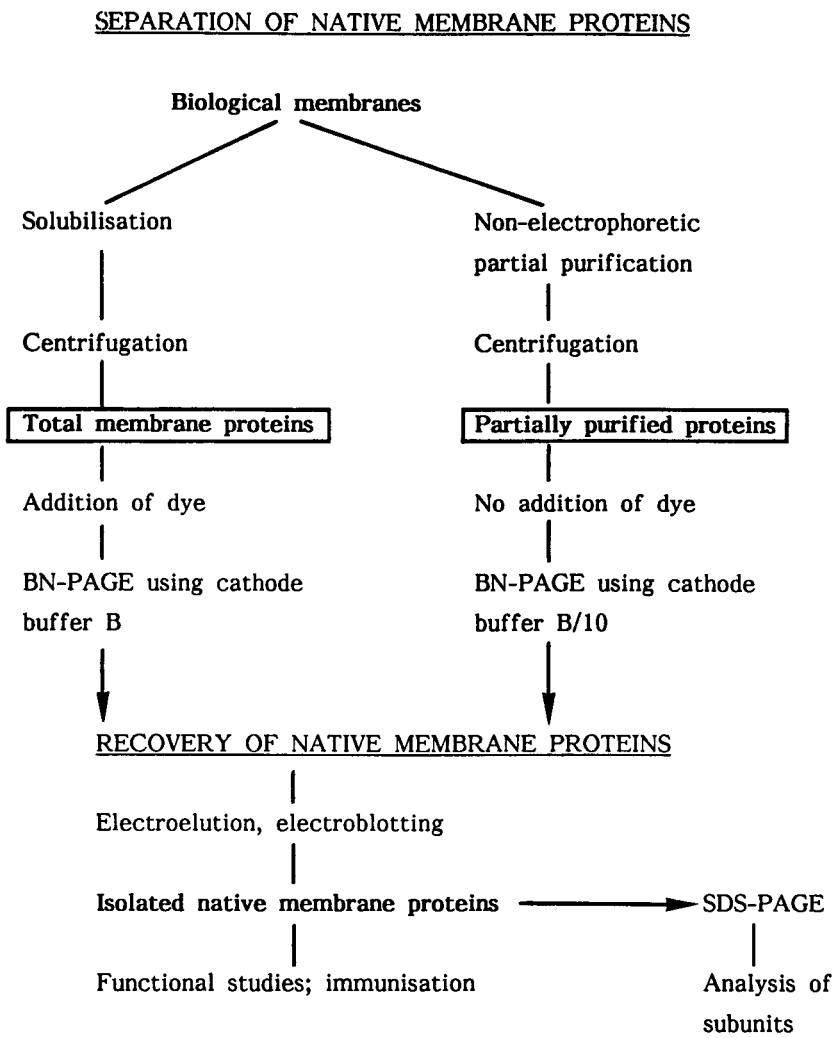

Fig. 1. Individual steps in isolation of native membrane proteins by native electrophoretic techniques. The processing of membrane protein extracts differs from that of prepurified membrane proteins by the different requirement of Coomassie dye in the sample itself and in the cathode buffer. The path described for partially purified proteins can be used for final purification of about 1 mg of a membrane protein from one preparative gel.

BN-PAGE Starting from Biological Membranes: Bovine Heart Mitochondria Used as an Example

1. Sediment a 100-μg aliquot of bovine heart mitochondria prepared according to Smith (*9*) by centrifugation (10 min, 10,000*g*). Centrifugation is recommended even if a concentrated protein suspension is available in order to remove any

compound that might precipitate the Coomassie dye that has to be added in step 4.
2. Solubilize the sediment by addition of 20 μL of solubilization buffer and 2.5 μL of dodecyl-maltoside (10%). The final dodecyl-maltoside concentration should neither exceed 2% (this would deteriorate the running front) nor (in the case of low protein amounts) fall below 0.2%.
3. Centrifuge 15 min at 100,000*g*.
4. Add Coomassie dye from the 5% Serva Blue G stock solution to the supernatant shortly before the sample is applied to BN-PAGE to adjust a detergent/Coomassie ratio of 4/1 (g/g). Addition of Coomassie is not required with samples containing < 0.05% dodecyl-maltoside.
5. Apply the sample to linear 5–13% acrylamide gels (1.6 mm) overlaid by a 4% gel with 5–10 mm pockets.
6. Use cathode buffer B (concentrated dye) for the electrophoresis.
7. Start electrophoresis, performed at 4°C, at 100 V until the sample is completely within the sample gel, and continue with voltage and current limited to 500 V and 15 mA, respectively. After 1/3 of the run, cathode buffer B may be removed by suction and replaced by cathode buffer B/10 for a better visualization of faint protein bands (*see* Notes 13 and 15).

BN-PAGE For Final Purification of Partially Purified Proteins, Exemplified with Crude Complex III from Bovine Heart (*10*)

1. Add 400 μL of 2*M* aminocaproic acid to 1.2 mL of a complex III solution (2 mg of total protein; ≥ 50% purity) in 0.05% Triton X-100, 100 m*M* NaCl, 10 m*M* Na-MOPS, pH 7.2 (*see* Note 14).
2. Mount a preparative gel (14 × 14 × 0.28 cm) into the electrophoresis chamber, and fill in cathode buffer B/10 (with low Coomassie dye concentration).
3. Then apply the total volume (1.6 mL of the complex III solution) to the gel.
4. Start electrophoresis, performed at 4°C, at 100 V until the developing blue band is completely within the sample gel, and continue with voltage and current limited to 500 V and 15 mA, respectively (*see* Note 15).
5. The main band representing highly pure complex III is cut out and electroeluted as described below.

Recovery of Native Proteins by Electroelution

1. Use H-shaped electroelutor/concentrator vessels made according to Hunkapiller et al. (*8*) (cf 1 under Electroelution of Denatured Proteins after Blue SDS-PAGE), cut appropriate

pieces out of thick-walled and mechanically stable dialysis membranes (*see* 2 under Electroelution of Denatured Proteins after Blue SDS-PAGE), allow them to swell in water, and seal the lower ends of both vertical tubes with these membranes.
2. Dip these ends into the anodic and cathodic sides of the electrophoresis tank filled with electroelution buffer, and remove any air bubbles below the membranes.
3. Excise visible blue spots or bands from the gel.
4. Squeeze large pieces of gel through a syringe directly into the cathodic arm of the electroelutor vessel, or slice smaller pieces with razor blades.
5. Then fill both arms of the H-shaped elutor vessel and the connecting horizontal tube with electroelution buffer.
6. Extract the proteins at 500 V for several hours or at 100–200 V overnight. The current should be limited to about 2 mA/elutor vessel, because the use of a wrong buffer might result in significantly higher current and damage of the elutor chamber. The proteins collect as a thin blue layer on the anodic dialysis membrane.
7. Remove the colorless supernatant as completely as possible.
8. Then agitate the blue layer by using a Pasteur pipet equipped with a soft silicon tube in order to suspend any aggregated protein and collect the dark blue protein suspension (*see* Note 16).
9. Add a small volume of water and repeat step 8.

Native electroelution is performed at 4°C.

Electroblotting of Native Membrane Proteins

1. Wet PVDF membranes for some seconds with methanol, and transfer the membranes to the electroblotting buffer. After sufficient equilibration, the membranes will sink.
2. Soak a 3-mm stack of papers with electroblotting buffer, and place it on the lower electrode (cathode).
3. The blue gel (without any pretreatment), the PVDF membrane, and a second 3-mm stack of papers soaked with electroblotting buffer then follow. Only thin gels (maximally 1.6 mm) run with cathode buffer B/10 (low dye concentration) should be used to avoid overloading of the membranes.
4. Mount then the anode, and put a 5-kg load on top.
5. Transfer proteins at 20 V for 2 h.
6. Background destaining can be achieved by 25% methanol, 10% acetic acid.

Semidry electroblotting of native proteins is performed at 4°C.

Two-Dimensional Denaturing Resolution: BN-PAGE/Tricine-SDS-PAGE (*see* Note 17)

1. Excise blue bands or 0.5-cm broad lanes from the first-dimension BN-PAGE.
2. Place them on a glass plate (with ears) within the stacking gel area; soak with 1% SDS, and 1% mercaptoethanol for 30 min, and after inversion, for a further 90 min.
3. Adjust spacers (0.7-mm spacers when BN-PAGE was performed with 1.6-mm gels), and put the second glass plate on top.
4. Remove mercaptoethanol as completely as possible; it would inhibit polymerization of acrylamide.
5. Pour a 16.5% separating gel (Tricine-SDS-PAGE; *see* Table 3) leaving a 2-cm gap to the first-dimension gel, and cover with water.
6. After polymerization, overlay a 1.5-cm 10% acrylamide Tricine-SDS-gel (*see* Table 3), and overlay by water.
7. Finally, embed the pieces of gel in a 10% acrylamide "native" gel, i.e., use the buffers for blue native gels (cf Table 5), but add 10% glycerol and 0.2% SDS.
8. Start second-dimension Tricine-SDS-PAGE (0.7-mm gels; total length of 14 cm) at maximally 250 V with the current limited to 40 mA. After two-thirds of the run, voltage may be raised to 300 V with the current limited to 45 mA. The run is then finished after about 6 h.

NOTES

1. The acrylamide-*bis*-acrylamide stock solution used in the original Laemmli procedure contains a similar percentage of crosslinker (2.7% C) as AB-mix S (3% C), but because of its lower concentration of total monomer (30% T) compared to AB-mix S (49.5% T), it cannot be used for casting high-percent acrylamide gels for the Tricine SDS-PAGE.
2. Gradient acrylamide gels are commonly used to cover a wide range of molecular masses. Uniform acrylamide gels are chosen if optimal separation within a narrow molecular-mass range is desired (cf Table 1). Use the Laemmli SDS-PAGE if proteins larger than 30 kDa are to be separated and smaller proteins are of no interest. Use Tricine SDS-PAGE if proteins below 30 kDa are of interest.
3. Use the colorless Laemmli and Tricine SDS techniques if electroblotting follows after the separation, except for electroblot-

ting of small proteins in the 1–3 kDa range (*see* Note 10). In this special case, use Blue Tricine SDS-PAGE. Use the Blue SDS techniques whenever protein extraction from gels is desired. If protein separation is possible both by using the Laemmli and the Tricine system, preferentially use the Blue Laemmli system, because the sensitivity of detection is higher than in the Blue Tricine system.

4. Gels for Blue Laemmli SDS-PAGE usually are uniform gels overlaid by a 4% sample (stacking) gel. After removal of the combs, the gels are covered with water and stored in the cold. Usually 0.7- or 1.6-mm gels are used.
5. Gels for Blue Tricine SDS-PAGE in the simplest form are composed of a separating gel overlaid by a 4% sample gel (Table 3). In contrast to the Laemmli SDS-PAGE, sample gel buffer and separation gel buffer are identical (gel buffer). If proteins smaller than 5 kDa are to be separated, the high-percent separating gel after polymerization is overlaid by a 10% gel (1 cm), and finally by the 4% sample gel. Some drops of water are overlaid at each step before polymerization to guarantee smooth surfaces. Usually 0.7-mm gels are used in Blue SDS-PAGE even for preparative purposes. Thin gels can be run faster, blue protein bands then are sharper, and detection is more sensitive.
6. The minimal protein load required for detection of blue-stained proteins during Blue SDS-PAGE is higher than for colorless SDS-PAGE using postelectrophoretic fixation and staining. One microgram per protein band usually is sufficient for detection of a protein band on a light screen when the sample is applied to 5-mm pockets of a 0.7-mm gel. Fast runs (cf Table 4) or shortened migration distances improve the sharpness of bands and detection. The maximal protein load is identical to conventional SDS-PAGE. Depending on the required resolution, up to 500 μg of a single, slightly contaminated protein or 1 mg of a protein mixture can be applied to a 14 × 14 × 0.16 cm gel.
7. Conductivity in Laemmli gels is much lower than in Tricine gels. Hence, the electrophoresis at about 200 V poses little heating problems in Laemmli SDS-PAGE, even if 1.6-mm gels are used. In Tricine SDS-PAGE, however, going beyond the voltage limits (Table 4) would raise the risk of cracking glass plates. The electrophoresis conditions are those for "runs at room temperature," i.e., for use of an apparatus without a special cooling device.
8. SDS from the electrode buffer does not accumulate in the electroeluate. Since the amount of SDS present in the vessel

is kept low, the electroeluted proteins can be used directly for immunization. Processing of proteins for fragmentation and N-terminal protein sequencing is described in (4).

9. The staining solution is also applied after Blue SDS-PAGE with subsequent electroblotting, although the PVDF membrane is already stained blue.
10. Transfer of large membrane proteins after (Blue) Tricine PAGE (running pH 8.5) is much better than from Laemmli gels (running pH 9.5) because of the larger difference to the pK of aminocaproic acid (pK = 10.7) used as a trailing ion. Despite that, low-percent acrylamide gels are required for efficient transfer of large-membrane proteins, e.g., 10% gels for proteins larger than 40 kDa. The second advantage of Tricine SDS-PAGE compared to the Laemmli SDS-PAGE concerns the acrylamide concentration of the gels. Two to 3% less acrylamide is required for separation of Tricine SDS-PAGE. The use of Blue SDS-PAGE instead of conventional SDS-PAGE offers great advantages for detection and protein recovery by electroelution. However, in electroblotting, advantages and disadvantages were noticed. Disadvantage: Coomassie occupies binding surface on the PVDF membrane, and at high protein load, more protein will pass the membrane. Advantage: Peptides in the 1–2 kDa range transferred from conventional SDS-PAGE usually are not retained on PVDF membranes. However, most of them are retained after transfer from blue SDS gels, especially if PVDF membranes specified for use in protein sequencing are used (high binding capacity; small pore size), and if 20% methanol is added to the electroblotting anode buffer.
11. Linear acrylamide gels overlaid with a 4% sample gel were used for BN-PAGE. Since proteins are separated according to size and migration ceases when an appropriate pore size of the gel is reached, uniform acrylamide gels will not work except for a narrow molecular mass range. Total gel size was 14 × 14 cm. The thickness of gels was 1.6 mm for analytical and 2.8 mm for preparative purposes. For analytical purposes, combs with 5–10 mm broad teeth were sufficient. Five to 13% acrylamide gels are optimal for the 10^5–10^6 dalton range. The composition of this gel type is shown in Table 5. Five to 18% acrylamide gels allow detection of 20-kDa proteins.
12. Any description of a special protein solubilization and purification procedure can only serve as a guide, not as a standard protocol. However, there are some generally valid aspects:
    a. First of all, a neutral or zwitterionic detergent has to be selected that solubilizes the desired protein without destroying the catalytic activity. Solubilization and stability

have to be tested by comparison of catalytic activity in the sample before and after solubilization. Dodecyl-maltoside is the preferred neutral detergent, but even dodecyl-maltoside may lead to dissociation of labile subunits at high detergent/protein ratios. It is therefore essential to find out the minimal detergent/protein ratio that keeps the membrane proteins of interest solubilized in the supernatant after centrifugation. A set of solubilization experiments performed on a small scale should include dodecyl-maltoside/protein ratios from 0.5 to 4 (g/g).

b. Solubilization of membrane proteins by neutral detergents mostly is ineffective without the presence of a dielectric. The use of salts, however, raises a problem when starting from biological membranes. High detergent concentrations (up to 2%) are usually required for solubilization of biological membranes in order to have a sufficient amount of the desired protein on the gel. The presence of large quantities of lipid/detergent micelles, however, interferes strongly with the separation of the proteins. Only when Coomassie dye is added to the sample at a ratio of about 1 g dye/4 g detergent, the mixed detergent micelles become negatively charged and are removed to the running front. The problem now is: Coomassie dye cannot be added to the sample if salt is present, because Coomassie is precipitated by millimolar concentrations of cations, especially by potassium and divalent cations. Lithium and sodium are tolerated best. The solution to the problem is the use of 500–750 m*M* of the zwitterionic compound 6-aminocaproic acid, which supports solubilization of membrane proteins similar to sodium chloride, but does not precipitate the dye. Additional advantages are: It inhibits serin proteases (2–5 m*M* are commonly used), it does not migrate in an electrical field at the running pH 7.5, and therefore, it does not contribute to heat production during the run.
c. Protein solubilization and protein stability depend also on the pH. It is recommended to try solubilization at neutral pH, because proteins are separated at pH 7.5.
d. One further problem with total membrane protein extracts besides the interference of salts concerns the presence of DNA. High DNA contents, for instance, of bacterial samples or of yeast French press particles, can plug the pores of the sample gel and prevent penetration of the proteins. The best way to reduce this problem is to break cells under mild conditions, e.g., by use of the French press at low pressure or by enzymatic cleavage of cell

walls. It is then possible to remove nuclei by differential centrifugation.

13. The use of 20–400 μg of total mitochondrial proteins (about 1–20 μg of the individual complexes) allows detection of the blue-stained membrane protein complexes during BN-PAGE. The protein determination should be performed before addition of aminocaproic acid, since aminocaproic acid disturbs all commonly used protein determination methods. Four hundred micrograms of total mitochondrial protein are the maximum load to a 1.6 × 10 mm^2 pocket.
14. Considering the interference of salts with Coomassie dye (*see* Note 12), the application of partially purified proteins to BN-PAGE seems to make no sense. However, these preparations containing salt usually contain low concentrations of free detergent and lipid/detergent micelles. Addition of Coomassie dye to the sample then is not required, and samples containing 100 m*M* NaCl can be applied. The Coomassie dye required for inducing the charge shift on the proteins is provided during the run by the cathode buffer with low Coomassie concentration (cathode buffer B/10). The separation of proteins from NaCl and the simultaneous staining of proteins occur gradually, and no precipitation of dye or protein is observed. Further guides are:
    a. The sample applied should not contain potassium or divalent cations that might precipitate the Coomassie dye and dye-binding proteins. Buffer exchange by gel filtration is recommended in these cases.
    b. The lower the salt concentration, the smoother the protein band will be, but solubility properties of the individual proteins have to be considered.
    c. Samples should not contain more than 0.2% detergent. However, there is also a critical lower limit of the detergent concentration. It is a safe way to keep the detergent concentration clearly above the critical micelle concentration (CMC).
15. All gels (1.6 and 2.8 mm) were run under identical conditions. Each run was started at 100 V until the sample was completely within the sample gel. Electrophoresis then was continued for 3–5 h with voltage and current limited to 500 V and 15 mA, respectively.
16. The protein concentration usually is higher than required for application to SDS-PAGE.
17. Blue Native Electrophoresis allows separation and detection of blue-stained protein bands. The identification of the proteins, however, has to be performed either by enzymatic

analysis after native electroelution or, if characteristic polypeptide patterns are known, by analysis of the subunit composition after second-dimension Tricine-SDS-PAGE. After second-dimension SDS-PAGE and use of sensitive detection methods (silver staining; immunodetection after Western blot), low abundant proteins can be identified that are not visible after BN-PAGE.

ACKNOWLEDGMENTS

This work was supported by grants to H. S. from the Deutsche Forschungsgemeinschaft.

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