Electrophoresis Analysis

This unit describes polyacrylamide gel electrophoresis of protein samples. Proteins are important constituents of foods. They not only provide essential nutrients but also contribute to the functional properties needed to develop desired textural and sensory qualities of manufactured foods. During the manufacturing process, proteins can undergo many changes. Electrophoresis is the method of choice to gain insight into changes in protein composition, hydrolysis, or any modifications that may occur. Applications of protein electrophoresis include sample comparison; purity evaluation; determination of physical characteristics such as molecular weight, isoelectric point, and subunit composition; and the purification of small amounts of protein for further analysis. These widely used techniques are easily performed using inexpensive equipment.

In protein electrophoresis, a sample is applied to a polyacrylamide gel and its protein components are separated by application of an electric field across the gel. Separation is dependent on the charge and size of the proteins in the sample. Different approaches to this method have been developed to suit a variety of purposes.

In Basic Protocol 1, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate denatured and fully reduced proteins on the basis of their molecular weight. Alternate Protocol 1 describes the preparation of gradient gels, which allow for separation of a wider range of molecular weights than conventional homogeneous gels. In Alternate Protocol 2, native discontinuous PAGE is used for separation that depends both on the size and intrinsic charge of the proteins. Alternate Protocol 3, SDS-PAGE in a Tris-tricine buffer system, describes the electrophoretic separation of proteins and peptides in the range of 1 to 20 kDa. In Basic Protocol 2, native isoelectric focusing (IEF) is used to separate proteins on the basis of their isoelectric point (pI). Alternate Protocol 4 offers a denaturing version of this technique.

Several protocols in this unit describe methods for visualizing results following electrophoresis. Support Protocol 1 renders the protein bands visible with Coomassie brilliant blue R250 dye. Support Protocol 2 describes silver staining, a more complicated technique that provides exceptional sensitivity. Basic Protocol 3 describes the specific staining for proteinases of samples separated in polyacrylamide gels. Alternate Protocols 5 and 6 describe gel electrophoresis to detect proteinase inhibitors and the determination of proteinase class, respectively. Finally, Support Protocol 3 describes the estimation of protein molecular weights by SDS-PAGE.

CAUTION: Many of the following protocols utilize acrylamide monomer, a neurotoxin and suspected carcinogen, as well as other chemical substances that require special handling. When working with acrylamide, general handling procedures include using double latex gloves and weighing the material in a hood while wearing a disposable dust mask. See *APPENDIX 2B* for other handling guidelines.

CAUTION: Voltages and currents used for electrophoresis are potentially lethal. Use properly shielded, safety-certified equipment.

NOTE: Cleanliness and reagent quality are particularly important for electrophoresis. Always wear gloves when preparing electrophoresis reagents and handling electrophoresis equipment. Clean all assemblies that will contact the gels or sample with a detergent designed for laboratory glassware and rinse well with distilled water. Always use the highest-quality reagents and the purest distilled or deoinized water available.

Characterization of Proteins

DENATURING DISCONTINUOUS PAGE (SDS-PAGE)

SDS-PAGE separates proteins according to their molecular weights. The technique is performed in polyacrylamide gels containing SDS. When proteins are treated with both SDS and a reducing agent such as dithiothreitol (DTT) or 2-mercaptoethanol, separations exclusively by molecular weight are possible. The most commonly used buffer system for SDS-PAGE is the Tris-glycine system described below. An example of an SDS-PAGE gel is given in Figure B3.1.1.

Materials

30% (w/v) acrylamide solution (see recipe)
4× running gel buffer (see recipe)
10% (w/v) SDS (*APPENDIX 2A*)
10% (w/v) ammonium persulfate (make fresh)
Tetramethylethylenediamine (TEMED)
Water-saturated butanol (see recipe)
Running gel overlay(see recipe)
4× stacking gel buffer (see recipe)
Protein sample
2× SDS sample buffer (see recipe)
SDS-PAGE tank buffer, pH 8.3 (see recipe)
Protein molecular weight standards appropriate for gel percentage (optional)

Standard or mini-format vertical electrophoresis apparatus (e.g., Hoefer SE600, SE260, or miniVE; Amersham Pharmacia Biotech) with associated accessories, including casting stand, glass plates, 1-mm-thick spacers, and comb
Side-arm vacuum flasks with stoppers
Boiling water bath or 100°C heating block
Long, narrow gel-loading micropipettor tips
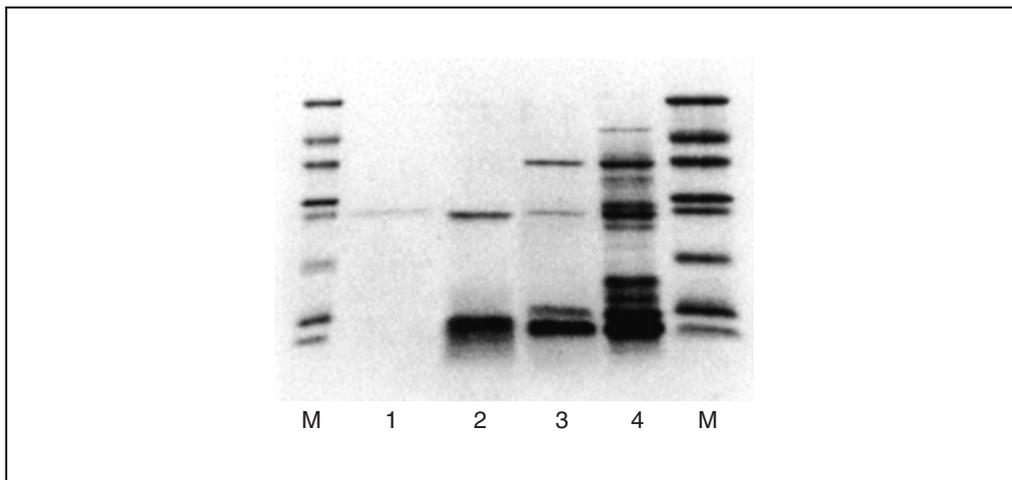Power supply capable of delivering constant current at voltages ≥250 V

Figure B3.1.1 A 15% SDS-polyacrylamide gel stained with Coomassie brilliant blue. Protein samples were assayed for the purification of a proteinase, cathepsin L, from fish muscle according to the method of Seymour et al. (1994). Lane 1, purified cathepsin L after butyl-Sepharose chromatography. Lane 2, cathepsin L complex with a cystatin-like proteinase inhibitor after butyl-Sepharose chromatography. Lane 3, sarcoplasmic fish muscle extract after heat treatment and ammonium sulfate precipitation. Lane 4, sarcoplasmic fish muscle extract. Lanes M, low-molecular-weight standards: aprotinin (M_r 6,500), α-lactalbumin (M_r 14,200), trypsin inhibitor (M_r 20,000), trypsinogen (M_r 24,000), carbonic anhydrase (M_r 29,000), gylceraldehyde-3-phosphate dehydrogenase (M_r 36,000), ovalbumin (M_r 45,000), and albumin (M_r 66,000) in order shown from bottom of gel. Lane 1 contains 4 µg protein; lanes 2 to 4 each contain ∼7 µg protein.

Additional reagents and equipment for staining with Coomassie brilliant blue (see Support Protocol 1) or silver (see Support Protocol 2), and for estimating protein molecular weight (optional; see Support Protocol 3)

Prepare running gel

1. Assemble a gel sandwich (consisting of glass plates and 1-mm-thick spacers) into a casting stand according to manufacturer's instructions.

   The glass plates should be cleaned with a laboratory glassware cleaner such as RBS-35 (Pierce), rinsed well, and dried.

   This protocol can be used for either standard (14 × 16–cm) or mini-format (8 × 10–cm) gels. The recipes can be adjusted for 0.75 or 1.5-mm-thick gels. Alternatively, precast gels for SDS-PAGE, which are available from a number of manufacturers, can be used, and protocol should be started at step 16.

2. Determine the appropriate acrylamide percentage of running gel solution (see Table B3.1.1), and mix solution in a side-arm vacuum flask according to Table B3.1.2, leaving out the ammonium persulfate and TEMED.

3. Stopper flask and apply a water vacuum for several minutes while shaking or stirring to deaerate the solution.

4. Add ammonium persulfate and TEMED and gently swirl flask to mix, being careful not to generate bubbles.

Table B3.1.1 Recommended Acrylamide Concentrations for Protein Separation by SDS-PAGE

% Acrylamide in running gel	Separation size range (kDa)
Single percentage:	
5%	36-200
7.5%	24-200
10%	14-200
12.5%	14-100[a]
15%	14-60[a]
Gradient:	
5%-15%	14-200
5%-20%	10-200
10%-20%	10-150[a]

[a]Larger proteins move into the gel but fail to separate significantly.

Table B3.1.2 Running Gel Solutions for 1-mm-Thick Gels[a]

Component	Final percentage[b]				
	5%	7.5%	10%	12.5%	15%
30% Acrylamide solution	6.7 ml	10 ml	13.3 ml	16.7 ml	20 ml
4× Running gel buffer	10 ml	10 ml	10 ml	10 ml	10 ml
10% SDS	0.4 ml	0.4 ml	0.4 ml	0.4 ml	0.4 ml
H_2O	22.7 ml	19.4 ml	16.1 ml	12.7 ml	9.4 ml
10% Ammonium persulfate[c]	200 µl	200 µl	200 µl	200 µl	200 µl
TEMED[c]	15 µl	15 µl	15 µl	15 µl	15 µl

[a]Final volume is 40 ml, sufficient for two standard (14 × 16–cm) or four mini-format (8 × 10–cm) gels.
[b]See Table B3.1.1 for guidelines on selecting appropriate percentage.
[c]Add after deaeration (step 3).

5. Pipet solution down the spacer and into gel sandwich to a level 3 to 4 cm from the top.

6. Overlay gel with a thin layer (100 to 500 µl) of water-saturated butanol and allow to polymerize.

   A very sharp liquid-gel interface will be visible when the gel has polymerized. This should be visible within 10 to 20 min. The gel should be fully polymerized after 1 to 2 hr.

7. Tilt casting stand to pour off butanol and rinse gel surface once with running gel overlay.

8. Overlay gel with running gel overlay and allow gel to sit while preparing stacking gel.

Prepare stacking gel

9. Prepare stacking gel solution according to Table B3.1.3 in a side-arm vacuum flask, leaving out the ammonium persulfate and TEMED.

10. Deaerate as in step 3.

11. Add ammonium persulfate and TEMED. Gently swirl flask to mix, being careful not to generate bubbles.

12. Pour off running gel overlay.

13. Add 1 to 2 ml stacking gel solution to gel sandwich to rinse the gel surface. Rock casting stand and pour off liquid.

14. Fill gel sandwich with stacking gel solution and insert a comb into the sandwich, taking care not to trap any bubbles below the comb teeth.

15. Allow gel to polymerize ≥60 min.

   A very sharp liquid-gel interface will be visible when the gel has polymerized. This should be visible within 10 to 20 min. The gel should be fully polymerized after 1 to 2 hr. In general, stacking gels should be cast just before use. However, the complete gel can be stored overnight at 4°C, with little effect on resolution, if covered with the comb in place.

Prepare sample

16. Combine equal volumes protein sample and 2× SDS sample buffer and incubate 90 sec in a boiling water bath or 100°C heating block.

   If the gels will be stained with Coomassie brilliant blue (see Support Protocol 1), a starting sample protein concentration of 10 to 20 mg/ml should be used. This will be diluted by the 2× SDS sample buffer to give 5 to 10 µg/µl. For complex mixtures, 50 µg protein (5 to 10 µl treated sample) per lane is recommended. For highly purified proteins, 0.5 to 5 µg per lane is usually adequate. Silver staining (see Support Protocol 2) requires 10- to 100-fold less protein per lane.

Table B3.1.3 Stacking Gel Solution (4% Acrylamide) for 1-mm-Thick Gels[a]

Component	Volume
30% Acrylamide solution	2 ml
4× Stacking gel buffer	3.75 ml
10% SDS	150 µl
H_2O	9 ml
10% Ammonium persulfate[b]	75 µl
TEMED[b]	7.5 µl

[a]Final volume is 15 ml, sufficient for two standard (14 × 16–cm) or four mini-format (8 × 10–cm) gels.
[b]Added after deaeration (step 10).

17. Place sample on ice until ready for use.

    The treated sample can be stored at –20°C for 6 months for future runs.

Load gel

18. Slowly remove comb from the gel, angling the comb up to avoid disturbing the well dividers

19. Rinse each well with SDS-PAGE tank buffer, invert the casting stand to drain the wells, and return the stand to an upright position.

20. Fill each well with SDS-PAGE tank buffer.

21. Using a micropipettor fitted with a long, narrow gel-loading tip, gently and slowly load 5 to 10 μl sample beneath the buffer in each well. Load every well with the same volume of sample. If a well is not needed, load it with 1× SDS sample buffer containing standard protein or no sample.

    This procedure ensures that each well behaves the same during separation. If a well is left empty, the adjacent samples will tend to spread during electrophoresis.

    When adding the sample, a sharp interface should be maintained between the sample and the SDS-PAGE tank buffer. Adding the sample too fast or erratically will lead to swirling and a diffuse loading zone. This will cause a loss of band sharpness.

    Depending on the design of the gel apparatus, it may be preferable to load the gel following installation of the gel into the apparatus and addition of buffer to the upper buffer chamber (steps 23 to 25).

22. If protein molecular weight standards are used, follow manufacturer's instructions for their preparation and load one or two wells with 5 to 10 μl standards.

    This volume should contain 0.2 to 1 μg of each standard component if the gel is to be stained with Coomassie brilliant blue, and ~10 to 50 ng of each component if the gel is to be silver stained.

Run gel

23. Fill lower buffer chamber of the electrophoresis apparatus with SDS-PAGE tank buffer.

24. Remove gel sandwich from casting stand and install it in the electrophoresis apparatus according to manufacturer's instructions. Make sure bottom of gel cassette is free of bubbles.

25. Carefully fill upper buffer chamber with SDS-PAGE tank buffer. Do not pour buffer into the sample wells because it will wash the sample out.

26. Put safety lid on the gel apparatus and connect it to a power supply.

    The cathode (black lead) is connected to the upper buffer chamber.

27. Set power supply to constant current and turn it on. Adjust current to 20 mA per gel.

    When running 0.75- or 1.5-mm-thick gels, the current should be adjusted accordingly; 0.75-mm-thick gels should be run at 15 mA per gel, and 1.5-mm-thick gels at 30 mA per gel.

28. Keep a record of the voltage and current readings to compare with future runs and to detect current leaks or incorrectly made buffers.

    The voltage should start at ~70 to 80 V, but will increase during the run.

Characterization of Proteins

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Under these conditions, a standard (14 × 16–cm) gel will take ~5 hr to run and a mini-format (8 × 10–cm) gel will take ~1.5 hr. If it is more convenient to run the gel for a longer period, e.g., 10 hr for a standard gel, the current should be cut in half (to 10 mA/gel). For a 15-hr run (i.e., overnight), the current should be cut to 7 mA/gel.

If an electrophoresis apparatus with active cooling capability is used, considerably higher currents (up to 50 mA per gel) can be used, with correspondingly shorter run times.

29. When the dye front reaches the bottom of the gel, turn power supply off and disconnect power cables.

30. Visualize protein bands by Coomassie brilliant blue (see Support Protocol 1) or silver staining (see Support Protocol 2). To estimate protein molecular weight, see Support Protocol 3.

LINEAR GRADIENT PAGE

Gradient gels, although more difficult to cast than single-concentration gels, fractionate a wider size range of proteins on a single gel. Furthermore, calculating molecular weights (see Support Protocol 3) is simplified because, unlike single-concentration gels, the relationship between the logarithm of the molecular weight of a protein and its mobility is linear over most of the fractionation range of a gradient gel. A gradient maker mixes a high- and a low-percentage acrylamide solution during the casting process, which results in a gel that spans a range of acrylamide percentages. The protocol below describes casting one gradient gel at a time. A multiple gel caster can cast multiple gradient gels simultaneously. Consult the manufacturer's instructions for casting several gradient gels at once.

Additional Materials (also see Basic Protocol 1)

Sucrose
Pump tubing
Side-outlet gradient maker for linear gradients (e.g., Hoefer SG50 for standard gels and Hoefer SG15 for mini-format gels; Amersham Pharmacia Biotech)
Peristaltic pump capable of delivering 1 to 6 ml/min

Set up gradient maker

1. Assemble a gel sandwich as described above (see Basic Protocol 1, step 1).

2. Connect a piece of pump tubing to the outlet tubing connector of a side-outlet gradient maker.

Table B3.1.4 Light Gradient Running Gel Solutions[a]

Component	Final percentage[b]				
	5%	7.5%	10%	12.5%	15%
30% Acrylamide solution	3.3 ml	5.0 ml	6.7 ml	8.3 ml	10.0 ml
4× Running gel buffer	5 ml	5 ml	5 ml	5 ml	5 ml
10% SDS	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml
H$_2$O	11.3 ml	9.6 ml	7.9 ml	6.3 ml	4.7 ml
10% Ammonium persulfate	180 µl	160 µl	130 µl	110 µl	80 µl
TEMED[c]	6.6 µl	6.6 µl	6.6 µl	6.6 µl	6.6 µl

[a]Final volume is 20 ml, sufficient for two standard (14 × 16–cm) or four mini-format (8 × 10–cm) 1-mm-thick gels.
[b]See Table B3.1.1 for guidelines on selecting appropriate percentage.
[c]Add just before pouring the gel (step 7).

3. Force a 1 to 200 μl pipet tip onto the other end of the tubing and, using a ring stand and clamp, position the tip 0.5 to 1 mm inside the top center of the gel sandwich. Use the clamp to hold the pipet tip firmly in place.

4. Attach tubing to a peristaltic pump.

Prepare gel solutions

5. Determine appropriate low and high percentages of the gradient gel (see Table B3.1.1). In separate flasks, mix all ingredients listed in Tables B3.1.4 and B3.1.5 for the respective light and heavy gradient running gel solutions, including ammonium persulfate. Do not add TEMED.

   A 5% to 20% or 10% to 20% gradient gel is recommended.

6. Gently swirl flasks to mix and place heavy gradient solution on ice.

   Deaeration is not needed in this protocol.

   The heavy gradient solution should be placed on ice to prevent polymerization. Polymerization can occur in high-concentration acrylamide solutions without addition of TEMED once ammonium persulfate has been added.

7. Add TEMED and gently swirl flasks to mix.

   Alternatively, gel solution can be added to the gradient maker before TEMED is added. TEMED (0.33 μl/ml gel solution) can be added just before opening the outlets and mixed by drawing the solution in and out of a disposable plastic pipet. If this technique is used, a large volume of heavy and light solutions can be prepared in advance and dispensed into the gradient maker for each individual gel. This is useful when casting several gels individually without using a multiple gel caster.

Pour gel

8. Pour heavy gradient solution into the chamber closest to the outlet (mixing chamber) of the gradient maker and add a small stir-bar. Use 9.3 ml per chamber for 1-mm-thick standard (14 × 16–cm) gels. Use 2.3 ml per chamber for 1-mm-thick mini-format (8 × 10–cm) gels.

   Volumes can be adjusted accordingly if pouring 0.75 or 1.5-mm-thick gels.

9. Open stopcock between the two chambers and allow a small amount of heavy gradient solution to flow through the channel to, but not into, the bottom of the reservoir chamber. Close stopcock.

10. Pour light gradient solution into the reservoir chamber.

Table B3.1.5 Heavy Gradient Running Gel Solutions[a]

Component	Final percentage[b]				
	10%	12.5%	15%	17.5%	20%
30% Acrylamide solution	6.7 ml	8.3 ml	10.0 ml	11.7 ml	13.3 ml
4× Running gel buffer	5 ml	5 ml	5 ml	5 ml	5 ml
10% SDS	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml
Sucrose	3 g	3 g	3 g	3 g	3 g
H_2O	6.46 ml	4.66 ml	3.06 ml	1.40 ml	—
10% Ammonium persulfate	100 μl	90 μl	40 μl	40 μl	40 μl
TEMED[c]	6.6 μl	6.6 μl	6.6 μl	6.6 μl	6.6 μl

[a]Final volume is 20 ml, sufficient for two standard (14 × 16–cm) or four mini-format (8 × 10–cm) 1-mm-thick gels.
[b]See Table B3.1.1 for guidelines on selecting appropriate percentage.
[c]Add just before pouring the gel (step 7).

11. Place the gradient maker on a magnetic stirrer and begin stirring.

12. Turn on the pump and open the outlet stopcock.

   The pump rate should be set so that casting takes from 5 to 10 min.

13. Open stopcock between the two chambers. Continue to pump until all liquid is in the gel sandwich.

14. Overlay gel with 100 µl water-saturated butanol and allow gel to polymerize.

15. Continue with casting the stacking gel and running the sample (see Basic Protocol 1, steps 7 to 30).

NATIVE DISCONTINUOUS PAGE

Under native conditions, polypeptides retain their higher-order structure and often retain enzymatic activity and interactions with other polypeptides. The migration of proteins under native conditions depends on many factors including size, shape, and native charge. One straightforward approach to native electrophoresis is to leave out the SDS and reducing agent (DTT or 2-mercaptoethanol) from the SDS-PAGE protocol (see Basic Protocol 1). Prepare all solutions without SDS, including tank buffer. Leave out both SDS and reductant from 2× sample buffer, and do not heat treat samples. Figure B3.1.2 shows an example of a native, discontinuous polyacrylamide gel.
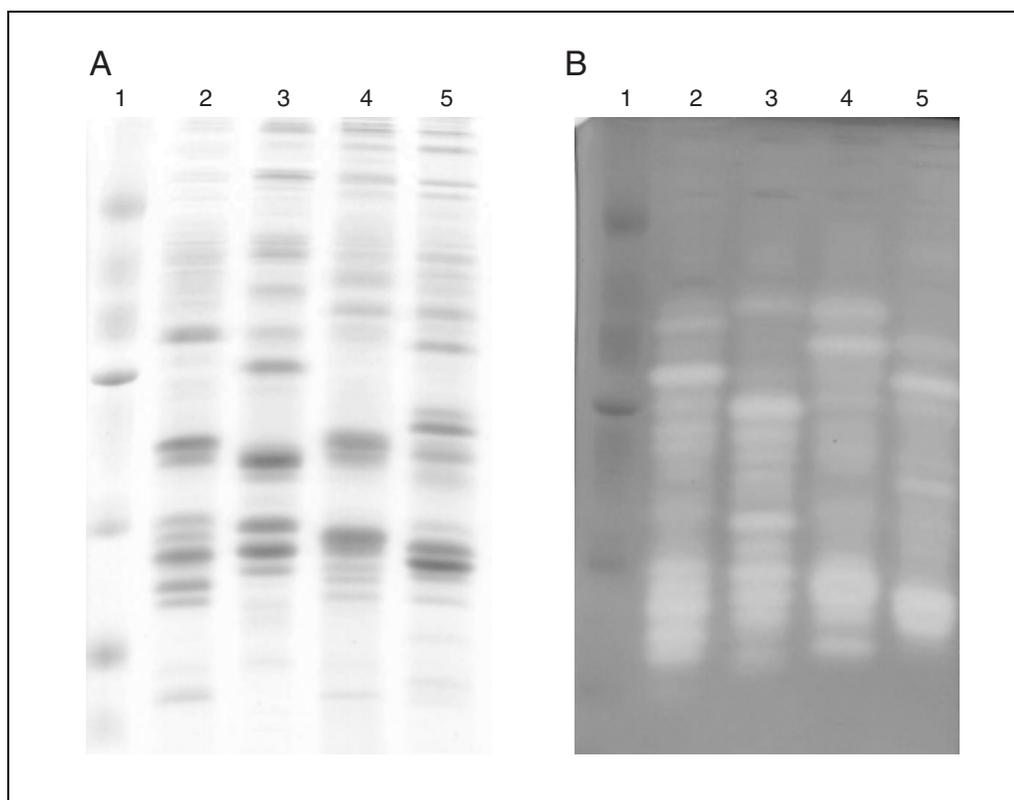

Figure B3.1.2 Native discontinuous polyacrylamide gels activity stained for proteinases. (**A**) Gel stained with Coomassie brilliant blue for total protein. (**B**) Gel assayed for proteinase activity using casein as a substrate. Samples are enzyme extracts of hepatopancreas from four shrimp species. Lane 1, molecular weight markers; Lane 2, *P. californiensis*; Lane 3 *P. vannamei*; Lane 4, *P. paulensis*, Lane 5, *P. schmitti*.

SDS-PAGE IN A TRIS-TRICINE BUFFER SYSTEM

The Tris-glycine discontinuous buffer system of Laemmli cannot be used for the separation of proteins with molecular weights <10 to 15 kDa. For the analysis of smaller proteins, an alternative Tris-tricine buffer system is used along with an acrylamide solution that has a high percentage of cross-linker. This technique should be used when separating peptides in the size range of 1 to 20 kDa.

Additional Materials *(also see Basic Protocol 1)*

   Ethylene glycol
   40% (w/v) acrylamide solution (see recipe)
   4× Tris-tricine gel buffer (see recipe)
   Upper (cathodic) Tris-tricine tank buffer (see recipe)
   Lower (anodic) Tris-tricine tank buffer (see recipe)

1. Prepare gel solutions and pour a gel for the Tris-tricine system as described above (see Basic Protocol 1, steps 1 to 15), but use the 16% running gel and 5% stacking gel solutions given in Table B3.1.6.

2. Prepare sample and load onto gel (see Basic Protocol 1, steps 16 to 22), but use upper (cathodic) Tris-tricine tank buffer to load the sample in steps 19 and 20.

   A different sample buffer may be recommended in some literature, but the author sees no difference when the standard sample buffer is used for this technique.

3. Run the gel (see Basic Protocol 1, steps 23 to 30), but fill the lower and upper buffer chambers of the electrophoresis apparatus with lower (anodic) and upper (cathodic) Tris-tricine tank buffers, respectively, and adjust current to 70 mA per gel.

   Under these conditions, a standard (14 × 16-cm) gel will take 5 to 6 hr to run and a mini-format (8 × 10-cm) gel will take 2 to 2.5 hr.

Table B3.1.6 Gel Solutions for Tris-Tricine System[a]

Component	16% Running gel	5% Stacking gel
Ethylene glycol	14 ml	—
40% Acrylamide solution	16 ml	—
30% Acrylamide solution	—	2.5 ml
4× Tris-tricine gel buffer	10 ml	3.75 ml
H$_2$O	—	8.7 ml
10% Ammonium persulfate[b]	160 µl	60 µl
TEMED[b]	20 µl	7.5 µl

[a]Sufficient for two standard (14 × 16–cm) or four mini-format (8 × 10–cm) 1-mm-thick gels.
[b]Add after deaeration.

NATIVE ISOELECTRIC FOCUSING

Isoelectric focusing (IEF) separates proteins according to their isoelectric point (pI). The technique is performed in thin polyacrylamide gels incorporating carrier ampholytes, which are small amphoteric compounds that form a pH gradient when voltage is applied across the gel. Proteins introduced into this pH gradient will migrate until they reach their isoelectric point. Each protein species in the sample can be visualized as a sharply focused band. IEF is best performed in a flatbed electrophoresis apparatus. This type of apparatus allows very effective cooling, which is necessary due to the high voltages employed for IEF. The following protocol is used to separate proteins under native conditions in a range from pH 3.5 to 9.5 in a commercially available precast IEF gel. An example of an IEF gel is given in Figure B3.1.3.

Materials

Protein sample
Kerosene or mineral oil
IEF anode solution: 1 M H_3PO_4 (*APPENDIX 2A*)
IEF cathode solution: 1 M NaOH (*APPENDIX 2A*)
Protein pI standards (prepared according to manufacturer's instructions)

Flatbed electrophoresis apparatus (e.g., Multiphor II; Amersham Pharmacia Biotech)
Thermostatic circulator
Precast IEF gel on plastic backing (e.g., Ampholine PAGplate, pH 3.5 to 9.5; Amersham Pharmacia Biotech)
IEF electrode strips (e.g., Amersham Pharmacia Biotech)
High-voltage power supply capable of delivering 1500 V while limiting both current and power
IEF sample applicator strip (e.g., Amersham Pharmacia Biotech)

Additional reagents and equipment for staining with Coomassie brilliant blue (see Support Protocol 1) or silver stain (see Support Protocol 2)

Prepare sample

1. Adjust sample concentration to 0.5 to 10 mg/ml for Coomassie brilliant blue staining or 0.05 to 1 mg/ml for silver staining.

   The composition at the sample buffer is not critical, but the ionic strength should be as low as possible. Buffers and salts should not be present at a concentration >50 mM.

   Sample solubility can occasionally be improved with the use of a nondenaturing, neutral detergent (e.g., 0.5% [w/v] Triton X-100, CHAPS, or octyl glucoside) or polyalcohol (e.g., 20% [w/v] glycerol or sorbitol) in both the sample and the gel.

2. If sample contains insoluble materials, centrifuge 5 min at maximum speed in a microcentrifuge and transfer supernatant to a clean tube.

Prepare apparatus and gel

3. Connect a flatbed electrophoresis apparatus to a thermostatic circulator and set the temperature to 10°C. Turn on the thermostatic circulator 10 min before starting the analysis.

4. Pipet ~1 ml kerosene or mineral oil onto the cooling plate of the electrophoresis apparatus.

5. Remove a precast IEF gel on plastic backing from its package and position it in the center of the cooling plate, allowing the kerosene or mineral oil to spread evenly underneath the gel. Make sure no bubbles are trapped beneath the gel.
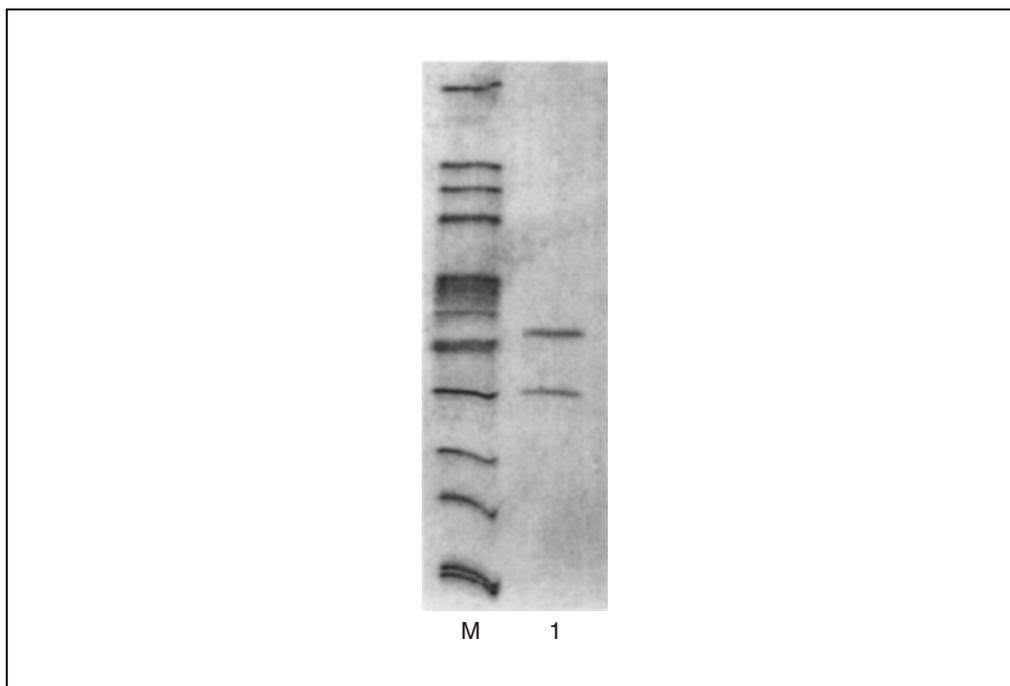
Figure B3.1.3 An isoelectric focusing (IEF) gel, pH 3 to 10. Lane 1, ~4 µg purified egg white cystatin. Lane M, broad-range pI standards: trypsinogen (pI 9.3), lentil lectin-basic band (pI 8.65), lentil lectin-middle band (pI 8.45), lentil lectin-acidic band (pI 8.15), myoglobin-basic band (pI 7.35; visible as a broad band), myoglobin-acidic band (pI 6.85), human carbonic anhydrase B (pI 6.55), bovine carbonic anhydrase (pI 5.85), α-lactoglobulin A (pI 5.20), soybean trypsin inhibitor (pI 4.55), and amyloglucosidase (pI 3.50) in order shown from top of gel. The pI values of the two purified egg white cystatin isomers were determined to be 6.6 (upper band) and 5.8 (lower band). Adapted from Akpinar (1998) with permission from author.

The gel can be used in one piece or, depending on the number of samples, cut into portions with scissors. If this is done, current and power limits should be reduced accordingly (e.g., if only half a gel is to be run, current and power limits should be set at 25 mA and 15 W, respectively, rather than 50 mA and 30 W). Unused portions of gel can be wrapped in plastic or placed in sealed plastic bags and stored at 4°C until use.

6. Soak one IEF electrode strip in ~3 ml IEF anode solution. Remove excess solution with lint-free tissues.

7. Repeat step 6 with a second IEF electrode strip using IEF cathode solution.

8. Apply electrode strips over the long edges of the gel, placing the electrode strip soaked with the anodic solution towards the anodic (+) side of the cooling plate.

9. Use sharp scissors to cut off the ends of the electrode strips that protrude beyond the ends of the gel.

Prerun gel (optional)

10. Place the electrode holder with the IEF electrodes on the electrophoresis apparatus and align electrodes over the center of the electrode strips. Connect the two electrodes to the base unit and place the lid on the apparatus.

11. Connect the leads to a high-voltage power supply. Set power supply to 1500 V, limiting the current at 50 mA and the power at 30 W. Run gel for 15 min.

    The voltage should start out between 300 and 600 V and rise steadily towards 1500 V during the prerun step.

    Prerunning the gel is optional, but can result in a higher-quality separation.

Characterization of Proteins

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Apply sample and run gel

12. Turn off power supply and disconnect leads. Take lid off apparatus and remove electrode holder.

13. Lay an IEF sample applicator strip across the gel towards either the anodic or cathodic edge. Check that contact between gel and applicator strip is uniform.

    The optimal position for sample application varies with sample type and must be determined empirically. This is best done using sample application pieces (e.g., Amersham Pharmacia Biotech), which are 5 × 10–mm pads of absorbent material that can be placed in multiple positions on a single gel.

14. Pipet 5 to 20 μl sample and an appropriate volume of protein pI standards (if desired) into the wells in the applicator strip.

    The protein standards should contain 0.2 to 1 μg of each standard component if the gel is to be stained with Coomassie brilliant blue and ~20 to 100 ng of each component if the gel is to be silver stained.

15. Repeat steps 10 and 11, running the gel for 1.5 hr.

    The running time depends on the pH range of the gel. For gels covering other pH ranges, the manufacturer's instructions should be consulted.

16. Stain gel with Coomassie brilliant blue or silver stain to visualize the proteins (see Support Protocols 1 and 2, respectively).

    A plot of pI versus distance migrated from the cathode end of the gel can be constructed for the pI standard. This can be used to estimate the pI of the proteins in the sample.

DENATURING ISOELECTRIC FOCUSING

In some cases, it is advantageous to perform IEF in the presence of 8 M urea. This denaturant renders some proteins more soluble under IEF conditions, allowing the analysis of samples that cannot be separated under native conditions. The following protocol separates proteins under denaturing conditions in a range from pH 3 to 10 using a commercially available dried polyacrylamide gel.

Additional Materials (also see Basic Protocol 2)

    Dried polyacrylamide gel on plastic backing (e.g., CleanGel IEF; Amersham Pharmacia Biotech)
    Denaturing IEF rehydration solution, pH 3 to 10 (see recipe)

    Rehydration tray (e.g., GelPool; Amersham Pharmacia Biotech)
    Rotary laboratory shaker
    Filter paper
    High-voltage power supply (capable of delivering 2500 V while limiting both current and power)

Rehydrate gel

1. Open gel package and remove a dried polyacrylamide gel on plastic backing.

    The dry gel can be used in one piece or, depending on the number of samples, cut into portions with scissors. Unused portions of gel can be wrapped in plastic or placed in sealed plastic bags and stored at –20°C until use.

2. Select the appropriate chamber of a rehydration tray. Clean tray with water and dry with lint-free tissues.

3. Pipet denaturing IEF rehydration solution into the chamber. For a full-size gel, use 10.4 ml. For portions of the gel, reduce volume accordingly (e.g., use 5.2 ml for half a gel).

4. Set the edge of the dried gel, with the gel surface downwards, into the rehydration solution and slowly lower it. Lift gel at the edges with forceps and slowly lower it down again to ensure an even distribution of liquid and to remove air bubbles.

5. Place rehydration tray on a rotary laboratory shaker and shake gently. Allow gel to rehydrate 4 hr to overnight.

   The gel must be used immediately following rehydration.

Prepare sample and run gel

6. Dissolve or dilute protein sample into denaturing IEF rehydration solution to a concentration of 1 to 3 mg/ml for Coomassie brilliant blue staining or 10 to 300 µg/ml for silver staining.

   The composition of the sample solution should be as similar as possible to the composition of the rehydration solution. Buffers and salts should not be present at a concentration >50 mM.

7. Continue to prepare sample and gel as described (see Basic Protocol 2, steps 2 to 5), except set the thermostatic circulator to 15°C, and remove gel from the rehydration tray and carefully dry gel surface with the edge of a sheet of filter paper before positioning it on the cooling plate.

   The gel surface should be absolutely dry.

8. Place an electrode holder with IEF electrodes on electrophoresis apparatus and align electrodes so that they rest on the outer edges of the gel. Connect the two electrodes to the base unit and place lid on apparatus.

   IEF electrode strips or wicks are not necessary.

9. Connect leads to a high-voltage power supply. Prerun gel for 20 min following the conditions given in Table B3.1.7.

10. Apply sample and run gel as described (see Basic Protocol 2, steps 12 to 16), except align electrodes in step 15 so that they rest on the outer edges of the gel and follow the recommended settings in Table B3.1.7.

Table B3.1.7 Running Conditions for Denaturing IEF

Step	Voltage (V)	Current (mA)[a]	Power (W)[a]	Time (min)
Prerun	700	12	8	20
Sample entrance	500	8	8	20
Isoelectric focusing	2000	14	14	90
Band sharpening	2500	14	18	10

[a]If only a portion of the gel is run, reduce current and power limits accordingly.

COOMASSIE BRILLIANT BLUE STAINING OF POLYACRYLAMIDE GELS

Coomassie brilliant blue staining is based on nonspecific binding of Coomassie brilliant blue dye to proteins. Separated proteins are simultaneously fixed and stained in the gel, and then destained to remove background staining prior to drying and documenting. The proteins are detected as blue bands on a clear background. The standard protocol can be used to stain conventional SDS-PAGE or native gels as well as IEF and Tris-tricine gels. When staining conventional SDS-PAGE or native gels, a fixing step is not required. A gel stained with Coomassie brilliant blue is shown in Figure B3.1.1.

Materials

Polyacrylamide gel (SDS-PAGE, IEF, native PAGE, or Tris-tricine SDS-PAGE) containing separated proteins (see Basic Protocols 1 and 2; see Alternate Protocols 1 to 4)

20% (w/v) trichloroacetic acid (TCA; for IEF gels only; store ≤1 month at room temperature)

Destaining solution I: 40% (v/v) methanol/7% (v/v) acetic acid (store ≤1 month at room temperature)

Glutaraldehyde fixing solution (see recipe; for Tris-tricine gels only)

0.025% (w/v) Coomassie brilliant blue staining solution (see recipe)

Destaining solution II: 5% (v/v) methanol/7% (v/v) acetic acid (store indefinitely at room temperature)

Glycerol

Covered tray

Laboratory shaker or rocker

Filter paper (e.g., Whatman 3MM) or porous cellophane sheets (e.g., Amersham Pharmacia Biotech).

Vacuum gel dryer (e.g., Hoefer GD2000; Amersham Pharmacia Biotech) attached to vacuum pump or air gel dryer with dryer with drying frames (e.g., Hoefer Easy Breeze; Amersham Pharmacia Biotech)

1a. *For IEF gels:* Place a polyacrylamide gel in a covered tray with enough 20% TCA to fully submerge the gel. Shake slowly 30 to 60 min on a laboratory shaker or rocker. Replace 20% TCA with destaining solution I and shake slowly for 3 min. Remove destaining solution I.

> *Do not leave a gel in 20% TCA for >60 min.*

1b. *For Tris-tricine gels:* Place a polyacrylamide gel in a covered tray with enough glutaraldehyde fixing solution to fully submerge the gel. Shake slowly 30 to 60 min on a laboratory shaker or rocker. Remove fixing solution.

1c. *For SDS-PAGE and native PAGE gels:* Place a polyacrylamide gel in a covered tray.

2. Add just enough Coomassie brilliant blue staining solution so that the gel floats freely in the tray. Shake slowly ~4 hr to overnight on a laboratory shaker or rocker.

> CAUTION: *Exposure to methanol and acetic acid vapors is minimized when using covered plastic trays. When covers are not used, these procedures should be done in a fume hood.*

> *For accelerated staining and destaining, the solutions can be heated to 45°C, which will reduce the time by 50%.*

3. Replace staining solution with destaining solution I. Shake slowly 30 min.

> *This removes the bulk of the excess stain.*

4. Remove destaining solution I and replace with destaining solution II. Change destaining solution II periodically until gel background is clear, typically ≤8 hr.

Alternatively, the waste volume can be minimized by the addition of paper tissue to one corner of the staining tray. Coomassie brilliant blue is removed from the gel without changing the destaining solution, minimizing the waste volume generated. The tissues are replaced when they are saturated with Coomassie brilliant blue. Caution should be used, however, because excessive destaining will lead to loss of band intensity.

5. Store gel in destaining solution II ≤1 week in a covered tray. To minimize cracking, add 4% (v/v) glycerol to the last destaining solution before drying the gel.

   For longer term storage, gels may be stored wet at 4°C. The wet gel should be wrapped in a piece of plastic wrap. This permits handling the gel without risk of breakage. The wrapped gel can be stored in a sealable bag for ≤1 year at 4°C. To preserve gels indefinitely, they may be either vacuum dried onto filter paper or air dried between sheets of cellophane.

6a. *For vacuum drying gels:* Place gel on a sheet of filter paper of the same size. Place gel and paper on a larger sheet of filter paper covering the metal screen of the platen of a vacuum gel dryer attached to a vacuum pump. Cover top of gel with plastic wrap, lower silicon cover flap, apply vacuum to seal flap, and turn on heater. Dry gel, typically <2 hr.

   During vacuum drying, the gel will feel cold relative to the surrounding platen if it is not completely dry. When the gel temperature is the same as the platen, the gel is dry and the vacuum and dryer can be turned off. Gel cracking can be caused by releasing the vacuum before the gel is dry.

6b. *For air drying gels:* Place gel between two sheets of porous cellophane and lock into the drying frame of an air dryer. Insert frame into air dryer and turn on fan and heater. Dry gel, typically <2 hr.

   Moisture evaporates through the cellophane leaving a flat, easy-to-store gel with a clear background.

   As an alternative to drying, gels may be photographed with a Polaroid or digital camera. Illumination should be provided by placing the gel on a light box. Gels may also be scanned with a transparency scanner.

SILVER STAINING OF POLYACRYLAMIDE GELS

Silver staining is approximately 50-fold more sensitive than Coomassie brilliant blue staining (see Support Protocol 1). It is a complex, multistep process, and many variables can influence the results. High-purity reagents and precise timing are essential for reproducible, high-quality results. Impurities in the gel and/or the water used for preparing the staining reagents can give poor staining results. The detection limit of this technique is from 1 to 5 ng protein per band. This protocol gives the best results when applied to standard SDS-PAGE and native gels. Silver staining IEF gels is less sensitive and high levels of background staining can be expected unless extra steps are taken to remove interfering carrier ampholytes. Tris-tricine gels can be silver stained according to this procedure, but a glutaraldehyde fixing solution (see Support Protocol 1), which prevents the loss of small peptides, cannot be used. Therefore, small peptides may diffuse out of the gel during staining and be lost.

Materials

Polyacrylamide gel (SDS-PAGE, IEF, native PAGE, or Tris-tricine SDS-PAGE) containing separated proteins (see Basic Protocols 1 and 2; see Alternate Protocols 1 to 4)
Silver stain fixing solution: 40% (v/v) ethanol/10% (v/v) acetic acid (make fresh)
Sensitizing solution (see recipe)
Silver solution (see recipe)

Characterization of Proteins

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Table B3.1.8 Silver Staining Protocol

Solution	Time (≤1-mm-thick gel)	Time (1.5-mm-thick gel)
Silver stain fixing solution[a]	30 min	30 min
Sensitizing solution	30 min	30 min
H_2O	3 × 5 min	3 × 10 min
Silver solution	20 min	30 min
H_2O	2 × 1 min	2 × 1 min
Developing solution[b]	3-5 min	5-10 min
Stop solution	10 min	10 min
H_2O	3 × 5 min	3 × 5 min
Preserving solution[c]	30 min	30 min

[a]Leave gel in this solution until a convenient time for completing procedure (≤1 week).
[b]Monitor development and change solution when protein bands are visible and background is just starting to darken.
[c]Store gel in this solution ≤1 week. The glycerol will prevent gel from cracking when dried.

Developing solution (see recipe)
Stop solution: 1.5% (w/v) Na₂EDTA (store ≤6 months at room temperature)
Preserving solution: 30% (v/v) ethanol/4% (v/v) glycerol (store ≤6 months at room temperature)

Covered tray
Laboratory shaker or rocker

Additional equipment for drying gel (see Support Protocol 1)

NOTE: Clean all equipment used for running and staining the gel with detergent and rinse thoroughly. Wear clean gloves when handling the electrophoresis apparatus, the gel, or the staining tray.

Place a polyacrylamide gel in a covered tray and process according to Table B3.1.8. Use 250 ml of each solution per standard (14 × 16–cm) gel and 100 ml per mini-format (8 × 10–cm) gel. Shake slowly on a laboratory shaker or rocker during each step. When staining an IEF gel, begin with a 30-min fixation step in 20% TCA (see Support Protocol 1) and then proceed with steps described in Table B3.1.8, adding three additional washes with water between the sensitizing step and silver step if needed. The timing of some steps differs according to the thickness of the gel as indicated in Table B3.1.8. If desired, dry gel as described (see Support Protocol 1, step 6).

ACTIVITY STAINING FOR PROTEINASE

Proteinases in complex protein samples are separated and detected by their activity using a technique called substrate-SDS-PAGE. After electrophoresis, the gel is incubated in a casein substrate solution and then stained with Coomassie brilliant blue. The presence of active proteinase is indicated by clear zones on a blue background. Information about the number of active components and their molecular weights is obtained by this technique. This method is useful when studying the presence, diversity, amount, and class of proteinases in food ingredients that affect properties such as texture. Other measurements of proteinase activity are addressed in *UNIT C2.1*. Figure B3.1.2 shows a discontinuous polyacrylamide gel activity stained for proteinase.

Materials

> Proteinase-containing protein sample
> 2 × nonreducing sample buffer (see recipe)
> 2% casein (added just before use) in 50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
> 0.1% (w/v) Coomassie brilliant blue staining solution (see recipe)
> Destaining solution: 40% (v/v) ethanol/10% (v/v) acetic acid (optional; store ≤1 month at room temperature)

> Additional reagents and equipment for SDS-PAGE (see Basic Protocol 1) and Coomassie brilliant blue staining and drying gel (see Support Protocol 1).

1. Dilute proteinase-containing protein sample 1:1 (v/v) in 2 × nonreducing sample buffer.

   The amount of sample used will depend on its proteolytic activity. The author recommends measuring this activity using azocasein as a substrate (García-Carreño, 1992). A sample with 10 mU of activity should be loaded.

2. Prepare and run duplicate SDS-polyacrylamide gels as described (see Basic Protocol 1, steps 1 to 29), using the diluted proteinase-containing protein sample and appropriate molecular weight standards. Do not boil the diluted sample.

   A mini-format (8 × 10–cm) gel is recommended.

   The duplicate gel will be used to calibrate molecular weights and visualize total protein.

3. Disassemble gel cassettes, place gels in separate covered trays, and immerse one gel in 50 ml of 2% casein in 50 mM Tris·Cl for 30 min at 4°C. Stain second gel with Coomassie brilliant blue as described (see Support Protocol 1, steps 1 to 5).

   This incubation allows the substrate to diffuse into the gel at reduced enzyme activity.

   The Coomassie staining solution is acidic enough to stop proteinase activity.

4. Move the casein gel to 25°C (room temperature) and incubate ≤90 min.

   Substrate is digested at sites where proteinases are located.

5. Wash casein gel briefly with water.

6. Place casein gel in covered tray containing enough 0.1% Coomassie brilliant blue staining solution to cover gel. Shake slowly 2 hr on a laboratory shaker or rocker.

   Clear zones on a blue background, indicative of casein hydrolysis by proteinases, should be observable at this stage in the casein gel. Molecular weight standards, proteins other than proteinases, and inhibitor proteins are recognized by their blue color, which is of a higher intensity than the background caused by staining of the undigested casein.

7. *Optional:* Wash 2 hr in destaining solution.

   This step will improve the contrast of the clear zones.

8. Dry gel or record results as described (see Support Protocol 1, step 6).

DETECTION OF PROTEINASE INHIBITORS

Proteinaceous proteinase inhibitors (present for example in legumes, eggs, and plasma) are separated and detected in complex mixtures by using a variation of the technique described in Basic Protocol 3. This technique is useful when studying the presence, diversity, amount, and specificity of proteinase inhibitors in food ingredients that affect protein digestion. An example of detection of proteinase inhibitors is given in Figure B3.1.4.

ALTERNATE PROTOCOL 5

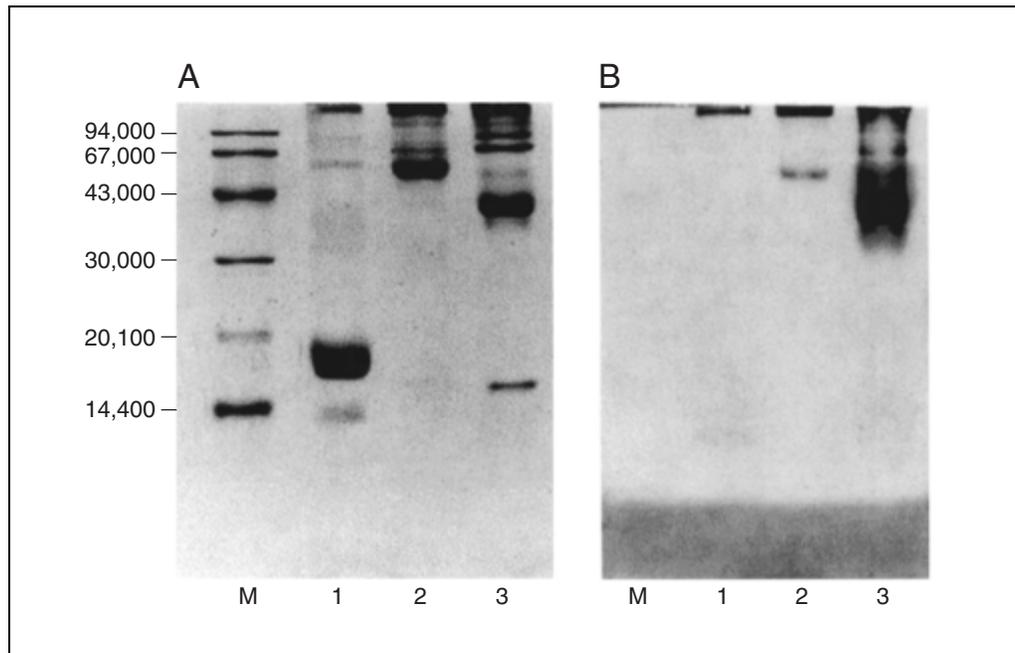**Characterization of Proteins**

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Figure B3.1.4 A 15% SDS-polyacrylamide gel assayed for proteinase inhibitors. (**A**) Gel stained with Coomassie brilliant blue for total protein. (**B**) Gel assayed for serine-proteinase inhibitory activity against trypsin. Food-grade proteinase inhibitors used in surimi manufacture were assayed. Lane 1, whey protein concentrate. Lane 2, bovine plasma proteins. Lane 3, egg white. Each lane contains 15 µg protein. Lane M, molecular weight standards. The dark bands in (B) indicate proteins with proteinase inhibitory activity. Numerous proteins in egg white (lane 3) are shown to have inhibitory activity against trypsin. Adapted from Weerasinghe (1995).

Additional Materials (also see Basic Protocol 3)

Proteinase inhibitor–containing protein sample
0.1 mg/ml proteinase solution: proteinase for which inhibition is to be assayed, diluted in 50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)

1. Run a proteinase inhibitor–containing protein sample on duplicate native polyacrylamide gels as described (see Basic Protocol 3, steps 1 to 2).

   An appropriate, known proteinase inhibitor may be run as a control.

2. Transfer one gel to a tray containing 50 ml of 0.1 mg/ml proteinase solution. Allow the enzyme to diffuse into the gel 30 min at 4°C. Stain second gel with Coomassie brilliant blue as described (see Support Protocol 1, steps 1 to 5).

3. Wash gel briefly with water.

4. Assay for protein-substrate hydrolysis as described (see Basic Protocol 3, steps 3 to 8).

   The presence of an inhibitor appears as a blue band on an otherwise clear background of hydrolyzed casein.

DETERMINATION OF PROTEINASE CLASS

Proteinase-containing samples are incubated with a variety of class- or enzyme-specific proteinase inhibitors, separated on a polyacrylamide gel, and activity stained as described in Basic Protocol 3. A clear zone will be evident in lanes where the proteinase is active (i.e., in the absence of inhibitor or in the presence of a mismatched inhibitor). This clear zone will be absent in the lane containing the properly matched proteinase inhibitor, which provides information about the class or type of proteinase detected in the band.

Additional Materials (also see Basic Protocol 3)

Proteinase-containing protein sample
Class- or enzyme-specific proteinase inhibitor solution, for example:
   200 mM phenylmethylsulfonyl fluoride (PMSF) in isopropanol
   20 mM $N\alpha$-p-tosyl-L-lysine chloromethyl ketone (TLCK) in 1 mM HCl
   10 mM N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) in methanol

1. Mix a proteinase-containing protein sample with 0.1 vol class- or enzyme-specific inhibitor solution. Include samples without inhibitor but with an appropriate amount of solvent used to prepare inhibitors, as well as known proteinases with their inhibitors as controls.

2. Incubate 1 hr at 25°C followed by overnight at 10°C.

3. Assay samples for proteinase activity as described (see Basic Protocol 3, steps 1 to 8).

ESTIMATION OF PROTEIN MOLECULAR WEIGHTS BY SDS-PAGE

Estimating the molecular weight of a protein is relatively straightforward with SDS-PAGE. Molecular weight standards are separated on a polyacrylamide gel along with the unknown or sample protein, and the migration distance of each is measured. This distance is converted to relative mobility (R_f), which is defined as the distance migrated by a protein divided by the distance migrated by a relative mobility marker. Usually the dye front is chosen as the relative mobility marker. Alternatively, a low-molecular-weight standard protein can be used as this marker. The R_f values of the standards are used to generate a standard curve that is compared to the unknown. The gel concentration should be chosen so that the standards produce a linear curve in the region of the unknown.

Materials

Processed and stained SDS-PAGE gel with molecular weight standards (see Basic Protocol 1; see Alternate Protocols 1 and 3)
Ruler with 0.1-cm markings
Calculator capable of two-variable statistics or computer with spreadsheet or graphing software

1. Measure migration distance of molecular weight standards and unknown protein(s) in a processed and stained SDS-polyacrylamide gel using a ruler with 0.1-cm markings. Measure the distance to the center of the protein band.

   This may be done with a dried gel, a photograph, or a digital image of a gel.

2. Calculate the R_f of each protein band and molecular weight standard by dividing the migration distance by that of the relative mobility marker. Use either the dye front or a low-molecular-weight standard as the relative mobility marker.

   Frequently, with gradient gels an internal protein marker is selected because the dye front has become diffuse or has run off the bottom of the gel.

   In one of the simplest approaches to estimating molecular weights, the migration distance into the gel is used without converting to R_f.

3. Plot R_f (x axis) versus the log[mol. wt.] (y axis) for each molecular weight standard.

With gradient gels, yet another measurement is frequently plotted on the x axis. By plotting acrylamide percentage (x axis) versus log[mol. wt.] (y axis), very good linearity is obtained. However, adequately straight calibration lines are much more simply determined using migration distance or R_f for the x axis.

4. Use a calculator or computer program to perform linear regression of the plot.

5. Use the equation of the regression line to estimate the size of the unknown protein.

The purpose of plotting the data and performing the regression is to generate a linear curve through the standards so that the size of the unknown can be estimated. Thus, a region of the plotted data that is reasonably linear should be chosen for performing the regression.

The general equation of a line is y = mx + b, where m is the slope and b is the y intercept. In this case the equation becomes log[mol. wt.] = (slope × R_f) + y intercept.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; *for suppliers, see* **SUPPLIERS APPENDIX**.

Acrylamide solution, 30% (w/v)

Mix 60 g acrylamide (final 30%) and 1.6 g bisacrylamide (final 0.8%) with water in a total volume of 200 ml. Filter the solution through a 0.45-μm filter. Store up to 3 months at 4°C in the dark.

Acrylamide solution, 40% (w/v), high cross-linker

Mix 75.2 g acrylamide (final 40%) and 4.8 g bisacrylamide (final 2.4%) with water in a total volume of 200 ml. Filter the solution through a 0.45-μm filter. Store ≤3 months at 4°C in the dark.

Coomassie brilliant blue staining solution, 0.025% (w/v)

Dissolve 0.5 g Coomassie brilliant blue R250 in 800 ml methanol (final 40%). Add 140 ml acetic acid and water to 2 liters. Store ≤1 month at room temperature.

Coomassie brilliant blue staining solution, 0.1% (w/v)

Dissolve 2 g Coomassie brilliant blue R250 in 800 ml ethanol (final 40%). Add 200 ml acetic acid (final 10%) and water to 2 liters. Store ≤1 month at room temperature.

Denaturing IEF rehydration solution, pH 3 to 10

Dissolve 5.04 g urea (final 8 M) and 788 μl carrier ampholyte mixture (e.g., Pharmalyte; Amersham Pharmacia Biotech; final 7.5%) in water to 10.5 ml. Add a reducing agent (60 mM dithiothreitol) and/or a detergent (0.5% [w/v] Triton X-100, CHAPS, or octyl glucoside), if desired. Make fresh.

Developing solution

Dissolve 25 g sodium carbonate (final 25%) in water to 1 liter. Store ≤2 months at room temperature. Within 1 hr of use, add 20 μl of 37% formaldehyde (final 0.0074%) per 100 ml solution.

Glutaraldehyde fixing solution

4 ml 25% glutaraldehyde (final 0.2%)
150 ml ethanol (final 30%)
13.61 g sodium acetate trihydrate (final 0.2 M)
Water to 500 ml
Prepare immediately before use

Lower (anodic) Tris-tricine tank buffer

Dissolve 121.1 g Tris base (final 0.2 M) and 10 g SDS (final 0.1%) in 500 ml water. Adjust pH to 8.9 with HCl. Dilute to 5 liters. Store ≤1 month at room temperature.

Nonreducing sample buffer, 2×

1.6 ml 4× stacking gel buffer (see recipe)
4 ml 10% (w/v) SDS (final 4%)
1.25 ml glycerol
1.0 mg bromphenol blue
Bring volume to 10.0 ml with water
Store 0.5-ml aliquots ≤ 6 months at –20°C

Running gel buffer, 4×

Dissolve 36.3 g Tris base (final 1.5 M) in 150 ml water. Adjust pH to 8.8 with HCl. Bring volume to 200 ml with water. Store ≤3 months at 4°C.

Running gel overlay

25 ml 4× running gel buffer (see recipe)
1 ml 10% (w/v) SDS (final 0.1%)
Water to 100 ml
Store ≤3 months at 4°C

SDS-PAGE tank buffer, pH 8.3

30.28 g Tris base (final 0.025 M)
144.13 g glycine (final 0.192 M)
10 g SDS (final 0.1%)
Bring to 10 liters with water
Make up directly in large reagent bottles
Store ≤1 month at room temperature

It is not necessary to check pH.

SDS sample buffer, 2×

2.5 ml 4× stacking gel buffer (see recipe)
4.0 ml 10% (w/v) SDS (final 4%)
2.0 ml glycerol (final 20%)
2.0 mg bromphenol blue (final 0.02%)
0.31 g dithiothreitol (DTT; final 0.2 M)
Bring to 10.0 ml with water
Store 0.5-ml aliquots ≤6 months at −20°C

If desired, 5% (v/v) 2-mercaptoethanol may be substitued for DTT. However, DTT is preferred.

Sensitizing solution

300 ml ethanol (final 30%)
68 g sodium acetate (final 6.8%)
2 g sodium thiosulfate (final 0.2%)
Water to 1 liter
Store stock solution ≤2 months at room temperature
Within 1 hr of use, add 0.5 ml of 25% glutaraldehyde (final 0.125%) per 100 ml solution

Silver solution

Dissolve 2.5 g silver nitrate (final 0.25%) in water to 1 liter. Store ≤2 months in a dark bottle at room temperature. Within 1 hr of use, add 40 µl of 37% formaldehyde (final 0.015%) per 100 ml solution.

Stacking gel buffer, 4×

Dissolve 3.0 g Tris base (final 0.5 M) in 40 ml water. Adjust to pH 6.8 with HCl. Bring volume to 50 ml with water. Store ≤3 months at 4°C.

Tris-tricine gel buffer, 4×

Dissolve 72.6 g Tris base (final 3 M) and 0.6 g of SDS (0.3% SDS) in 150 ml water. Adjust pH to 8.45 with HCl. Bring volume to 200 ml with water. Store ≤3 months at 4°C.

Upper (cathodic) Tris-tricine tank buffer

Mix 12.11 g Tris base (final 0.1 M), 17.92 g tricine (final 0.1 M), and 1 g SDS (final 0.1%) with water in a total volume of 1 liter. Do not adjust pH. Store ≤1 month at room temperature.

Water-saturated butanol

Combine 50 ml *n*-, *t*-, or *i*-butanol with 5 ml water. Shake and allow phases to separate. Use top phase (butanol) for overlaying gels. Store indefinitely at room temperature.

COMMENTARY

Background Information

Electrophoresis is the process of moving charged molecules in solution by applying an electrical field across the mixture. Because molecules in an electrical field move with a speed dependent on their charge, shape, and size, electrophoresis has been extensively developed for molecular separations.

As an analytical tool, electrophoresis is simple, relatively rapid, and has unparalleled resolving power. It is used chiefly for analysis and purification of very large molecules such as proteins and nucleic acids. Highly sensitive detection methods have been developed to monitor and analyze electrophoretic separations.

Electrophoresis of macromolecules is normally carried out by applying a sample to a solution stabilized by a porous gel matrix. Under the influence of an applied voltage, different species of molecules in the sample move through the matrix at different velocities. At the end of the separation, the different species are detected as bands at different positions in the matrix. A matrix is required because the electric current passing through the electrophoresis solution generates heat, which causes diffusion and convective mixing of the bands in the absence of a stabilizing medium.

The procedures described in this unit all utilize a polyacrylamide gel matrix. The gel forms when a dissolved mixture of acrylamide and bisacrylamide cross-linker monomers polymerizes into long chains that are covalently cross-linked. The gel structure is held together by the cross-linker. Polymerization of acrylamide is a free-radical reaction that is initiated and catalyzed, respectively, by the addition of ammonium persulfate and TEMED.

When the gel solution is poured into a glass-plate sandwich, the top of the solution forms a meniscus. If measures are not taken to prevent this, the gel will polymerize with a curved top, which will cause the separated sample bands to have a similar curved pattern. To eliminate the meniscus, a thin layer of water-saturated butanol is floated on the surface of the gel mixture before it polymerizes. After polymerization, the butanol layer is poured off, leaving the upper surface of the gel flat. The butanol also excludes oxygen, which would otherwise inhibit polymerization on the gel surface.

SDS Polyacrylamide gel electrophoresis

The most widely used method for protein electrophoresis is the SDS-PAGE system of Laemmli (1970); see Basic Protocol 1. This method separates proteins according to their molecular weights. The intrinsic electrical charge of the sample proteins is not a factor in the separation due to the presence of SDS in the sample and the gel. SDS is an anionic detergent that denatures proteins by wrapping around the polypeptide backbone in a ratio of approximately 1.4 g SDS per gram protein. The bound SDS masks the charge of the proteins themselves, forming anionic complexes with constant net negative charge per unit mass. The SDS also disrupts hydrogen bonds, blocks hydrophobic interactions, and partially unfolds

the protein molecules, minimizing differences in molecular form by eliminating the secondary and tertiary structures. The proteins unfold completely in the presence of a reducing agent such as DTT. The reducing agent cleaves the disulfide bonds, which can form between cysteine residues, and the polypeptides become flexible rods of negative charges with equal charge densities, or charge per unit length. Treating proteins with both SDS and a reducing agent can result in separations exclusively by molecular weight.

There is an approximately linear relationship between the logarithm of the molecular weight and the relative distance of migration of the SDS-polypeptide micelle. This linear relationship is valid only for a certain molecular weight range that is determined by the polyacrylamide percentage. The linear separation range can be increased by employing gels cast with a linear gradient of acrylamide percentage (see Alternate Protocol 1). Guidelines for selecting an appropriate acrylamide percentage or gradient for a particular protein molecular weight range are included in Table B3.1.1.

The SDS-PAGE system of Laemmli (1970) utilizes a discontinuous buffer system, meaning that the counter ion of the Tris buffer is different between the tank buffer and the buffer in the gel. The tank buffer contains glycine ions, whose electrophoretic mobility is less than that of the proteins in the sample, and the gel contains chloride ions, whose electrophoretic mobility is higher than that of the proteins in the sample. The sample first passes through a stacking gel of relatively low acrylamide concentration, where the proteins concentrate into a thin zone between the low mobility and the high mobility ion. This stacking effect results in sharp protein bands and exceptional resolution.

This SDS-PAGE system is a modification of a discontinuous system described by Ornstein (1964) and Davis (1964), which was originally devised to separate proteins under nondenaturing (native) conditions (see Alternate Protocol 2). Under native conditions, the migration rate of a protein is dependent on both its intrinsic charge and its size. The molecular weight of the protein therefore cannot be directly determined by its migration in a single gel. The resolution of nondenaturing electrophoresis is generally not as high as SDS-PAGE, but the technique is useful when one wishes to retain the native structure or enzymatic activity of a protein to assay following electrophoresis.

In the SDS-PAGE system of Laemmli, smaller proteins comigrate with SDS micelles. This prevents the separation of proteins smaller than ~10 kDa. A modified buffer system described by Schägger and von Jagow (1987) was developed to allow separations of peptides and smaller proteins. A modification of this technique (see Alternate Protocol 3) incorporates 35% (v/v) ethylene glycol and a high proportion of bisacrylamide cross-linker (6% of the total monomer) in the running gel to further optimize separation of small peptides.

In the discontinuous systems described in this unit (see Basic Protocol 1; see Alternate Protocols 1, 2, and 3), the choice between a standard gel and a mini-format gel must be made. Mini-format gels are now more commonly run than standard gels due to the higher speed with which they can be run and the increased ease of handling smaller gels. Standard gels are used when higher resolution or greater separation distance is required.

Isoelectric focusing

IEF is an electrophoretic method that separates proteins according to their pIs. Proteins are amphoteric molecules; they carry either positive, negative, or zero net charge, depending on the pH of their surroundings. The net charge of a protein is the sum of all the negative and positive charges of its amino acid side chains and amino and carboxyl termini. The pI is the specific pH at which the net charge of the protein is zero. Proteins are positively charged at pH values below their pI, and negatively charged at pH values above their pI.

The presence of a pH gradient is critical to the IEF technique. In a pH gradient, under the influence of an electric field, a protein will move to the position in the gradient where its net charge is zero. A protein with a positive net charge will migrate toward the cathode, becoming progressively less positively charged as it moves through the pH gradient until it reaches its pI. A protein with a negative net charge will migrate toward the anode, becoming less negatively charged until it also reaches zero net charge. If a protein should diffuse away from its pI, it immediately gains charge and migrates back. This is the *focusing* effect of IEF, which concentrates proteins at their pI and allows proteins to be separated on the basis of very small charge differences.

The degree of resolution is determined by electric field strength. IEF is therefore performed at high voltages (typically >1000 V). When the proteins have reached their final

positions in the pH gradient, there is very little ionic movement in the system, resulting in a very low final current (typically <1 mA).

The methods for IEF described in this unit (see Basic Protocol 2; see Alternate Protocol 4) are dependent on a carrier ampholyte–generated pH gradient. Carrier ampholytes are small, soluble, amphoteric molecules with a high buffering capacity near their pI. Commercial carrier ampholyte mixtures are comprised of hundreds of individual polymeric species with pIs spanning a specific pH range. When a voltage is applied across a carrier ampholyte mixture, the carrier ampholytes with the lowest pI (and the most negative charge) move toward the anode, and the carrier ampholytes with the highest pI (and the most positive charge) move toward the cathode. The other carrier ampholytes align themselves between the extremes, according to their pIs, and buffer their environment to the corresponding pHs. The result is a continuous pH gradient.

IEF can be run in either a native or denaturing mode. Native IEF is the more convenient option, as precast native IEF gels are available in a variety of pH gradients. This method is also preferred when native protein is required, as when activity staining is to be employed. The use of native IEF, however, is often limited by the fact that many proteins are not soluble in water at low ionic strength, or have low solubility close to their isoelectric point. In these cases, denaturing IEF is employed. Urea is the denaturant of choice, as this reagent can solubilize many proteins not otherwise soluble under IEF conditions. Detergents and reducing agents are often used in conjunction with urea for more complete unfolding and solubilization. Urea is not stable in aqueous solution, so precast IEF gels are not manufactured with urea. Rehydrating a dried gel with urea, carrier ampholytes, and other additives is a convenient alternative.

Protein visualization

After an electrophoresis run is complete, the gel must be analyzed to answer analytical or experimental questions. As most proteins are not directly visible, the gel must be processed to determine the location and amount of the separated proteins. The most common analytical procedure is staining. Proteins are usually stained with Coomassie brilliant blue or silver nitrate.

Coomassie brilliant blue staining (see Support Protocol 1) is based on the binding of the dye Coomassie brilliant blue R250, which binds nonspecifically to virtually all proteins. The gel is impregnated with a solution of the dye. Dye that is not bound to protein is allowed to diffuse out of the gel during the destain steps. Although Coomassie brilliant blue staining is less sensitive than silver staining, it is widely used due to its convenience. Coomassie brilliant blue binds to proteins stoichiometrically, so this staining method is preferable when relative amounts of protein are to be determined by densitometry.

When staining IEF gels with Coomassie brilliant blue, the gel is first fixed in a trichloroacetic acid solution to leach out the carrier ampholytes, which would otherwise cause background staining. When staining small peptides run on Tris-tricine gels, the gel is first fixed in a solution containing glutaraldehyde, which cross-links the peptides and prevents them from diffusing out of the gel during subsequent staining steps.

Silver staining is the most sensitive method for permanent visible staining of proteins in polyacrylamide gels. This sensitivity, however, is obtained at the expense of high susceptibility to interference from a number of factors. Precise timing, high quality reagents, and cleanliness are essential for reproducible, high-quality results. In silver staining, the gel is impregnated with soluble silver ion and developed by treatment with formaldehyde, which reduces silver ion to metallic silver, which is insoluble and visible. This reduction is promoted in the presence of proteins, leading to visible bands on the gel. There are many variations of the silver staining process. The method described (see Support Protocol 2) is based on the method of Heukeshoven and Dernick (1985) and has been selected for its overall convenience, sensitivity, reproducibility, and speed.

Activity staining

Techniques have also been developed for the specific visualization of particular classes of enzymes following electrophoretic separation in a gel. These techniques are often referred to as "activity staining," as the intrinsic activity of the enzyme is used, either to produce a colored product or to produce a clear zone on a colored background within the gel. A method for visualizing proteinases based on the work of García-Carreño and Haard (1993) and García-Carreño et al. (1993) is presented (see Basic Protocol 3).

Following electrophoresis of samples that are not heat treated, the gel is impregnated with a good general protein substrate (casein). Pro-

teinases within the gel are allowed to digest the casein, and the gel is stained with Coomassie brilliant blue. The casein in the gel produces a uniform blue background stain, except where it has been digested by proteinases in the gel to produce small peptides that diffuse out of the matrix. Proteinases are thus visualized as clear bands against a blue background.

This technique can be applied following SDS-PAGE, as most proteinases are monomeric, and hence minimally affected by SDS (García-Carreño et al., 1993). The technique described is applicable to most proteinases, which are active at neutral to alkaline pH. A method for characterizing proteinase inhibitors (see Alternate Protocol 5), a growing are of study in food science (García-Carreño, 1996; García-Carreño and Hernandez-Cortes, 2000; García-Carreño et al., 2000) is also included. Recently, a separation technique for acid proteinases with high pIs was published (Díaz-López et al., 1998).

Critical Parameters and Troubleshooting

Many factors influence the quality of electrophoretic separations, including gel preparation, reagent quality, instrument assembly, electrophoresis conditions, and the nature and quantity of the sample.

Preparing and running the gel

To have a high-quality gel, acrylamide polymerization must be complete and uniform. Polymerization may be inhibited by low temperatures, oxygen, insufficient or degraded catalyst, and low acrylamide concentrations. Any of these factors can prevent complete polymerization or, in extreme cases, prevent polymerization entirely. Insufficient polymerization results in poorly defined sample wells and distorted bands. Air bubbles trapped under the comb teeth can inhibit polymerization locally, also resulting in band distortions. Insufficient polymerization along spacers can cause the gel to run faster towards the edges, producing a localized "frown" effect. Polymerization may also be uneven across the entire gel, resulting in distortions in the final electrophoresis result.

The following suggestions should minimize problems of incomplete polymerization. Warm all refrigerated gel solutions to room temperature prior to use and always deaerate the gel solution 5 to 10 min with at least a water aspirator. It is helpful to warm the gel solution to 20° to 25°C after deaeration and allow it to polymerize at or slightly above room temperature. Check the ammonium persulfate for freshness. Fresh ammonium persulfate will crackle when water is added; if it doesn't, use a fresh bottle. Check the freshness of the acrylamide stock solution. Old acrylamide can also inhibit polymerization. If the polymerization problem persists, increase the ammonium persulfate and TEMED concentrations by 50%. Increasing the catalyst concentration is particularly useful when working with low acrylamide concentrations.

Too rapid polymerization can result from high temperatures or too much catalyst. If polymerization is too rapid, the gel may not polymerize evenly, or may polymerize before the gel is completely poured. To prevent these problems, decrease the amount of ammonium persulfate and TEMED by 30%.

Reagents used in preparing electrophoresis gels and buffers must be of high quality. Many reagent manufacturers supply specifically designated "electrophoresis quality" reagents that are prepared to be largely free of interfering contaminants. Use such reagents whenever possible. Also take care when preparing the buffers and other solutions used in electrophoresis. Mistakes in concentration or pH adjustment can result in a slowly running gel or a low-quality electrophoretic separation.

Many electrophoresis problems can be attributed to improper instrument assembly. Sealing gaskets must be seated correctly and spacers must be aligned or buffer leakage may result. If the level of upper buffer drops too far, electrical continuity is interrupted and protein migration is stopped. Air bubbles trapped between the glass plates at the bottom of the gel cassette can be large enough to block current locally and cause a distortion in the gel pattern. Care should be taken to make sure the bottom of the gel cassette is free of bubbles.

Electrophoresis generates heat, and if the gel runs faster than this heat can dissipate, it will not run evenly. Running the gel too fast is the primary cause of "smiling," where the center of the gel runs faster than the edges. The use of an electrophoresis unit that allows the gel cassette to be completely submerged in the lower buffer allows more efficient dissipation of heat and can allow the gel to run faster without smiling. The use of external cooling (connecting the electrophoresis unit to a thermostatic circulator) results in still more effective dissipation of heat.

Preparing the sample

To be analyzed effectively by electrophoresis, a protein sample must be well solubilized, undegraded, free of particulate material, and loaded at an appropriate concentration. Poor solubilization is manifested by vertical streaks or smears rather than distinct bands. In the case of SDS-PAGE, heating briefly in SDS-containing treatment buffer is usually sufficient to solubilize all of the proteins in the sample. Some proteins, however, aggregate during heating to 100°C. In these cases, it is better to solubilize at lower temperatures (40° to 80°C).

Many proteins are poorly soluble under conditions prevailing in nondenaturing electrophoresis or IEF. Solubility can occasionally be improved with the use of a nondenaturing, neutral detergent (e.g., 0.5% [w/v] Triton X-100, CHAPS, or octyl glucoside) or polyalcohol (e.g., 20% [w/v] glycerol or sorbitol) in both the sample and the gel.

Degradation by endogenous proteinases can result in smearing, loss of high-molecular-weight proteins, or loss or splitting of bands. Most proteinases are inactivated by heating in the presence of SDS, so if SDS-PAGE is being employed, the sample should be heated as soon as possible. Up until the heating step (if one is employed) samples should be kept on ice to slow proteolysis. If proteolysis remains a problem, proteinase inhibitors (e.g., phenylmethyl-sulfonyl fluoride, leupeptin, pepstatin) should be used during sample preparation.

Protein can be prevented from entering the gel cleanly by particulate and unsolubilized material, which will remain in the sample wells. Samples containing particulate material should therefore always be centrifuged prior to electrophoresis.

When too much protein is loaded onto a gel, bands may spread and not resolve well. The staining may saturate, producing indistinct bands. Too little protein will result in bands of interest being faint or absent. The optimal amount to load varies widely depending on the complexity of the sample and the method of staining. It is often useful to load several different dilutions of a sample on the same gel to determine the optimal amount.

Whereas SDS-PAGE and other discontinuous techniques are generally quite tolerant of sample impurities and buffer and ionic variations, the quality of the sample and the nature of the solution it is loaded in have a strong influence on the quality of an IEF separation. The sample must be as free as possible of salts, buffers, and other small charged molecules, otherwise the proteins will not focus into sharp bands.

Staining the gel

Coomassie brilliant blue staining, although approximately 50-fold less sensitive that silver staining, is considerably simpler to perform and less subject to interference or error than silver staining. Insufficient staining intensity may be the result of not staining long enough, in which case, the gel should be left longer in the staining solution. The blue background should be removed by destaining until the background is clear.

Silver staining, on the other hand, is highly susceptible to interference from a variety of sources. Exceptional cleanliness must be practiced in preparing the electrophoresis unit and in handling the gel. All equipment used for running and staining the gel must be cleaned with detergent and thoroughly rinsed. Clean gloves should be worn when handling the electrophoresis apparatus, the gel, or the staining tray. High-quality water must be used to prepare the reagents, as impurities have a strong effect on silver staining. For best results, water with a resistivity ≥ 5 MΩ should be used. The reagents used for silver staining should be of the highest quality possible.

Temperature has a strong effect on silver staining, with higher temperatures promoting faster development and darker background. For optimal reproducibility, the timing of silver staining steps should be precisely controlled. The timing of the wash steps is particularly important. Faint bands or poor development could be the result of poor reagent quality or improperly prepared reagents. It could also be the result of washing too long between the silver step and the developing step. An excessively dark background could also be the result of poor reagent quality or improperly prepared reagents. It may also be the result of developing too long, the use of impure water or detergent, or the presence of other residues in the staining tray.

Proteinases and proteinase inhibitors in a sample should be analyzed for their sensitivity to SDS concentration before activity staining is carried out. Their activity should be measured in the presence and absence of 0.1% SDS before proceeding with electrophoresis. The author has characterized many proteinases under such conditions without a loss of activity. Most SDS-sensitive enzymes regain their activity if the gel is washed in the buffer used to dissolve the

substrate before proceeding with the activity staining.

Anticipated Results

Properly executed, SDS-PAGE should be able to resolve a protein sample into up to 100 distinct protein species. Proteins separated by nondenaturing PAGE appear more diffuse and exhibit less overall resolution than proteins separated under denaturing conditions; however, biological activity is often maintained. Gradient gels provide superior protein-band sharpness and resolve a larger size range of proteins; however, they are more difficult to prepare. Molecular weight calculations are simplified with gradient gels because of the extended linear relationship between size and protein position within the gel.

IEF has a similar resolving power to SDS-PAGE, but it has less applicability due to the limited solubility of many proteins under IEF conditions.

With Coomassie brilliant blue staining one should be able to detect ~50 to 100 ng protein in a normal band. The lower detection limit of silver staining is ~1 ng protein. Once the gel is stained, it can be photographed or dried on a transparent backing for a record of the position and intensity of each band.

Time Considerations

Preparation of electrophoresis running and stacking gels requires 2 to 3 hr. Running the gel requires 1.5 hr for mini-format gels or IEF gels and 5 hr for standard gels. Visualization of the electrophoresis result takes a full day for Coomassie brilliant blue staining and 3 hr for silver staining. Activity staining a gel for proteinase requires ≥6 hr (including the destaining solution wash) after running the polyacrylamide gel. The determination of proteinase class requires an additional overnight incubation before the gel can be run.

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Good general references on protein electrophoresis that contain detailed descriptions of several important techniques.

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Electrophoresis Analysis

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